



# **Draft Assessment Report**

## **Evaluation of Active Substances**

Plant Protection Products

Prepared according to **Regulation (EC) 1107/2009**  
as it applies in Great Britain (GB PPP)

### **Cinmethylin (BAS 684 H)**

#### **Volume 3 – B.7 (AS)**

#### **Residues Data**

Great Britain

November 2020

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**Version History**

| <b>When</b>   | <b>What</b> |
|---------------|-------------|
| November 2020 | Initial DAR |
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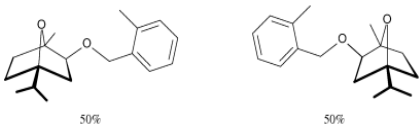
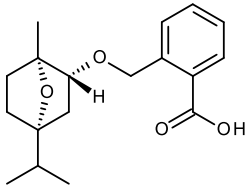
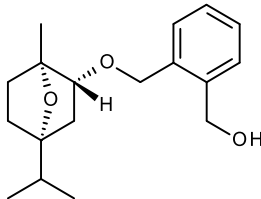
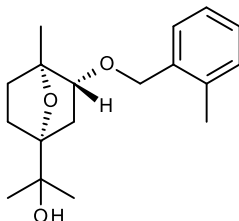
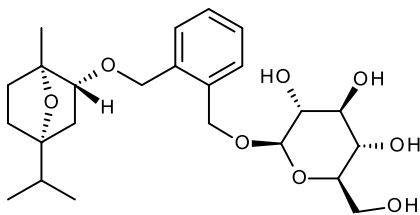
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## B.7. RESIDUE DATA

Metabolites and their derivatives (including their codes, chemical names and structural formulas) relevant for the residues evaluation (identified or used as reference standards but not found) are shown in Table 7-1.

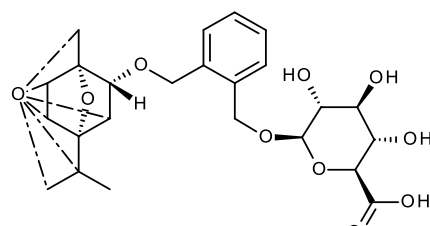
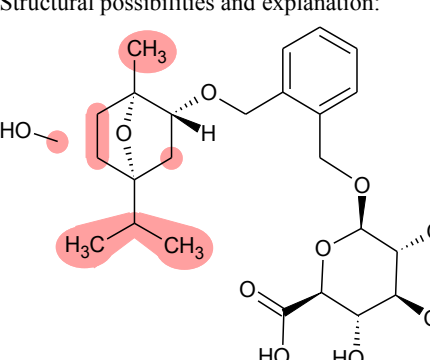
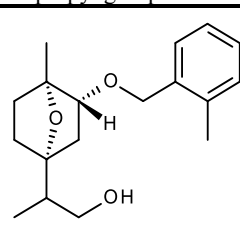
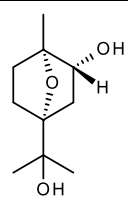
Table 7-1 Metabolites (and their derivatives) either identified or used as reference standards (but not found), and their structures, names and codes

| Code Number<br>(Reg. Number)<br>(SD/WL-Code)  | Chemical Name   | Molecular Structure<br>(Only a single enantiomer/diastereomer is shown<br>for metabolites with stereochemistry) | Occurrence in<br>residues studies   |
|---|---|---|---|
| BAS 684 H (900202)<br>(SD95481)<br>(WL95481)<br>(IN-YA168)<br>(IN-42326)<br>(N.B. 5103-156) | (1R,2SR,4SR)-1,4-epoxy-p-menth-2-yl 2-methylbenzyl ether  |                               | Hen<br>Goat<br>Wheat<br>Carrot<br>Rotational crops<br>Reference item<br>used but not<br>found in<br>soybean, peanut<br>and rice<br>1980s goat   |
| M684H001 (6055521)<br>(SD202193)  | 2-({[(1SR,2RS,4RS)-1-methyl-4-(propan-2-yl)-7-oxabicyclo[2.2.1]hept-2-yl]oxy}methyl)benzoic acid                            |                              | Hen<br>Goat   |
| M684H002 (6055479)<br>(SD207856)  | [2-({[(1SR,2RS,4RS)-1-methyl-4-(propan-2-yl)-7-oxabicyclo[2.2.1]hept-2-yl]oxy}methyl)phenyl]methanol                        |                             | Livestock<br>Wheat (after<br>deconjugation)<br>Oilseed rape<br>(after<br>deconjugation)<br>Soybean<br>Peanut<br>Reference item<br>used but not<br>found in rice and<br>goat<br>1980s goat |
| M684H004 (6055480)<br>(SD/WL 205588)  | 2-({[(1SR,3RS,4SR)-4-methyl-3-[(2-methylbenzyl)oxy]-7-oxabicyclo[2.2.1]hept-1-yl]propan-2-ol                                |                             | Wheat (after<br>deconjugation)<br>Soybean<br>Peanut<br>Reference item<br>used but not<br>found in rice<br>1980s goat  |
| M684H005 (6067256)  | [2-({[(1SR,2RS,4RS)-1-methyl-4-(propan-2-yl)-7-oxabicyclo[2.2.1]heptan-2-yl]oxy}methyl)phenyl]methyl beta-D-glucopyranoside |                             | Wheat Oilseed<br>rape<br>Carrot   |

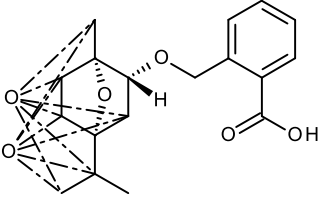
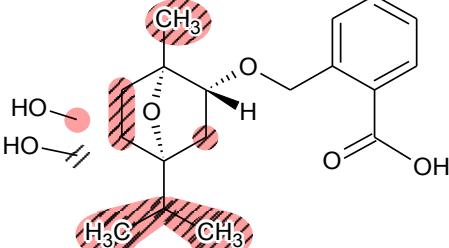
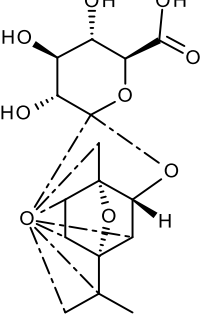
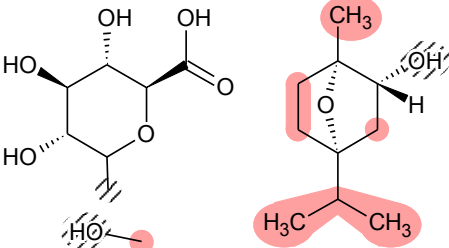
| Code Number<br>(Reg. Number)<br>(SD/WL-Code) | Chemical Name   | Molecular Structure<br>(Only a single enantiomer/diastereomer is shown<br>for metabolites with stereochemistry)                                 | Occurrence in<br>residues studies |
|--|---|---|-----------------------------------|
| M684H006 (6067258)                           | [2-({[(1S,2RS,4RS)-1-methyl-4-(propan-2-yl)-7-oxabicyclo[2.2.1]heptan-2-yl]oxy}methyl)phenyl)methyl (carboxyacetyl)-beta-D-glucopyranoside 6-O- |   | Wheat<br>Oilseed rape<br>Carrot   |
| M684H007                                     | —   | <p>Markush structure:</p> <p>Structural possibilities and explanation:</p> <p>Hydroxylation and malonylglucosylation at the aromatic moiety</p> | Wheat<br>Oilseed rape             |
| M684H008                                     | —   | <p>Markush structure:</p> <p>Structural possibilities and explanation:</p> <p>Hydroxylation and glucosylation at the aromatic moiety</p>        | Wheat<br>Oilseed rape             |
| M684H009<br>(73032)<br>(SD213341)            | N-(2-methylbenzoyl)glycine  |   | Goat                              |
| M684H010 (111609)<br>(SD207859)              | 2-(hydroxymethyl)benzoic acid   |   | Hen                               |

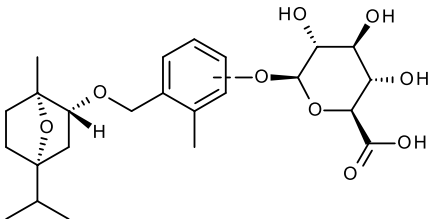
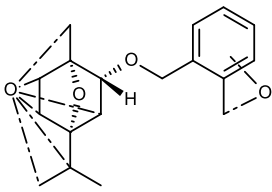
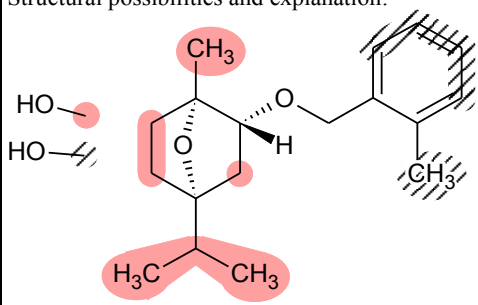
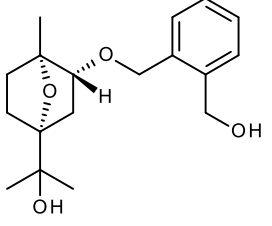
| Code Number<br>(Reg. Number)<br>(SD/WL-Code) | Chemical Name   | Molecular Structure<br>(Only a single enantiomer/diastereomer is shown<br>for metabolites with stereochemistry) | Occurrence in<br>residues studies |
|--|---|---|-----------------------------------|
| M684H011 (6055478)<br>(SD207574)             | 2-([[(1SR,2RS,4RS)-4-(2-hydroxypropan-2-yl)-1-methyl-7-oxabicyclo[2.2.1]hept-2-yl]oxy)methyl)benzoic acid                               |   | Hen<br>Goat<br>1980s goat         |
| M684H012 (6074715)                           | [2-([[(1SR,2RS,4RS)-1-methyl-4-(propan-2-yl)-7-oxabicyclo[2.2.1]heptan-2-yl]oxy)methyl]phenyl)methyl beta-D-glucopyranosiduronic acid   |   | Goat                              |
| M684H013 (6055481)<br>(SD207852)             | 2-([[(1SR,2RS,4RS)-4-(1-hydroxypropan-2-yl)-1-methyl-7-oxabicyclo[2.2.1]hept-2-yl]oxy)methyl)benzoic acid                               |   | 1980s goat                        |
|  |   |   |                                   |
| M684H015                                     | 3-methyl-4-([[(1SR,2RS,4RS)-1-methyl-4-(propan-2-yl)-7-oxabicyclo[2.2.1]heptan-2-yl]oxy)methyl]phenyl 6-O-(carboxyacetyl)hexopyranoside |   | Wheat<br>Oilseed rape             |
| M684H016                                     | 2-methyl-3-([[(1SR,2RS,4RS)-1-methyl-4-(propan-2-yl)-7-oxabicyclo[2.2.1]heptan-2-yl]oxy)methyl]phenyl 6-O-(carboxyacetyl)hexopyranoside |   | Wheat<br>Oilseed rape             |

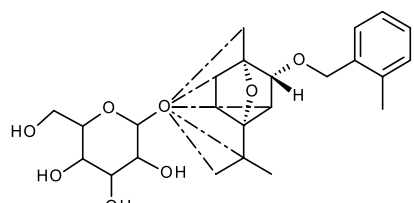
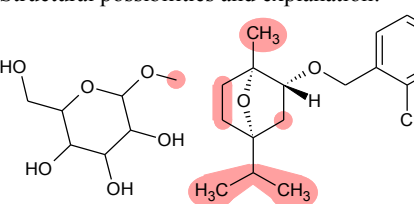
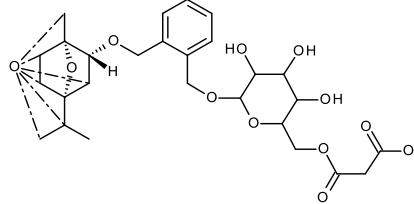
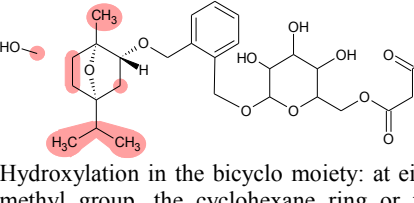
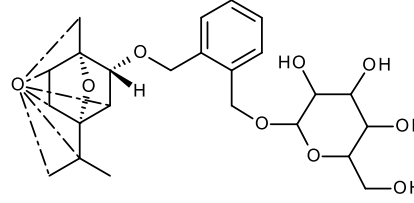
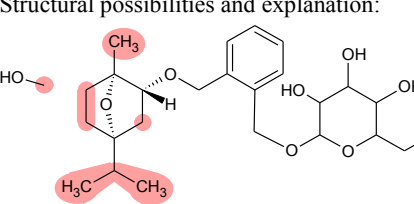
| Code Number<br>(Reg. Number)<br>(SD/WL-Code) | Chemical Name   | Molecular Structure<br>(Only a single enantiomer/diastereomer is shown<br>for metabolites with stereochemistry)  | Occurrence in<br>residues studies  |
|--|---|--|--|
| M684H017 (6066765)<br>(SD 211648)            | 2-methyl-3-({[(1SR,2RS,4RS)-1-methyl-4-(propan-2-yl)-7-oxabicyclo[2.2.1]heptan-2-yl]oxy}methyl)phenol |  | Wheat (after deconjugation)<br>Oilseed rape (after deconjugation)<br>Soybean<br>Peanut (proposed structure) 1980s goat |
| M684H018 (6067259)<br>(SD/WL 211647)         | 4-methyl-3-({[(1SR,2RS,4RS)-1-methyl-4-(propan-2-yl)-7-oxabicyclo[2.2.1]heptan-2-yl]oxy}methyl)phenol |  | Wheat (after deconjugation)<br>Soybean<br>Peanut<br>Reference item used but not found in rice 1980s goat               |
| M684H019 (6066766)<br>(SD/WL 211368))        | 3-methyl-4-({[(1SR,2RS,4RS)-1-methyl-4-(propan-2-yl)-7-oxabicyclo[2.2.1]heptan-2-yl]oxy}methyl)phenol |  | Wheat (after deconjugation)<br>Soybean<br>Peanut<br>Reference item used but not found in rice 1980s goat               |
| M684H021                                     | –   | <p>Markush structure:</p> <p>The “O” means hydroxy group.<br/>Structural possibilities and explanation:</p> <p>Double hydroxylation at two positions in the bicyclo moiety: at the methyl group, the cyclohexane ring or the iso-propyl group in any possible combination.</p> | Hen  |

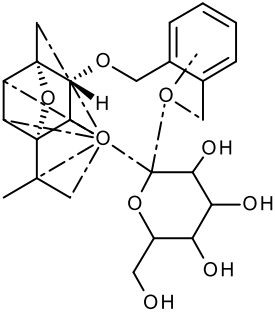
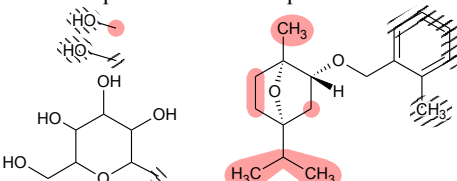
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|--|---|--|---|
| M684H022                                     | —   | <p>Markush structure:</p>  <p>The “O” means hydroxy group.<br/>Structural possibilities and explanation:</p>  <p>Single hydroxylation in the bicyclo moiety: either at the methyl group, the cyclohexane ring or the iso-propyl group.</p> | Goat  |
| M684H024 (6059085)<br>(SD 207430)            | 2-[(1R,3R,4S)-4-methyl-3-[(2-methylphenyl)methoxy]-7-oxabicyclo[2.2.1]heptan-1-yl]propan-1-ol |    | Wheat (after deconjugation)<br>Reference item used but not found in soybean<br>Peanut<br>1980s goat |
| M684H026 (6059081)                           | (1S,2R,4R)-4-(2-hydroxypropan-2-yl)-1-methyl-7-oxabicyclo[2.2.1]heptan-2-ol                   |    | Hen<br>Goat   |

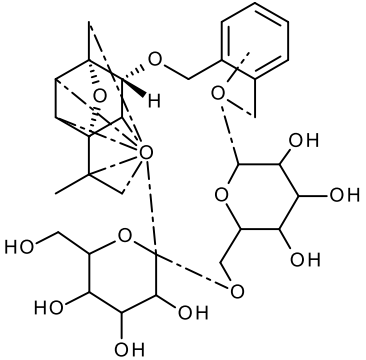
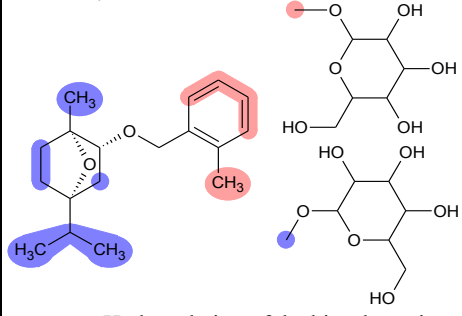
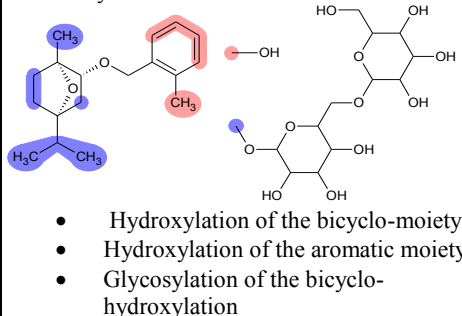
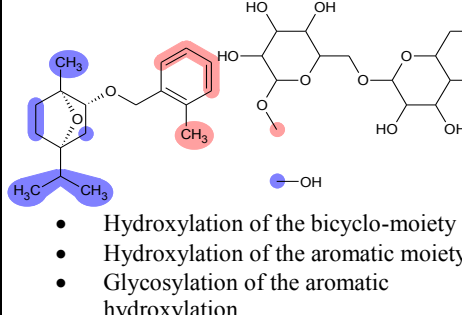


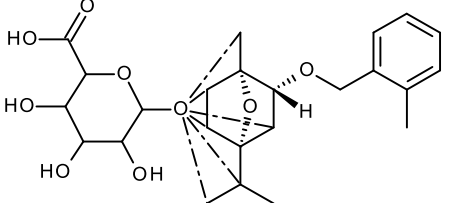
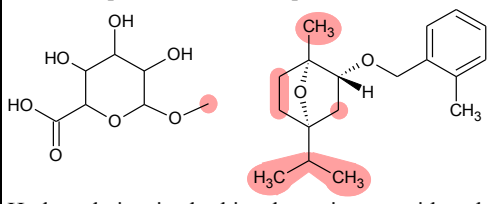
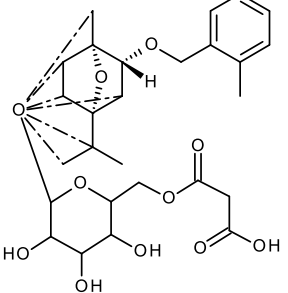
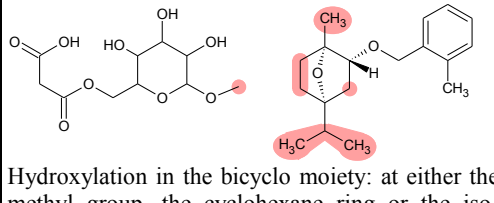
| Code Number<br>(Reg. Number)<br>(SD/WL-Code) | Chemical Name | Molecular Structure<br>(Only a single enantiomer/diastereomer is shown<br>for metabolites with stereochemistry)  | Occurrence in<br>residues studies |
|--|---------------|--|-----------------------------------|
| M684H027                                     | —             | <p>Markush structure:</p>  <p>The “O” means hydroxy group.<br/>Structural possibilities and explanation:</p>  <p>Double hydroxylation at two positions in the bicyclo moiety: at the methyl group, the cyclohexane ring or the iso-propyl group in any possible combination.</p> | Hen                               |
| M684H029                                     | —             | <p>Markush structure:</p>  <p>Structural possibilities and explanation:</p>  <p>Hydroxylation in the bicyclo moiety at either the methyl group, the cyclohexane ring or the iso-propyl group.<br/>Subsequent glycosylation of any of the available hydroxy groups.</p>       | Goat                              |

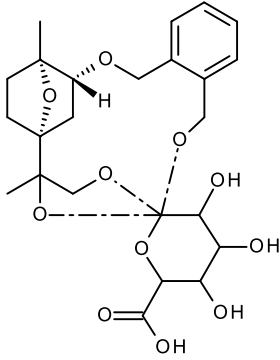
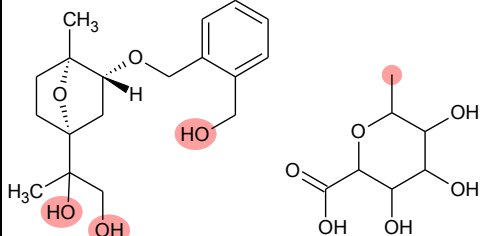
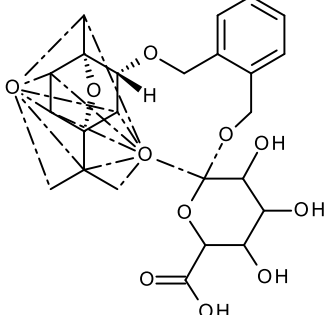
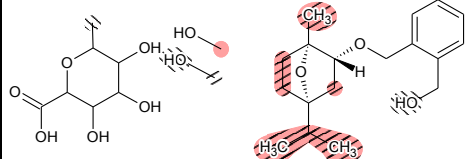
| Code Number<br>(Reg. Number)<br>(SD/WL-Code) | Chemical Name   | Molecular Structure<br>(Only a single enantiomer/diastereomer is shown<br>for metabolites with stereochemistry)   | Occurrence in<br>residues studies   |
|--|---|---|---|
| M684H034                                     | —   |   | Goat  |
| M684H039                                     | —   | <p>Markush structure:</p>  <p>The “O” means hydroxy group.<br/>Structural possibilities and explanation:</p>  <p>One hydroxylation in the bicyclo moiety: at either the methyl group, the cyclohexane ring or the isopropyl group.<br/>One hydroxylation in the aromatic moiety.</p> | Hen<br>Oilseed rape   |
| M684H044 (6059083)<br>(SD/WL 207855)         | 2-[(1RS,3RS,4SR)-3-{[2-(hydroxymethyl)phenyl]methoxy}-4-methyl-7-oxabicyclo[2.2.1]heptan-1-yl]propan-2-ol |   | Wheat (after deconjugation)<br>Oilseed rape (after deconjugation)<br>Soybean<br>Peanut (proposed structure)<br>Rice (tentative)<br>1980s goat |

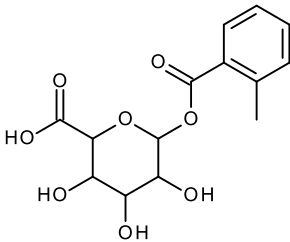
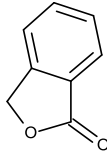
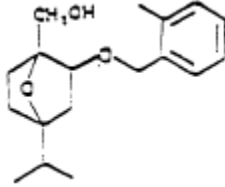
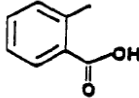
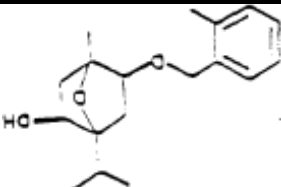
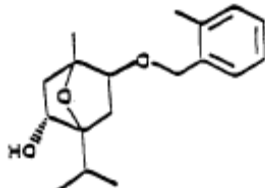
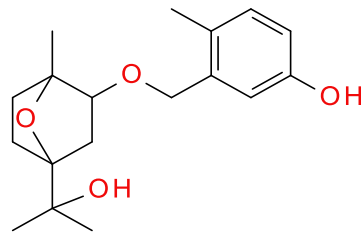
| Code Number<br>(Reg. Number)<br>(SD/WL-Code) | Chemical Name | Molecular Structure<br>(Only a single enantiomer/diastereomer is shown<br>for metabolites with stereochemistry)   | Occurrence in<br>residues studies |
|--|---------------|---|-----------------------------------|
| M684H046                                     | —             | <p>Markush structure:</p>  <p>Structural possibilities and explanation:</p>  <p>Hydroxylation and subsequent glycosylation in the bicyclo moiety: at either the methyl group, the cyclohexane ring or the iso-propyl group.</p>         | Oilseed rape                      |
| M684H047                                     | —             | <p>Markush structure:</p>  <p>Structural possibilities and explanation:</p>  <p>Hydroxylation in the bicyclo moiety: at either the methyl group, the cyclohexane ring or the iso-propyl group.<br/>The “O” means hydroxy group.</p>  | Wheat<br>Oilseed rape<br>Carrot   |
| M684H048                                     | —             | <p>Markush structure:</p>  <p>The “O” means hydroxy group.<br/>Structural possibilities and explanation:</p>  <p>Hydroxylation in the bicyclo moiety: at either the methyl group, the cyclohexane ring or the iso-propyl group.</p> | Wheat<br>Oilseed rape<br>Carrot   |

| Code Number<br>(Reg. Number)<br>(SD/WL-Code) | Chemical Name | Molecular Structure<br>(Only a single enantiomer/diastereomer is shown<br>for metabolites with stereochemistry)   | Occurrence in<br>residues studies |
|--|---------------|---|-----------------------------------|
| M684H050                                     | —             | <p>Markush structure:</p>  <p>For the non-glycosylated hydroxy group, the “O” means hydroxy group.</p> <p>Structural possibilities and explanation:</p>  <p>One hydroxylation in the bicyclo moiety: at either the methyl group, the cyclohexane ring or the iso-propyl group.</p> <p>One hydroxylation in the aromatic moiety.</p> <p>Subsequent glycosylation of one of the two hydroxy groups.</p> | Carrot                            |

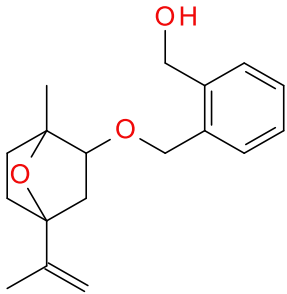
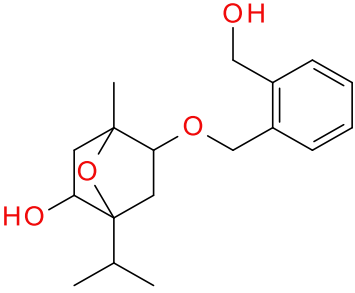
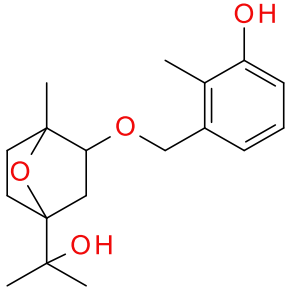
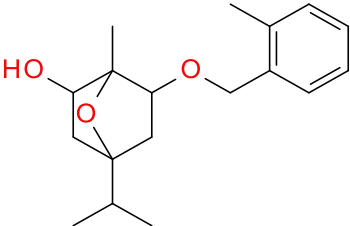
| Code Number<br>(Reg. Number)<br>(SD/WL-Code) | Chemical Name | Molecular Structure<br>(Only a single enantiomer/diastereomer is shown<br>for metabolites with stereochemistry)   | Occurrence in<br>residues studies |
|--|---------------|---|-----------------------------------|
| M684H051                                     | —             | <p>Markush structure:</p>  <p>Structural possibilities and explanation:</p> <p>Possibility 1</p>  <ul style="list-style-type: none"> <li>• Hydroxylation of the bicyclo moiety</li> <li>• Hydroxylation of the aromatic moiety</li> <li>• Glycosylation of both hydroxylations</li> </ul> <p>Possibility 2</p>  <ul style="list-style-type: none"> <li>• Hydroxylation of the bicyclo-moiety</li> <li>• Hydroxylation of the aromatic moiety</li> <li>• Glycosylation of the bicyclo-hydroxylation</li> <li>• Glycosylation of the saccharide</li> </ul> <p>Possibility 3</p>  <ul style="list-style-type: none"> <li>• Hydroxylation of the bicyclo-moiety</li> <li>• Hydroxylation of the aromatic moiety</li> <li>• Glycosylation of the aromatic hydroxylation</li> <li>• Glycosylation of the saccharide</li> </ul> | Oilseed rape<br>Carrot            |

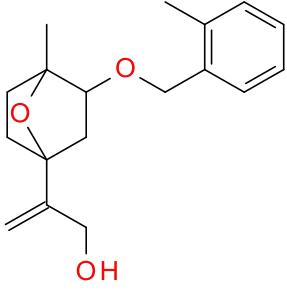
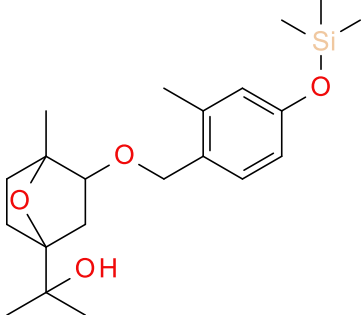
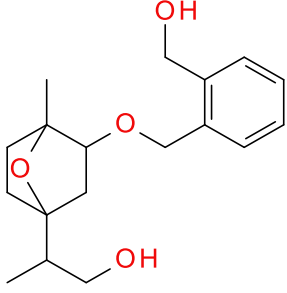
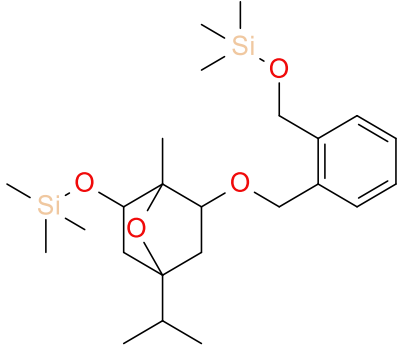
| Code Number<br>(Reg. Number)<br>(SD/WL-Code) | Chemical Name | Molecular Structure<br>(Only a single enantiomer/diastereomer is shown<br>for metabolites with stereochemistry)  | Occurrence in<br>residues studies |
|--|---------------|--|-----------------------------------|
| M684H052                                     | —             | <p>Markush structure:</p>  <p>Structural possibilities and explanation:</p>  <p>Hydroxylation in the bicyclo moiety: at either the methyl group, the cyclohexane ring or the isopropyl group.<br/>Subsequent glucuronidation of the hydroxy group.</p>         | Goat                              |
| M684H055                                     | —             | <p>Markush structure:</p>  <p>Structural possibilities and explanation:</p>  <p>Hydroxylation in the bicyclo moiety: at either the methyl group, the cyclohexane ring or the isopropyl group.<br/>Subsequent malonylglucosilation of the hydroxy group.</p> | Wheat<br>Oilseed rape             |

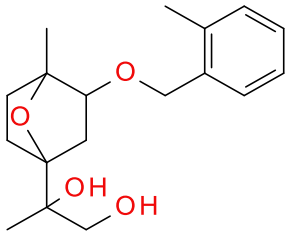
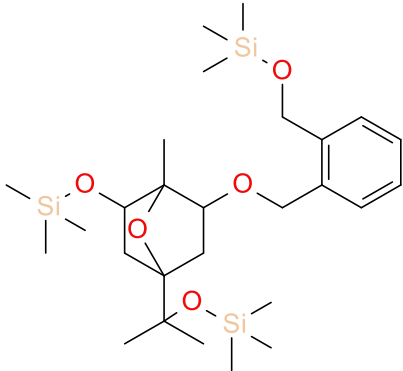
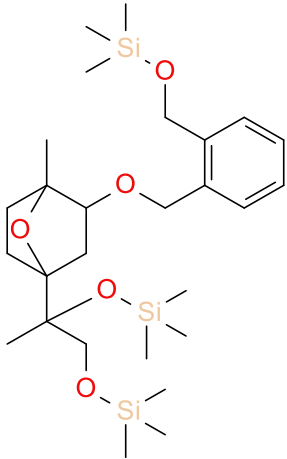
| Code Number<br>(Reg. Number)<br>(SD/WL-Code) | Chemical Name | Molecular Structure<br>(Only a single enantiomer/diastereomer is shown<br>for metabolites with stereochemistry)   | Occurrence in<br>residues studies |
|--|---------------|---|-----------------------------------|
| M684H056                                     | —             | <p>Markush structure:</p>  <p>For the unconjugated hydroxy group the “O” means hydroxy group.<br/>Structural possibilities and explanation:</p>  <p>Double hydroxylation of the iso-propyl group of the bicyclo moiety and hydroxylation of the methyl group in the aromatic moiety and subsequent glucuronidation of one of the hydroxy groups.</p>   | Goat                              |
| M684H057                                     | —             | <p>Markush structure:</p>  <p>For the unconjugated hydroxy group the “O” means hydroxy group.<br/>Structural possibilities and explanation:</p>  <p>Double hydroxylation in the bicyclo moiety: at either the methyl group, the cyclohexane ring or the iso-propyl group in any possible combination. Subsequent glucuronidation of one hydroxy group: either in the bicyclo moiety or at the hydroxy group in the aromatic moiety.</p> | Goat                              |

| Code Number<br>(Reg. Number)<br>(SD/WL-Code) | Chemical Name   | Molecular Structure<br>(Only a single enantiomer/diastereomer is shown<br>for metabolites with stereochemistry) | Occurrence in<br>residues studies   |
|--|---|---|---|
| M684H058                                     | 1-O-(2-methylbenzoyl) hexopyranuronic acid  |                               | Hen   |
| M684H059<br>(18851)<br>(SD637)               | 2-benzofuran-1(3H)-one  |                                | Hen<br>1980s goat<br>Reference item<br>used but not<br>found in<br>rotational crops                 |
| SD 207322                                    | 7-oxabicyclo[2.2.1]heptane-1-methanol,4-(1-methylethyl)-2-((2-methylphenyl)methoxy)-,exo-   |                               | Reference item<br>used but not<br>found in soybean<br>and peanut                                    |
| SD 751                                       | Benzoic acid, 2-methyl-   |                              | Soybean   |
| SD 207850                                    | 7-oxabicyclo[2.2.1]heptan-2-ol, 4-methyl-1-(1-methylethyl)-5-((2-methylphenyl)methoxy)-, exo, exo-  |                             | Soybean<br>Peanut   |
| SD 211646                                    | 7-oxabicyclo[2.2.1]heptan-2-ol,4-methyl-1-(1-methylethyl)-5-((2-methylphenyl)methoxy)-,2-endo,5-exo-  |                             | Reference item<br>used but not<br>found in soybean<br>and peanut                                    |
| SD 211733<br>WL 211733                       | 3-[[4-(1-hydroxy-1-methyl-ethyl)-1-methyl-7-oxabicyclo[2.2.1]heptan-2-yl]oxymethyl]-4-methyl-phenol<br>(without stereochemistry)<br><br>3-[[ (2S)-4-(1-hydroxy-1-methyl-ethyl)-1-methyl-7-oxabicyclo[2.2.1]heptan-2-yl]oxymethyl]-4-methyl-phenol<br>(with stereochemistry) |                             | Soybean<br>(proposed<br>structure)<br>Reference item<br>used but not<br>round in rice<br>1980s goat |

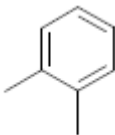
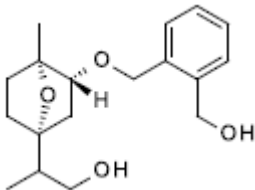


| Code Number<br>(Reg. Number)<br>(SD/WL-Code) | Chemical Name   | Molecular Structure<br>(Only a single enantiomer/diastereomer is shown<br>for metabolites with stereochemistry) | Occurrence in<br>residues studies         |
|--|---|---|---|
| SD 211892                                    | <p>[2-[(4-isopropenyl-1-methyl-7-oxabicyclo[2.2.1]heptan-2-yl)oxymethyl]phenyl]methanol<br/>(without stereochemistry)</p> <p>[2-[[2-(2S)-4-isopropenyl-1-methyl-7-oxabicyclo[2.2.1]heptan-2-yl)oxymethyl]phenyl]methanol<br/>(with stereochemistry)</p>                               |                               | Soybean<br>Peanut<br>1980s goat           |
| SD 211731                                    | <p>5-[[2-(hydroxymethyl)phenyl]methoxy]-1-isopropyl-4-methyl-7-oxabicyclo[2.2.1]heptan-2-ol<br/>(without stereochemistry)</p> <p>(5S)-5-[[2-(hydroxymethyl)phenyl]methoxy]-1-isopropyl-4-methyl-7-oxabicyclo[2.2.1]heptan-2-ol<br/>(with stereochemistry)</p>                         |                              | Soybean<br>Peanut<br>(proposed structure) |
| SD 213323                                    | <p>3-[[4-(1-hydroxy-1-methyl-ethyl)-1-methyl-7-oxabicyclo[2.2.1]heptan-2-yl]oxymethyl]-2-methyl-phenol<br/>(without stereochemistry)</p> <p>3-[[2-(2S)-4-(1-hydroxy-1-methyl-ethyl)-1-methyl-7-oxabicyclo[2.2.1]heptan-2-yl]oxymethyl]-2-methyl-phenol<br/>(with stereochemistry)</p> |                             | Peanut (proposed structure)<br>1980s goat |
| SD 211732                                    | <p>4-isopropyl-1-methyl-6-(o-tolylmethoxy)-7-oxabicyclo[2.2.1]heptan-2-ol<br/>(without stereochemistry)</p> <p>(6S)-4-isopropyl-1-methyl-6-(o-tolylmethoxy)-7-oxabicyclo[2.2.1]heptan-2-ol<br/>(with stereochemistry)</p>   |                             | 1980s goat                                |

| Code Number<br>(Reg. Number)<br>(SD/WL-Code) | Chemical Name  | Molecular Structure<br>(Only a single enantiomer/diastereomer is shown<br>for metabolites with stereochemistry) | Occurrence in<br>residues studies |
|--|--|---|-----------------------------------|
| SD 213325                                    | 2-[4-methyl-3-(o-tolylmethoxy)-7-oxabicyclo[2.2.1]heptan-1-yl]prop-2-en-1-ol<br>(without stereochemistry)<br><br>2-[(3S)-4-methyl-3-(o-tolylmethoxy)-7-oxabicyclo[2.2.1]heptan-1-yl]prop-2-en-1-ol<br>(with stereochemistry)   |                               | 1980s goat                        |
| SD 213327                                    | 2-[4-methyl-3-[(2-methyl-4-trimethylsilyloxy-phenyl)methoxy]-7-oxabicyclo[2.2.1]heptan-1-yl]propan-2-ol<br>(without stereochemistry)<br><br>2-[(3S)-4-methyl-3-[(2-methyl-4-trimethylsilyloxy-phenyl)methoxy]-7-oxabicyclo[2.2.1]heptan-1-yl]propan-2-ol<br>(with stereochemistry)                                   |                              | 1980s goat                        |
| SD 214014                                    | 2-[3-[[2-(hydroxymethyl)phenyl]methoxy]-4-methyl-7-oxabicyclo[2.2.1]heptan-1-yl]propan-1-ol<br>(without stereochemistry)<br><br>2-[(3S)-3-[[2-(hydroxymethyl)phenyl]methoxy]-4-methyl-7-oxabicyclo[2.2.1]heptan-1-yl]propan-1-ol<br>(with stereochemistry)   |                             | 1980s goat                        |
| SD 213324                                    | [4-isopropyl-1-methyl-6-[[2-(trimethylsilyloxymethyl)phenyl]methoxy]-7-oxabicyclo[2.2.1]heptan-2-yl]oxy-trimethyl-silane<br>(without stereochemistry)<br><br>[(6S)-4-isopropyl-1-methyl-6-[[2-(trimethylsilyloxymethyl)phenyl]methoxy]-7-oxabicyclo[2.2.1]heptan-2-yl]oxy-trimethyl-silane<br>(with stereochemistry) |                             | 1980s goat                        |

| Code Number<br>(Reg. Number)<br>(SD/WL-Code) | Chemical Name   | Molecular Structure<br>(Only a single enantiomer/diastereomer is shown<br>for metabolites with stereochemistry) | Occurrence in<br>residues studies |
|--|---|---|-----------------------------------|
| SD 214013                                    | 2-[4-methyl-3-(o-tolylmethoxy)-7-oxabicyclo[2.2.1]heptan-1-yl]propane-1,2-diol<br>(without stereochemistry)<br><br>2-[(3S)-4-methyl-3-(o-tolylmethoxy)-7-oxabicyclo[2.2.1]heptan-1-yl]propane-1,2-diol<br>(with stereochemistry)  |                               | 1980s goat                        |
| SD 214012                                    | trimethyl-[[1-methyl-4-(1-methyl-1-trimethylsilyloxy-ethyl)-6-[[2-(trimethylsilyloxymethyl)phenyl]methoxy]-7-oxabicyclo[2.2.1]heptan-2-yl]oxy]silane<br>(without stereochemistry)<br><br>trimethyl-[[[(6S)-1-methyl-4-(1-methyl-1-trimethylsilyloxy-ethyl)-6-[[2-(trimethylsilyloxymethyl)phenyl]methoxy]-7-oxabicyclo[2.2.1]heptan-2-yl]oxy]silane<br>(with stereochemistry) |                              | 1980s goat                        |
| SD 214011                                    | trimethyl-[[2-[[1-methyl-4-[1-methyl-1,2-bis(trimethylsilyloxy)ethyl]-7-oxabicyclo[2.2.1]heptan-2-yl]oxymethyl]phenyl]methoxy]silane<br>(without stereochemistry)<br><br>trimethyl-[[2-[[[(2S)-1-methyl-4-[1-methyl-1,2-bis(trimethylsilyloxy)ethyl]-7-oxabicyclo[2.2.1]heptan-2-yl]oxymethyl]phenyl]methoxy]silane<br>(with stereochemistry)                                 |                             | 1980s goat                        |

| Code Number<br>(Reg. Number)<br>(SD/WL-Code) | Chemical Name   | Molecular Structure<br>(Only a single enantiomer/diastereomer is shown<br>for metabolites with stereochemistry) | Occurrence in<br>residues studies                              |
|--|---|---|--|
| SD 214010                                    | 2-[3-[[2-(hydroxymethyl)phenyl]methoxy]-<br>4-methyl-7-oxabicyclo[2.2.1]heptan-1-<br>yl]prop-2-en-1-ol<br>(without stereochemistry)<br><br>2-[(3S)-3-[[2-<br>(hydroxymethyl)phenyl]methoxy]-4-<br>methyl-7-oxabicyclo[2.2.1]heptan-1-<br>yl]prop-2-en-1-ol<br>(with stereochemistry)  |   | 1980s goat   |
| SD 214009                                    | trimethylsilyl 2-[(4-isopropyl-1-methyl-6-<br>trimethylsilyloxy-7-<br>oxabicyclo[2.2.1]heptan-2-<br>yl)oxymethyl]benzoate<br>(without stereochemistry)<br><br>trimethylsilyl 2-[[2S)-4-isopropyl-1-<br>methyl-6-trimethylsilyloxy-7-<br>oxabicyclo[2.2.1]heptan-2-<br>yl]oxymethyl]benzoate<br>(with stereochemistry)   |   | 1980s goat   |
| SD 214008                                    | trimethylsilyl 2-[[1-methyl-4-(1-methyl-1-<br>trimethylsilyloxy-ethyl)-6-<br>trimethylsilyloxy-7-<br>oxabicyclo[2.2.1]heptan-2-<br>yl]oxymethyl]benzoate<br>(without stereochemistry)<br><br>trimethylsilyl 2-[[2S)-1-methyl-4-(1-<br>methyl-1-trimethylsilyloxy-ethyl)-6-<br>trimethylsilyloxy-7-<br>oxabicyclo[2.2.1]heptan-2-<br>yl]oxymethyl]benzoate<br>(with stereochemistry) |   | 1980s goat   |
| Reg. No. 545654                              | 2-Benzofuran-1,3-dione  |   | Reference item<br>used but not<br>found in<br>rotational crops |

| Code Number<br>(Reg. Number)<br>(SD/WL-Code) | Chemical Name   | Molecular Structure<br>(Only a single enantiomer/diastereomer is shown<br>for metabolites with stereochemistry) | Occurrence in<br>residues studies  |
|--|---|---|--|
| Reg. No. 4108046                             | 1,2-Xylene  |                                | Reference item<br>used but not<br>found in<br>rotational crops                                   |
| Reg. No. 6059084                             | 2-[(1RS,3RS,4SR)-3-{[2-(hydroxymethyl)phenyl]methoxy}-4-methyl-7-oxabicyclo[2.2.1]heptan-1-yl]propan-1-ol |                               | Reference item<br>used but not<br>found in oilseed<br>rape and carrot<br>cleavage<br>experiments |

### B.7.1. STORAGE STABILITY OF RESIDUES

Studies on storage stability of parent BAS 684 H and its main plant metabolite M684H005 are summarised below. No separate investigation on the storage stability of the other major plant metabolite M684H006 has been performed as the metabolite is determined as its precursor M684H005 in the residue analytical method.

No data on storage stability are needed for animal matrices as no livestock feeding studies were required and none conducted. (see section 7.4).

**Report:** CA 6.1/1  
Spangler C., 2018 a  
Investigation of the storage stability of BAS 684 H in plant matrices  
2016/1029128

**Guidelines:** OECD 506, EPA 860.1380, EEC 7032/VI/97 rev. 5

**GLP:** yes

A storage stability study at  $\leq -18^\circ\text{C}$  was carried out with BAS 684 H in plant matrices. No specific details have been provided on the form of the samples, the study report states that samples are handled in the same way as routine residue samples. For magnitude of residues studies samples are shipped as whole commodities under deep frozen conditions, then the specimens are homogenized using dry ice and the resulting homogenate stored deep frozen until analysis. The applicant provided the following additional information:

*The samples used for the storage stability studies are generally obtained from different sources (e.g. local supermarkets). The samples are deep frozen ( $\leq -18^\circ\text{C}$ ) and then homogenized at the specimen management unit according to BASF internal standard operating procedures (SOPs). The SOPs which are followed for samples used for storage stability studies are the same as those used for samples for residue studies. The frozen samples are homogenized under dry ice and the resulting samples are stored under frozen conditions ( $\sim \leq 18^\circ\text{C}$ ) in the dark until further used for storage stability studies.*

This information is considered acceptable, the storage stability studies are conducted in a similar way to the magnitude of residues studies, therefore no further information is required.

The test compound was added to untreated sample matrices at a level of 0.10 mg/kg. The samples were kept in PP-containers at  $\leq -18^\circ\text{C}$  in the dark for up to 24 months. After time intervals of 0, 1, 3, 6, 9, 12, 18 and 24 months ( $0, 30 \pm 2, 90 \pm 5, 180 \pm 7, 280 \pm 7, 365 \pm 7, 545 \pm 7$  and  $730 \pm 7$  days), samples were removed from storage and analysed for BAS 684 H.

Specimens were analysed with BASF method No L0337/01 which allows the quantitation of BAS 684 H residues to a limit of quantitation of 0.01 mg/kg in all matrices. Method validation data (presented in Section B5) are available for the same matrices as tested in this storage stability study.

For matrix oilseed rape seed, another earlier version of BASF method No L0337/01 was used for storage intervals of 9-24 months ( $280 \pm 7$  days,  $365 \pm 7$  days,  $545 \pm 7$  days and  $730 \pm 7$  days) because at the time of sample weigh-in for these time intervals and the spare samples method L0337/01 was still under development and a different sample weigh-in and partly different clean-up procedure was intended to be used. This version of BASF method No L0337/01 was validated within the storage stability study. A summary of results of the validation is shown in Section B5 (Analytical Methods). Both versions of the method are fully validated in accordance with SANCO 3029/99 rev. 4 with an LOQ of 0.01 mg/kg.

The non-corrected results of the freezer storage stability of BAS 684 H from plant matrices after the various storage periods are summarised in Table 7-2. The procedural recoveries of the freshly fortified untreated samples are also reported to demonstrate the effectiveness of the method at the time of analysis. Only one recovery has been completed at each fortification level therefore mean values have not been calculated. As all the procedural recoveries are within the range 70 – 110 % at both the LOQ and 10 x LOQ (with the exception of oilseed rape seed at the 9-month time point) and the method is fully validated in accordance with SANCO 3029/99 rev.4 no additional data are required.

**Table 7-2 Stability of BAS 684 H residues in plant matrices following storage at  $\leq -18$  °C**

| Matrix   | Storage period (months) | Residue level in freezer storage stability sample (mg/kg) | Residue level in freezer storage stability sample (% of fortification level) | Spiking level (mg/kg) and procedural recovery for freshly spiked sample (%) |              |
|--|-------------------------|---|--|---|--------------|
| Barley whole plant without roots<br>(high water content) | 0                       | 0.090, 0.092  | 90.3, 91.8 (91.0)  | 0.01<br>0.1   | 75.3<br>97.0 |
|  | 1                       | 0.098, 0.093  | 97.8, 93.0 (95.4)  | 0.01<br>0.1   | 94.0<br>96.8 |
|  | 3                       | 0.095, 0.096  | 94.8, 95.8 (95.3)  | 0.01<br>0.1   | 103<br>106   |
|  | 6                       | 0.089, 0.088  | 89.3, 87.8 (88.5)  | 0.01<br>0.1   | 100<br>90.7  |
|  | 12                      | 0.089, 0.083  | 88.5, 83.3 (85.9)  | 0.01<br>0.1   | 88.6<br>87.8 |
|  | 18                      | 0.091, 0.092  | 91.0, 91.8 (91.4)  | 0.01<br>0.1   | 85.1<br>89.4 |
|  | 24                      | 0.086, 0.086  | 86.0, 85.8 (85.9)  | 0.01<br>0.1   | 84.3<br>90.8 |
| Bean pods with seeds<br>(high water content)             | 0                       | 0.083, 0.082  | 83.0, 82.3 (82.6)  | 0.01<br>0.1   | 90.3<br>81.8 |
|  | 1                       | 0.084, 0.083  | 84.3, 82.8 (83.5)  | 0.01<br>0.1   | 90.5<br>88.8 |
|  | 3                       | 0.083, 0.086  | 83.0, 85.8 (84.4)  | 0.01<br>0.1   | 96.2<br>94.4 |
|  | 6                       | 0.079, 0.083  | 79.3, 82.5 (80.9)  | 0.01<br>0.1   | 90.9<br>85.9 |
|  | 12                      | 0.082, 0.086  | 81.8, 86.0 (83.9)  | 0.01<br>0.1   | 82.3<br>91.1 |
|  | 18                      | 0.079, 0.078  | 78.5, 77.8 (78.1)  | 0.01<br>0.1   | 81.8<br>79.5 |
|  | 24                      | 0.083, 0.081  | 82.5, 81.3 (81.9)  | 0.01<br>0.1   | 83.3<br>84.3 |
| Oilseed rape seed<br>(high oil content)                  | 0                       | 0.066, 0.076  | 65.5, 75.5 (70.5)  | 0.01<br>0.1   | 70.3<br>72.8 |
|  | 1                       | 0.078, 0.077  | 77.5, 77.0 (77.3)  | 0.01<br>0.1   | 80.0<br>77.3 |
|  | 3                       | 0.075, 0.074  | 75.5, 74.2 (74.9)  | 0.01<br>0.1   | 77.5<br>75.5 |
|  | 6                       | 0.084, 0.079  | 84.3, 79.5 (81.9)  | 0.01<br>0.1   | 88.3<br>87.8 |

|   |    |              |                   |             |              |
|---|----|--------------|-------------------|-------------|--------------|
|   | 9  | 0.067, 0.067 | 66.6, 66.6 (66.6) | 0.01<br>0.1 | 68.2<br>68.8 |
|   | 12 | 0.077, 0.074 | 76.9, 74.1 (75.5) | 0.01<br>0.1 | 78.5<br>83.5 |
|   | 18 | 0.071, 0.071 | 70.9, 71.3 (71.1) | 0.01<br>0.1 | 87.4<br>72.9 |
|   | 24 | 0.071, 0.078 | 70.6, 78.4 (74.5) | 0.01<br>0.1 | 77.5<br>77.8 |
| Bean dried seed<br><br>(high protein content) | 0  | 0.089, 0.086 | 88.5, 85.8 (87.1) | 0.01<br>0.1 | 85.5<br>85.0 |
|   | 1  | 0.075, 0.086 | 75.3, 85.8 (80.5) | 0.01<br>0.1 | 84.3<br>85.5 |
|   | 3  | 0.086, 0.084 | 86.3, 84.3 (85.3) | 0.01<br>0.1 | 78.3<br>87.1 |
|   | 6  | 0.087, 0.089 | 87.3, 88.5 (87.9) | 0.01<br>0.1 | 110<br>96.7  |
|   | 12 | 0.086, 0.093 | 86.3, 92.8 (89.5) | 0.01<br>0.1 | 94.8<br>95.6 |
|   | 18 | 0.089, 0.085 | 88.8, 84.8 (86.8) | 0.01<br>0.1 | 85.4<br>87.9 |
|   | 24 | 0.081, 0.085 | 80.8, 84.5 (82.6) | 0.01<br>0.1 | 79.0<br>86.3 |
| Wheat grain<br><br>(high starch content)      | 0  | 0.087, 0.092 | 87.3, 91.8 (89.5) | 0.01<br>0.1 | 92.3<br>91.0 |
|   | 1  | 0.098, 0.094 | 98.3, 93.8 (96.0) | 0.01<br>0.1 | 95.0<br>101  |
|   | 3  | 0.087, 0.087 | 87.3, 86.5 (86.9) | 0.01<br>0.1 | 92.9<br>92.9 |
|   | 6  | 0.095, 0.086 | 95.0, 85.8 (90.4) | 0.01<br>0.1 | 94.2<br>98.5 |
|   | 12 | 0.094, 0.095 | 93.5, 95.3 (94.4) | 0.01<br>0.1 | 101<br>98.1  |
|   | 18 | 0.088, 0.091 | 87.8, 91.0 (89.4) | 0.01<br>0.1 | 89.4<br>87.4 |
|   | 24 | 0.090, 0.086 | 89.5, 85.8 (87.6) | 0.01<br>0.1 | 84.5<br>85.3 |
| Grapes<br><br>(high acid content)             | 0  | 0.083, 0.083 | 82.5, 82.5 (82.5) | 0.01<br>0.1 | 87.3<br>80.0 |
|   | 1  | 0.080, 0.084 | 79.5, 83.8 (81.6) | 0.01<br>0.1 | 91.5<br>86.0 |
|   | 3  | 0.080, 0.083 | 79.5, 83.0 (81.3) | 0.01<br>0.1 | 85.1<br>87.1 |
|   | 9  | 0.073, 0.073 | 73.0, 72.5 (72.8) | 0.01<br>0.1 | 82.8<br>79.5 |
|   | 12 | 0.087, 0.087 | 86.8, 87.0 (86.9) | 0.01<br>0.1 | 92.3<br>90.3 |
|   | 18 | 0.077, 0.078 | 77.3, 78.0 (77.6) | 0.01<br>0.1 | 81.8<br>86.9 |
|   | 24 | 0.081, 0.081 | 80.5, 80.8 (80.6) | 0.01<br>0.1 | 79.3<br>84.5 |
| Wheat straw<br><br>(no specified group)       | 0  | 0.096, 0.096 | 96.3, 95.6 (95.9) | 0.01<br>0.1 | 104<br>101   |
|   | 1  | 0.097, 0.101 | 96.9, 101 (99.1)  | 0.01<br>0.1 | 105<br>100   |
|   | 3  | 0.092, 0.090 | 91.6, 90.3 (90.9) | 0.01<br>0.1 | 100<br>101   |
|   | 9  | 0.088, 0.088 | 87.8, 87.5 (87.7) | 0.01<br>0.1 | 84.0<br>90.0 |

|  |    |              |                   |             |              |
|--|----|--------------|-------------------|-------------|--------------|
|  | 12 | 0.10, 0.098  | 105, 97.5 (101)   | 0.01<br>0.1 | 99.1<br>102  |
|  | 18 | 0.099, 0.098 | 99.4, 97.8 (98.6) | 0.01<br>0.1 | 78.6<br>81.8 |
|  | 24 | 0.088, 0.088 | 87.5, 88.1 (87.8) | 0.01<br>0.1 | 90.6<br>88.8 |

Residues of BAS 684 H are considered stable in all tested plant matrices for 24 months under frozen conditions ( $\leq -18^{\circ}\text{C}$ ). This accommodates the time period that the samples are stored for in the supporting residue trials (see section 7.3 for further details, maximum of 502 days). As residues of BAS 684 H have been shown to be stable in all five commodity categories (high water, high oil, high protein, high starch and high acid) it can be assumed that BAS 684 H residues are stable in all other commodities for the same duration of time under the same storage conditions (24 months at  $\leq -18^{\circ}\text{C}$ ).

#### *Stability of extracts*

No specific information is provided within the storage stability study report relating to the storage of extracts, however within the method validation it is stated that sample solutions are stable for 7 days when stored refrigerated at  $4^{\circ}\text{C}$  in the dark. The time period encompasses almost all extract storage periods within the magnitude of residues trials (see Section 7.3 for full details, approximately 0 – 7 days across all samples) except for the studies Ale 2017a and Klimek, 2018b where extracts were stored for a maximum of 14 days and 22 days respectively. Within these studies the stability of the analyte in final volume solutions was proven by procedural recovery samples which were stored for the same period of time between extraction and LC-MS/MS analysis therefore this is considered acceptable and no further data are required at this time.

**Report:** CA 6.1/2  
Eilers B., 2020  
Investigation of the storage stability of M684H005 in plant matrices  
2020/2005975

**Guidelines:** OECD 506 (Oct. 2007), EPA 860.1380, EEC 7032/VI/97 rev. 5

**GLP:** yes

A storage stability study at  $\leq -18^{\circ}\text{C}$  was carried out with M684H005 in plant matrices. No specific details have been provided on the form of the samples, the study report states that samples are handled in the same way as routine residue samples. For magnitude of residues studies samples are shipped as whole commodities under deep frozen conditions, then the specimens are homogenized using dry ice and the resulting homogenate stored deep frozen until analysis. The applicant provided the following additional information:

*The samples used for the storage stability studies are generally obtained from different sources (e.g. local supermarkets). The samples are deep frozen ( $\leq -18^{\circ}\text{C}$ ) and then homogenized at the specimen management unit according to BASF internal standard operating procedures (SOPs). The SOPs which are followed for samples used for storage stability studies are the same as those used for samples for residue studies. The frozen samples are homogenized under dry ice and the resulting samples are stored under frozen conditions ( $- \leq 18^{\circ}\text{C}$ ) in the dark until further used for storage stability studies.*

This information is considered acceptable, the storage stability studies are conducted in a similar way to the magnitude of residues studies, therefore no further information is required.

The test compound was added to untreated sample matrices at a level of 0.1 mg/kg. The samples are planned to be kept in PP-containers at  $\leq -18^{\circ}\text{C}$  in the dark for a period of 32 months. After time intervals of 0, 1, 3, 6, 12, 18, 24 and 32 months ( $0, 30 \pm 4, 90 \pm 5, 180 \pm 4, 365 \pm 5, 545 \pm 5, 730 \pm 5$  and  $970 \pm 5$  days), samples were removed from storage and analysed for M684H005.

Specimens were analysed with BASF method No L0337/02 which allows the quantitation of M684H005 residues to a limit of quantitation 0.01 mg/kg in all matrices. It is noted that the method validation data are presented for wheat whole plant, wheat straw, wheat grain, sunflower seeds, citrus fruit, bean dried seed and lettuce heads. These are not the same matrices as analysed in the storage stability study, no data have been provided for rape seeds, grapes or kale. However, as commodities have been tested from the same matrix groups, no matrix effects have been noted, procedural recoveries are acceptable at LOQ and 10 x LOQ and the study report states no residues of the analyte at or above the limit of quantitation were detected which proves that no interferences of the specimen material with the analytical procedure occurred, the data provided are



considered acceptable. The full method validation data are presented in Section B5 (Analytical Methods) and the method is fully validated in accordance with SANCO 3029/99 rev. 4 with an LOQ of 0.01 mg/kg.

Unlike the usual procedure for storage stability studies, this study had to be started before the development of the analytical method was finished. Therefore, the specimens which were to be stored over longer intervals were prepared and stored first. The specimens for the short intervals were only prepared when the method was available.

The non-corrected results of the freezer storage stability of M684H005 from plant matrices after the various storage periods are summarised in Table 7-3. The procedural recoveries of the freshly fortified untreated samples are also reported to demonstrate the effectiveness of the method at the time of analysis. Only one recovery has been completed at each fortification level therefore mean values have not been calculated. As all the procedural recoveries are within the range 70 – 110 % at both the LOQ and 10 x LOQ (with the exception of wheat grain spiked with 0.01 mg/kg M648H005 at the 6 month time point) and the method is fully validated in accordance with SANCO 3029/99 rev.4 no additional data are required.

Table 7-3 Stability of M684H005 residues in plant matrices following storage at  $\leq -18^{\circ}\text{C}$

| Matrix  | Storage period (months) | Residue level in freezer storage stability sample (mg/kg) | Residue level in freezer storage stability sample (% of fortification level) | Spiking level (mg/kg) and procedural recovery for freshly spiked sample (%) |              |
|---|-------------------------|---|--|---|--------------|
| Wheat whole plant without roots<br>(high water content) | 0                       | 0.090, 0.086, 0.086, 0.084, 0.090                         | 89.6, 86.0, 86.4, 83.6, 90.4 (87.2)  | 0.01<br>0.1   | 82.8<br>87.6 |
|   | 1                       | 0.086, 0.092  | 85.6, 92.0 (88.8)  | 0.01<br>0.1   | 85.2<br>86.4 |
|   | 3                       | 0.095, 0.099  | 95.8, 99.8 (97.8)  | 0.01<br>0.1   | 108<br>97.2  |
|   | 6                       | 0.084, 0.083  | 84.9, 83.7 (84.3)  | 0.01<br>0.1   | 85.6<br>88.4 |
|   | 12                      | 0.091, 0.091  | 91.8, 91.8 (91.8)  | 0.01<br>0.1   | 92.7<br>93.5 |
|   | 18                      | 0.088, 0.089  | 88.1, 89.8 (89.0)  | 0.01<br>0.1   | 94.3<br>91.5 |
|   | 24                      | 0.089, 0.088  | 89.8, 88.5 (89.2)  | 0.01<br>0.1   | 91.2<br>93.6 |
|   | 32                      | 0.090, 0.093  | 90.2, 93.8 (92.0)  | 0.01<br>0.1   | 90.8<br>94.4 |
| Wheat straw<br>(no specified group)                     | 0                       | 0.082, 0.078, 0.080, 0.078, 0.080                         | 81.5, 78.2, 79.5, 77.7, 80.2 (79.4)  | 0.01<br>0.1   | 85.2<br>78.5 |
|   | 1                       | 0.076, 0.078  | 75.5, 77.5 (76.5)  | 0.01<br>0.1   | 82.8<br>76.5 |
|   | 3                       | 0.077, 0.074  | 77.7, 74.2 (76.0)  | 0.01<br>0.1   | 83.0<br>80.8 |
|   | 6                       | 0.072, 0.075  | 71.9, 75.0 (73.5)  | 0.01<br>0.1   | 81.8<br>83.3 |
|   | 12                      | 0.071, 0.076  | 71.4, 76.2 (73.8)  | 0.01<br>0.1   | 83.9<br>83.2 |
|   | 18                      | 0.071, 0.071  | 71.2, 71.2 (71.2)  | 0.01<br>0.1   | 83.4<br>77.7 |
|   | 24                      | 0.071, 0.069  | 71.4, 69.2 (70.3)  | 0.01<br>0.1   | 84.2<br>81.7 |
|   | 32                      | 0.069, 0.067  | 69.4, 67.7 (68.5)  | 0.01<br>0.1   | 88.7<br>88.0 |
| Wheat grain<br>(high starch content)                    | 0                       | 0.076, 0.084, 0.074, 0.079, 0.085                         | 75.6, 84.4, 74.4, 78.8, 85.2 (79.7)  | 0.01<br>0.1   | 75.6<br>78.0 |
|   | 1                       | 0.082, 0.084  | 81.6, 84.0 (82.8)  | 0.01<br>0.1   | 81.6<br>86.8 |
|   | 3                       | 0.078, 0.088  | 78.9, 88.5 (83.7)  | 0.01  | 88.0         |

|   |    |                                      |  |             |              |
|---|----|--------------------------------------|--|-------------|--------------|
|   |    |                                      |  | 0.1         | 87.2         |
|   | 6  | 0.073, 0.075                         | 73.3, 75.3 (74.3)                      | 0.01<br>0.1 | 68.0<br>70.4 |
|   | 12 | 0.079, 0.080                         | 79.3, 80.9 (80.1)                      | 0.01<br>0.1 | 78.7<br>83.1 |
|   | 18 | 0.080, 0.079                         | 80.1, 79.7 (79.9)                      | 0.01<br>0.1 | 77.1<br>79.5 |
|   | 24 | 0.085, 0.084                         | 85.3, 84.9 (85.1)                      | 0.01<br>0.1 | 82.8<br>79.2 |
|   | 32 | 0.084, 0.090                         | 84.1, 90.6 (87.3)                      | 0.01<br>0.1 | 77.6<br>87.6 |
| Rape seed<br>(high oil<br>content)                      | 0  | 0.096, 0.088, 0.088,<br>0.10, 0.095  | 95.6, 88.4, 88.0, 100,<br>94.8 (93.3)  | 0.01<br>0.1 | 92.0<br>86.4 |
|   | 1  | 0.083, 0.089                         | 82.8, 89.2 (86.0)                      | 0.01<br>0.1 | 86.4<br>86.0 |
|   | 3  | 0.083, 0.082                         | 82.8, 82.0 (82.4)                      | 0.01<br>0.1 | 96.1<br>84.4 |
|   | 6  | 0.089, 0.085                         | 89.2, 85.2 (87.2)                      | 0.01<br>0.1 | 87.2<br>82.8 |
|   | 12 | 0.088, 0.092                         | 88.4, 92.4 (90.4)                      | 0.01<br>0.1 | 90.4<br>90.8 |
|   | 18 | 0.107, 0.104                         | 107, 104 (105.5)                       | 0.01<br>0.1 | 103<br>101   |
|   | 24 | 0.097, 0.103                         | 96.9, 103 (100.0)                      | 0.01<br>0.1 | 95.2<br>96.8 |
|   | 32 | 0.088, 0.091                         | 88.0, 91.2 (89.6)                      | 0.01<br>0.1 | 86.8<br>84.0 |
| Grapes<br>(high acid<br>content)                        | 0  | 0.080, 0.076, 0.079,<br>0.077, 0.075 | 80.0, 76.4, 78.8, 77.2,<br>75.2 (77.5) | 0.01<br>0.1 | 81.2<br>76.0 |
|   | 1  | 0.074, 0.073                         | 73.6, 72.8 (73.2)                      | 0.01<br>0.1 | 72.0<br>71.2 |
|   | 3  | 0.076, 0.073                         | 76.5, 73.3 (74.9)                      | 0.01<br>0.1 | 90.4<br>82.0 |
|   | 6  | 0.080, 0.078                         | 80.5, 78.1 (79.3)                      | 0.01<br>0.1 | 81.2<br>79.2 |
|   | 12 | 0.080, 0.079                         | 80.5, 79.3 (79.9)                      | 0.01<br>0.1 | 80.4<br>83.2 |
|   | 18 | 0.083, 0.085                         | 83.7, 85.7 (84.7)                      | 0.01<br>0.1 | 85.9<br>85.1 |
|   | 24 | 0.087, 0.087                         | 87.7, 87.7 (87.7)                      | 0.01<br>0.1 | 81.6<br>82.8 |
|   | 32 | 0.087, 0.085                         | 87.3, 85.3 (86.3)                      | 0.01<br>0.1 | 84.8<br>89.2 |
| Bean, dried<br>seed<br><br>(high<br>protein<br>content) | 0  | 0.080, 0.081, 0.081,<br>0.079, 0.082 | 80.0, 81.2, 80.8, 78.8,<br>81.6 (80.5) | 0.01<br>0.1 | 81.2<br>77.2 |
|   | 1  | 0.078, 0.086                         | 78.4, 85.6 (82.0)                      | 0.01<br>0.1 | 80.4<br>86.8 |
|   | 3  | 0.084, 0.078                         | 84.0, 78.4 (81.2)                      | 0.01<br>0.1 | 89.3<br>85.2 |
|   | 6  | 0.076, 0.076                         | 76.4, 76.4 (76.4)                      | 0.01<br>0.1 | 85.6<br>86.4 |
|   | 12 | 0.081, 0.084                         | 81.2, 84.0 (82.6)                      | 0.01<br>0.1 | 84.8<br>85.6 |
|   | 18 | 0.086, 0.086                         | 85.6, 86.4 (86.0)                      | 0.01<br>0.1 | 82.3<br>82.7 |
|   | 24 | 0.095, 0.091                         | 95.3, 90.8 (93.0)                      | 0.01<br>0.1 | 99.2<br>88.8 |
|   | 32 | 0.097, 0.098                         | 97.3, 97.7 (97.5)                      | 0.01        | 92.4         |

|  |    |                                      |  |             |              |
|--|----|--------------------------------------|--|-------------|--------------|
|  |    |                                      |  | 0.1         | 88.0         |
| Kale (curly)<br>whole plant<br>no roots<br><br>(high water<br>content) | 0  | 0.075, 0.086, 0.091,<br>0.088, 0.085 | 75.2, 86.4, 91.2, 87.6,<br>84.8 (85.0) | 0.01<br>0.1 | 88.4<br>83.2 |
|  | 1  | 0.090, 0.082                         | 90.4, 82.0 (86.2)                      | 0.01<br>0.1 | 90.0<br>80.0 |
|  | 3  | 0.080, 0.082                         | 80.4, 81.6 (81.0)                      | 0.01<br>0.1 | 93.7<br>80.4 |
|  | 6  | 0.076, 0.079                         | 75.6, 79.2 (77.4)                      | 0.01<br>0.1 | 80.8<br>79.6 |
|  | 12 | 0.075, 0.074                         | 74.8, 74.0 (74.4)                      | 0.01<br>0.1 | 82.4<br>86.0 |
|  | 18 | 0.071, 0.071                         | 70.8, 71.2 (71.0)                      | 0.01<br>0.1 | 86.3<br>85.5 |
|  | 24 | 0.064, 0.065                         | 64.0, 65.2 (64.6)                      | 0.01<br>0.1 | 87.2<br>84.0 |
|  | 32 | 0.055, 0.056                         | 55.2, 56.4 (55.8)                      | 0.01<br>0.1 | 91.2<br>90.0 |

Residues of M684H005 are considered stable in wheat whole plant without roots, wheat grain, rape seed, grapes and bean dried seed for 32 months under frozen conditions. For the matrices wheat straw and kale whole plant without roots a decline in the residues of M684H005 is observed over the 32 month period. In wheat straw this decline is from 79.4 – 68.5 % and in kale whole plant without roots 85.0 – 55.8 %.

It can be concluded that residues of M684H005 are considered sufficiently stable in wheat straw for 32 months as the decline observed is only slightly over 10 %. The low value at day 0 of 79.4 % could be attributed to the analytical method as the procedural recoveries are also low; within a range of 76.5 – 88.7 % over the whole study period.

It can be concluded that residues of M684H005 are considered just sufficiently stable in kale whole plant without roots for 24 months, within this period the observed decline was from 85.0 – 64.6 % (absolute decline was 20.4 %). The slightly low value at day 0 of 85.0 % could be attributed to the analytical method as the procedural recoveries are within a similar range of 93.7 – 80.0 % over the period of the study. As the procedural recoveries are within a similar range it is considered appropriate to compare the decrease to the time zero (i.e. 85.0 %) rather than 100 %. A significant decline from 64.6 – 55.8 % is observed between 24 and 32 months therefore M684H005 is not considered stable in kale whole plant without roots for 32 months. To further support the stability for 24 months in kale it is noted that for wheat whole plant without roots, which is also considered a high-water commodity, residue levels are stable of 32 months frozen storage. It is also recommended that for a future use on high-water commodities (especially leafy commodities similar to kale) it is preferable that samples are analysed before 24 months due to some observed degradation being recorded.

As residues of M684H005 have been shown to be stable in all five commodity categories (high water, high oil, high protein, high starch and high acid) it can be assumed that M684H005 residues are stable in all other commodities for the same duration of time under the same storage conditions (24 months at  $\leq -18^{\circ}\text{C}$ ).

This 24 month period accommodates the time period that the majority of samples are stored for in the supporting residue trials (see Section 7.3 for further details, range of 274 – 872 days), the exception being the trials in CA 6.3.2/1, Ale 2017a where the samples were stored for a maximum of 872 days before analysis of M684H005 and M684H006. However, as these residue trials are in wheat, and acceptable storage stability has been shown in wheat whole plant, wheat grain and wheat straw for 32 month (970 days) it is considered acceptable to rely on these data. This is further discussed in Section 7.3.

It is considered appropriate that storage stability data have only been provided for the metabolite M684H005. In an ideal situation storage stability data would have been provided for metabolite M684H006 however, conclusions can be made based on the whole data package. The metabolite M684H005 is the precursor to M684H006. This is supported by the plant metabolism studies (see proposed pathway diagrams) and the *in-vitro* livestock studies where both metabolites M684H005 and M684H006 are rapidly cleaved to form M684H002 under physiological conditions. Also, as the content of M684H006 is determined as M684H005 no additional data are required at this time to support the storage stability of both metabolites.

*Stability of extracts*

No specific information is provided within the storage stability study report relating to the storage of extracts, however within the method validation it is stated that the differences in recoveries between days 0 and 7 (0 and 8 for sunflower seeds) were all below 20 % for all matrices stored in the dark at  $5 \pm 3$  °C indicating these analytes are stable in final volumes for at least 7 days. The time period encompasses almost all extract storage periods within the magnitude of residues trials (see Section 7.3 for full details, approximately 0 – 7 days across all samples) except for the study Klimek, 2018b where extracts from whole plant without roots were stored for a maximum of 22 days. As this matrix is not considered further within the risk assessment, and within this study the oilseed rape seed extracts were stored for < 7 days this is considered acceptable and no further data are required at this time.

**B.7.2. METABOLISM, DISTRIBUTION AND EXPRESSION OF RESIDUES**

The applicant submitted studies investigating the metabolism of BAS 684 H in plant (wheat, oilseed rape and carrot) and in animal (poultry, goat and fish) matrices which are compliant with current OECD guidelines. Additionally, the applicant provided studies performed in the 1980s on metabolism in plants (soybean, peanut and rice) and in animals (goats), the majority of which have not been performed under GLP and have major deviations to current OECD guidelines (see conclusions).

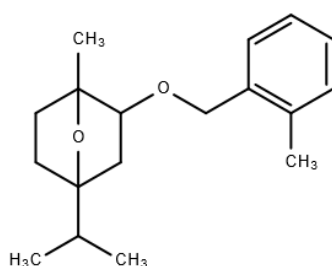
The BAS 684 H molecule is a two-ring structure, as shown in Figure 7-1. It consists of a cyclohexane ring and a phenyl ring which are connected by an ether bridge.

The test items were a mixture of  $^{14}\text{C}$ -BAS 684 H,  $^{13}\text{C}$ -BAS 684 H and unlabelled BAS 684 H. The molecular structures and the positions of the labels are shown in Figure 7-2.

The metabolism and distribution of BAS 684 H in plants and livestock was investigated using the active substance radiolabelled in the cyclohexane ring or in the phenyl ring. These labelling positions are considered appropriate to provide sufficient information.

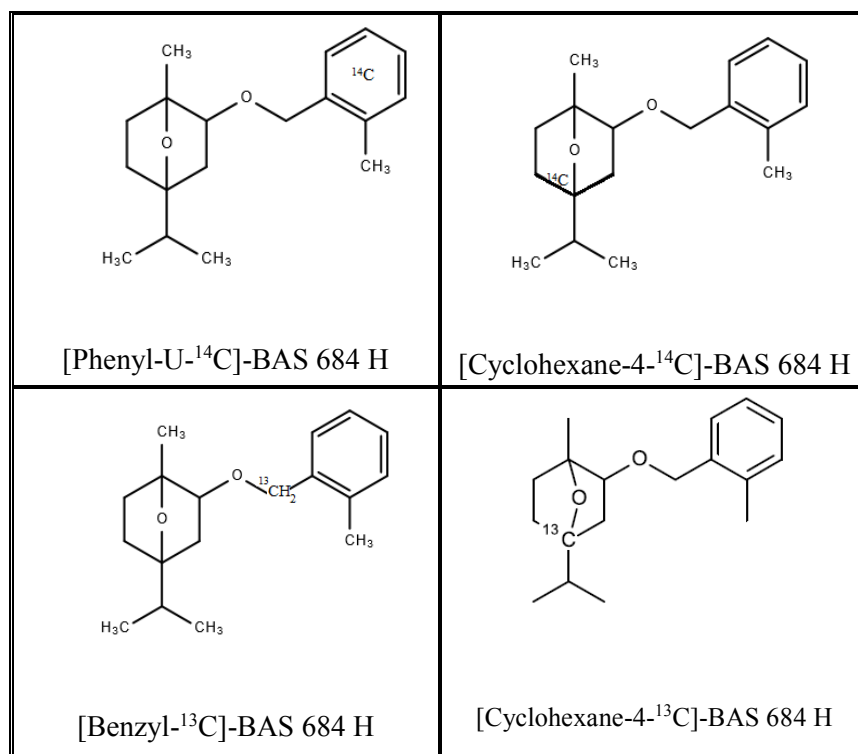
Unless otherwise stated, % TRR values are calculated using the TRR calculated as the sum of ERR (extractable radioactive residue) and RRR (residual radioactive residue) after extraction of the residues (TRR calculated). Significant differences between the TRR calculated and the TRR measured by combustion prior to extraction (TRR measured) have been considered in further detail where these have occurred.

Figure 7-1 Structure of non-radiolabelled BAS 684 H



(1RS, 2R, 4SR)-1,4-epoxy-p-menth-2-yl 2-methylbenzyl-ether

Figure 7-2 Structures of radiolabelled BAS 684 H



### B.7.2.1. Plants

#### B.7.2.1.1. Wheat

|                    |  |
|--------------------|--|
| <b>Report:</b>     | CA 6.2.1/001<br>Rosenbaum-Stieber C., 2018<br>Metabolism of <sup>14</sup> C-BAS 684 H in wheat<br>741150<br>2017/1004405   |
| <b>Guidelines:</b> | EPA 860.1000, EPA 860.1300: Nature of the Residue in Plants Livestock, PMRA Residue Chemistry Guidelines Section 97.2 Nature of the Residue - Plants - Livestock (Canada), EEC 7028/VI/95 rev. 3 Appendix A (EU): Metabolism and distribution in plants, JMAFF 59<br>NohSan No 4200, Test No. 501: Metabolism in crops |
| <b>GLP:</b>        | yes  |

#### Materials and methods

##### Materials

##### 1. C-label BAS 684 H (CAS No. 87818-31-3)

|                              |  |
|------------------------------|--|
| <b>Description:</b>          | Phenyl-U- <sup>14</sup> C (spec. activity of a.s. 17.1 MBq/mg) |
| <b>Lot/Batch #:</b>          | 1147-2001  |
| <b>Radiochemical Purity:</b> | 98.9%  |
| <b>Chemical Purity:</b>      | 97.0%  |

##### 2. C-label BAS 684 H (CAS No. 87818-31-3)

|                              |   |
|------------------------------|---|
| <b>Description:</b>          | Cyclohexane-4- <sup>14</sup> C (spec. activity of a.s. 7.75 MBq/mg) |
| <b>Lot/Batch #:</b>          | 1146-1001   |
| <b>Radiochemical Purity:</b> | 99.4%   |
| <b>Chemical Purity:</b>      | 99.3%   |

3. C-label BAS 684 H (CAS No. 87818-31-3)

**Description:** Benzyl-<sup>13</sup>C  
**Lot/Batch #:** 1159-1012  
**Chemical Purity:** 99.6%

4. BAS 684 H (CAS No. 87818-31-3)

**Description:** Unlabelled BAS 684 H  
**Lot/Batch #:** COD-001950  
**Chemical Purity:** 96.3%

*Methods*

The metabolism and distribution of BAS 684 H in/on spring wheat (variety *Thassos*) was investigated using BAS 684 H radiolabelled in the cyclohexane ring (cyclohexane-label) or in the phenyl ring (phenyl-label). The study was carried out in 2015-2018 at the Agricultural Research Centre of BASF SE in Limburgerhof, Germany. Plants were grown in a climatic chamber, which simulated natural climatic conditions of a typical wheat growing area. The experiments with both labels were carried out within the same time period.

Spring wheat seeds were sown into twenty-four boxes (4 rows per box) filled with sandy loam soil. The containers were located in climatic chambers for application of BAS 684 H. For each radiolabel, twelve containers were used, the containers have an external dimension of 0.4m x 0.6m and an internal dimension of 0.365 m x 0.56 m.

For both labels (preparation 1 and preparation 2), the crops were treated once with a single foliar application of BAS 684 H, at growth stage BBCH 29. The test item was applied as an emulsifiable concentrate EC formulation at a target rate of 500 g a.s./ha (1N for cereal crops) at BBCH 29 (timings in line with the proposed post-emergence GAP). The actual application rates were 499.3 g ai/ha (phenyl label) and 501.4 g ai/ha (cyclohexane label). A summary of the applications in the study are given in Table 7-4.

Preparation 1 (phenyl label): For the preparation of the application formulation of the phenyl label: phenyl-U-<sup>14</sup>C (dissolved in toluene), benzyl-<sup>13</sup>C and unlabelled BAS 684 H were mixed to obtain a ratio of approximately 1:1:1.

Preparation 2 (cyclohexane label): For the preparation of the application formulation of the cyclohexane label, cyclohexane-4-<sup>14</sup>C (dissolved toluene) and unlabelled BAS 684 H were mixed in an approximate ratio of 1:1.

On the day of application, the mixtures were taken up in water and blank formulation assisted by ultrasonication. The purity of the application solution was confirmed using HPLC and the isotopic pattern as well as the identity was determined and verified by HPLC-MS analysis.

The structural formulae of the labelled BAS 684 H molecules are given in Figure 7-2.

Table 7-4 Study design: plant uptake part (wheat)

| Label                                      | <sup>14</sup> C-Phenyl-label<br>(with <sup>13</sup> C-Benzyl-label) |    | <sup>14</sup> C-Cyclohexane-label |    |
|--|---|----|-----------------------------------|----|
| <b>Intended use rate [g a.s./ha]</b>       | 500   |    | 500                               |    |
| <b>Actual application rate [g a.s./ha]</b> | 499.3   |    | 501.4                             |    |
| <b>Application number</b>                  | 1   |    | 1                                 |    |
| <b>Application growth stage</b>            | BBCH29  |    | BBCH29                            |    |
| <b>Sampled matrices</b>                    | forage, grain, straw  |    | forage, grain, straw              |    |
| <b>Sampling [DALA] <sup>1)</sup></b>       | forage  | 11 | forage                            | 13 |
|  | grain   | 56 | grain                             | 56 |
|  | straw   | 56 | straw                             | 56 |

1) days after last application

The wheat plant population was thinned at growth stage BBCH 59 (wheat forage). Therefore, two plants per row were removed and collected.

Samples of immature wheat plants (BBCH 59) were taken 11 or 13 days after application and designated as wheat forage. Mature wheat plants were harvested at growth stage BBCH 89 (56 days after treatment) and separated into wheat straw and wheat grains. The ears of wheat and straw were cut off. Straw was chopped and the ears were separated into grain (wheat grain) and chaff using a thresher. Chaff and chipped straw were combined (wheat straw). Samples were then stored in a freezer at  $\leq -18^{\circ}\text{C}$ . Wheat forage, straw and grain were extracted up to 369 days after sampling and the extracts were analysed up to 384 days after extraction. The period from sampling to analysis was up to 609 days.

### *Description of analytical procedures*

Combustion of solid samples: Homogenised solid plant samples were weighed and combusted by means of an automatic sample oxidiser. The  $^{14}\text{CO}_2$  was trapped by an absorption and scintillation liquid and the collected radioactivity was measured by liquid scintillation counting (LSC).  $^{14}\text{C}$  standards were combusted to determine the recovered radioactivity and the measurements were corrected accordingly (factors ranging from 0.991 to 1.079). The residues after the enzyme solubilisations were analysed similarly.

The limit of quantitation in mg eq/kg was calculated from the twofold background radioactivity level (dpm/g matrix) divided by the corresponding specific radioactivity. For the quantitation of radioactive residues in liquid samples a liquid scintillation counter (LSC) was used.

### Homogenisation and solvent extraction (ERR: extractable radioactive residues):

Prior to solvent extraction plant samples (forage, grain and straw) were homogenised with a mill along with dry ice. After sublimation of the dry ice (overnight in a freezer), the samples were weighed and stored in a freezer.

Forage, straw and wheat grain were then extracted with methanol (3x) and water (2x) using a Polytron. Each extraction was performed for 5 minutes using a homogeniser. After each extraction step, solid material was separated from extract by centrifugation and filtration, and the supernatants of methanol and water extracts were each combined. Aliquots of the extracts were radio-assayed and further aliquots of the extracts obtained from wheat forage and straw (both labels) were analysed by HPLC. Further aliquots of the extracts obtained from wheat grain were not analysed by HPLC due to low amounts of radioactivity (total extractable radioactive residues (ERR) 0.005 mg eq/kg and 0.007 mg eq/kg for the phenyl-label and cyclohexane-label respectively).

Solubilisation of the RRR (Residual Radioactive Residue): The RRR of all wheat matrices and both labels were further investigated. All incubations were carried out in buffered enzyme solutions (at  $37^{\circ}\text{C}$ ) and stopped by addition of acetonitrile.

The RRR after solvent extraction (after methanol and water extraction) of all wheat matrices was extracted twice with 1% ammonia solution using a Polytron for 5 min at 7000 rpm in an ice bath.

The ammonia solubilisates were pooled and where appropriate analysed by HPLC. The residues were separated from the extracts by centrifugation followed by filtration with a filter paper. Thereafter the residues were allowed to dry at room temperature and homogenised using a tube mill. Residues with sufficient amount of radioactivity were resuspended in a buffer solution and further solubilised with enzymes at  $37^{\circ}\text{C}$ .

The resulting residues with sufficient amounts of radioactivity after ammonia treatment of wheat forage and straw (both labels) were dried, washed with water (phenyl label only, designated as water rinse), suspended in 0.1 M acetate buffer and incubated with macerozyme and cellulase at  $37^{\circ}\text{C}$  for approximately 22 h to solubilise cell walls. The residues after macerozyme / cellulase incubation were taken up in 1/15 M phosphate buffer and incubated with tyrosinase / laccase at  $37^{\circ}\text{C}$  for approximately 12-24 h to solubilise lignin. For solubilisation of polysaccharides, the residues after incubation with tyrosinase / laccase were taken up in 1/15 M phosphate buffer and incubated with  $\alpha$ -amylase /  $\beta$ -amylase / amyloglycosidase at  $37^{\circ}\text{C}$  for approximately 23-72 h. For wheat forage roots (both labels), the residue after  $\alpha$ -amylase /  $\beta$ -amylase / amyloglycosidase treatment was not further investigated. For wheat straw (both labels), the residue after  $\alpha$ -amylase /  $\beta$ -amylase / amyloglycosidase treatment was dissolved in an artificial gastric juice containing pepsin and incubated at  $37^{\circ}\text{C}$  for 3-23 h. Finally, the residue after pepsin treatment was incubated in an artificial intestine fluid containing pancreatin at  $37^{\circ}\text{C}$  for approximately 22 h. Aliquots of the solubilisates (as well as of the water rinse) and residues thereof were radio-assayed. Further aliquots of the resulting ammonia solubilisates obtained from wheat forage and straw, the water rinse obtained from wheat straw of the phenyl label and the macerozyme solubilisates from wheat straw (both



labels) were analysed by HPLC-MS. Furthermore, due to very low levels of radioactive residues in wheat grains ( $\leq 0.001$  mg eq/kg), only wheat forage and straw were consecutively solubilised with macerozyme, tyrosinase and amylase. The results are summarised in Table 7-10.

Components of the residue were identified by HPLC-MS (LC13 and LC02Z (see details below), NMR, as well as by co-chromatography and comparison of retention times by HPLC methods LC13 and LC02Z.

HPLC method LC13: A Phenomenex Synergi Polar RP column (250 x 4.6 mm, 4  $\mu$ m) was used with a phenyl pre-column. A gradient elution was used (mobile phase A: ammonium formate (20 mM; pH 6); mobile phase B: acetonitrile).

HPLC method LC02Z: A YMC Pro C18 RS column (250 x 4.6 mm, 5  $\mu$ m) was used with a C18 (4 x 3 mm) pre-column. A gradient elution was used (mobile phase A: water:formic acid (1000:1); mobile phase B: acetonitrile:formic acid (1000:1)).

Table 7-5 How identification of metabolites was achieved

| Metabolite | Initial identification   |
|------------|--|
| BAS 684 H  | HPLC-MS in wheat forage methanol extract and co-chromatography by two sufficiently dissimilar techniques (LC02Z and LC13)                              |
| M684H005   | HPLC-MS and NMR in wheat straw methanol extract  |
| M684H006   | HPLC-MS and NMR in wheat straw methanol extract  |
| M684H007   | HPLC-MS in wheat forage methanol extract and wheat straw methanol extract  |
| M684H008   | HPLC-MS in wheat forage methanol extract   |
| M684H015   | HPLC-MS in wheat straw methanol extract  |
| M684H016   | HPLC-MS in wheat straw methanol extract  |
| M684H054   | HPLC-MS in wheat straw methanol extract  |
| M684H055   | HPLC-MS in wheat straw methanol extract  |
| M684H047   | HPLC-MS in wheat straw methanol extract<br>Co-chromatography with external sample from oilseed rape metabolism study (Section B.7.2.1.2, CA 6.2.1/002) |
| M684H048   | HPLC co-chromatography with external sample from oilseed rape metabolism study using two sufficiently dissimilar techniques (LC02Z and LC13)           |

HPLC-MS has been used to identify all metabolites except M684H048 and this is a technique capable of positive structural identification. HPLC co-chromatography using two sufficiently dissimilar techniques has been used to identify M684H048. Following initial identification through the techniques above, metabolites were identified in all other matrices by co-chromatography (HPLC method LC13) with reference standards and isolated fractions of identified compounds. Therefore the techniques used to identify the metabolites are considered sufficient.

**Cleavage experiments:** Cleavage experiments were conducted with the methanol extract of wheat forage (phenyl label) and with individual fractions of the methanol extracts of wheat straw (cyclohexane label). The purpose of these experiments was to investigate the stability of conjugated metabolites to support the development of the residue analytical method. An aliquot of the methanol phase was concentrated and then fractionated by SPE with water/acetonitrile mixtures and acetonitrile. Aliquots of the wheat forage methanol extract (phenyl label) or isolated fractions of the wheat straw methanol extract were concentrated nearly to dryness and suspended in the incubation media according to the conditions summarised in Table 7-6. All final extracts were subject to HPLC-MS.

Table 7-6 Cleavage experiments with methanol extracts and isolated fractions thereof

| Method  | Conditions   |
|---|--|
| <b>Hydrolysis of ester bonds</b><br>Hydrochloric acid treatment | Suspension in 2 M hydrochloric acid for 2 h or overnight at room temperature, neutralisation with ammonia (25%) to ~ pH 7                            |
| <b>Hydrolysis of ester bonds</b><br>Ammonia treatment           | Suspension in 1 mL acetonitrile and 2 mL 25% ammonia for 2 h or overnight at room temperature, neutralisation with 37% hydrochloric acid to pH 6 – 8 |



|  |  |
|--|--|
|  | or<br>Suspension in 10 mL ammonia (25%) for 2 h, neutralisation with acetic acid (99%) and hydrochloric acid (2 M) to ~ pH 7                       |
| <b>Hydrolysis of <math>\beta</math>-glucosidic bonds</b><br>$\beta$ -glucosidase treatment | Suspension in acetate buffer (0.1M, ~ pH 5), treatment with $\beta$ -glucosidase (3590 U/g residue) over night at 37°C and 180 rpm                 |
| <b>Hydrolysis of <math>\beta</math>-glucosidic bonds</b><br>Rumen fluid treatment          | Suspension in 5 mL rumen fluid, shaking for 24 h at 39°C in the dark under nitrogen atmosphere, stopping of incubation by addition of acetonitrile |

Table 7-7 How identification of aglycones was achieved after deglucosilation

| Aglycone<br>deglucosilation | after | Initial identification                  |
|-----------------------------|-------|---|
| M684H002                    |       | HPLC-MS in rumen-fluid treated fraction |
| M684H004                    |       | HPLC-MS in rumen-fluid treated fraction |
| M684H017                    |       | HPLC-MS in rumen-fluid treated fraction |
| M684H018                    |       | HPLC-MS in rumen-fluid treated fraction |
| M684H019                    |       | HPLC-MS in rumen-fluid treated fraction |
| M684H024                    |       | HPLC-MS in rumen-fluid treated fraction |

### Results and discussion

#### Total radioactive residue (TRR)

The calculated total radioactive residues (TRR) with the Phenyl-label were highest in straw (DALA 56) at 5.954 mg eq/kg, lower in forage at 2.632 mg eq/kg, and lowest in grain with 0.009 mg eq/kg. A similar distribution was seen with the cyclohexane-label (TRR highest in straw: 9.732 mg eq/kg, lower in forage at 2.678 mg eq/kg and grain at 0.012 mg eq/kg). Notably the TRR in straw was much higher with the Cyclohexane-label compared with the Phenyl-label, indicating a difference in composition of the detectable residue. This is discussed further below. A summary of the TRRs are presented in Table 7-8. There are no significant differences between the TRR measured and TRR calculated for each label and matrix.

Table 7-8 Total radioactive residue after foliar spray application of BAS 684 H

| Matrix<br>[BBCH]         | DALA <sup>1)</sup> | TRR<br>measured (LSC) <sup>2)</sup> [mg eq/kg] | TRR<br>calculated <sup>3)</sup> [mg eq/kg] |
|--------------------------|--------------------|--|--|
| <b>Phenyl label</b>      |                    |  |  |
| <b>forage</b> [59]       | 11                 | 2.877  | 2.632                                      |
| <b>straw</b> [89]        | 56                 | 5.709  | 5.954                                      |
| <b>grain</b> [89]        | 56                 | 0.010  | 0.009                                      |
| <b>Cyclohexane label</b> |                    |  |  |
| <b>forage</b> [59]       | 13                 | 2.693  | 2.678                                      |
| <b>straw</b> [89]        | 56                 | 9.777  | 9.732                                      |
|                          |                    | 8.675  | 8.271                                      |
| <b>grain</b> [89]        | 56                 | 0.011  | 0.012                                      |

1) days after last application, 2) TRR measured directly via combustion LSC, 3) TRR calculated as the sum of ERR (extractable radioactive residue) and RRR (residual radioactive residue) after extraction of the residues

#### Extractability of radioactive residues

The extractabilities of <sup>14</sup>C residues from wheat forage, straw and grain are summarised in Table 7-9.

High extractability of  $^{14}\text{C}$  residue was seen in forage (>95% TRR for total extract for both labels) and straw (20 - 90% TRR for total extract for both labels). The majority of the radioactivity was extracted with methanol (>50% TRR) while subsequent water extraction resulted in additional extraction of 4 - 36% TRR. Solvent extraction left an RRR (residual radioactive residue) in forage of <5% TRR, while the RRR in straw amounted to 14.2% TRR (1.379 mg eq/kg, Cyclohexane-label) and 14.3 % TRR (0.853 mg eq/kg, Phenyl-label). The RRR was therefore further investigated by enzyme treatment as discussed below. No significant label specific differences were seen for forage and straw.

There was no significant difference in extractability in grain between both labels. For the Phenyl-label 53.9% TRR was extractable, 31.2% TRR of which by water extraction and 22.7% TRR by methanol. Similarly, for the Cyclohexane-label 60.7% TRR was extracted, 33.1% of extracted by water and methanol removed 27.6% TRR. The RRR after solvent extraction amounted to 46.1% TRR (Phenyl-label, 0.004 mg eq/kg) and 39.3% TRR (Cyclohexane-label, 0.005 mg eq/kg) and thus was subject to further investigation by enzyme treatment.

Table 7-9 Extractability of radioactive residues from forage, straw and grain

| Matrix            | DALA <sup>1)</sup> | TRR<br>calculated<br><sup>2)</sup> | distribution of radioactive residues |             |                                 |             |                         |             |                   |          |
|-------------------|--------------------|------------------------------------|--------------------------------------|-------------|---------------------------------|-------------|-------------------------|-------------|-------------------|----------|
|                   |                    |                                    | methanol<br>extracts <sup>3)</sup>   |             | water<br>extracts <sup>3)</sup> |             | Total ERR <sup>2)</sup> |             | RRR <sup>2)</sup> |          |
|                   |                    | mg eq/kg                           | % TRR                                | mg<br>eq/kg | % TRR                           | mg<br>eq/kg | % TRR                   | mg<br>eq/kg | % TRR             | mg eq/kg |
| Phenyl-label      |                    |                                    |                                      |             |                                 |             |                         |             |                   |          |
| forage            | 11                 | 2.632                              | 91.3                                 | 2.402       | 4.9                             | 0.129       | 96.2                    | 2.531       | 3.8               | 0.100    |
| straw             | 56                 | 5.954                              | 51.4                                 | 3.062       | 34.2                            | 2.038       | 85.7                    | 5.101       | 14.3              | 0.853    |
| grain             | 56                 | 0.009                              | 22.7                                 | 0.002       | 31.2                            | 0.003       | 53.9                    | 0.005       | 46.1              | 0.004    |
| Cyclohexane-label |                    |                                    |                                      |             |                                 |             |                         |             |                   |          |
| forage            | 13                 | 2.678                              | 90.7                                 | 2.430       | 4.4                             | 0.118       | 95.2                    | 2.548       | 4.8               | 0.130    |
| straw             | 56                 | 9.732                              | 62.2                                 | 6.053       | 23.6                            | 2.301       | 85.8                    | 8.353       | 14.2              | 1.379    |
| grain             | 56                 | 0.012                              | 27.6                                 | 0.003       | 33.1                            | 0.004       | 60.7                    | 0.007       | 39.3              | 0.005    |

<sup>1)</sup> days after last application, <sup>2)</sup> TRR calculated as the sum of ERR + RRR was set to 100% TRR, <sup>3)</sup> pool of combined repetitive extracts

#### *Solubilisation of radioactive residues*

The residues after solvent extraction of all matrices were solubilised with ammonia. Due to very low levels of radioactive residues in wheat grains, only wheat forage and straw were consecutively solubilised with macerozyme, tyrosinase and amylase. Wheat straw was additionally incubated with artificial gastric juice containing pepsin and artificial intestinal fluid containing pancreatin. The results of the ammonia incubations and enzyme solubilisations of the residue after solvent extraction are summarised in Table 7-10. Generally, low amounts of radioactive residues were released from forage (both labels) by ammonia and enzyme incubations, which accounted in sum for up to 3.5 % TRR. These residues are likely to be associated into the cell structure (e.g. starch or lignin associated).

For Phenyl-labelled wheat grain, 0.001 mg eq/kg (15.6% TRR) were released by ammonia only.

For Cyclohexane-labelled wheat grain, 0.001 mg eq/kg (12.1% TRR) were released by ammonia only.

Table 7-10 Characterisation of the radioactive residues after solvent extraction in wheat samples/ Solubilisation of the RRR of wheat matrices Summary of solubilised components in wheat

| Designation  | Matrix                          |                                |                                |                                 |                                |                                |
|--|---------------------------------|--------------------------------|--------------------------------|---------------------------------|--------------------------------|--------------------------------|
|  | Phenyl label                    |                                |                                | Cyclohexane label               |                                |                                |
|  | Forage<br>[mg eq/kg]<br>[% TRR] | Straw<br>[mg eq/kg]<br>[% TRR] | Grain<br>[mg eq/kg]<br>[% TRR] | Forage<br>[mg eq/kg]<br>[% TRR] | Straw<br>[mg eq/kg]<br>[% TRR] | Grain<br>[mg eq/kg]<br>[% TRR] |
| <i>Residue after solvent extraction</i>                        | <i>0.100</i><br><i>3.8</i>      | <i>0.853</i><br><i>14.3</i>    | <i>0.004</i><br><i>46.1</i>    | <i>0.130</i><br><i>4.8</i>      | <i>1.379</i><br><i>14.2</i>    | <i>0.005</i><br><i>39.3</i>    |
| Ammonia solubilisate   | 0.034<br>1.3                    | 0.468<br>7.9                   | 0.001<br>15.6                  | 0.050<br>1.9                    | 0.772<br>7.9                   | 0.001<br>12.1                  |
| Rinse water  | 0.006<br>0.2                    | 0.051<br>0.9                   | not applied                    | not applied                     | not applied                    | not applied                    |
| Macerozyme solubilisate  | 0.019<br>0.7                    | 0.107<br>1.8                   | not applied                    | 0.028<br>1.1                    | 0.308<br>3.2                   | not applied                    |
| Tyrosinase solubilisate  | 0.006<br>0.2                    | 0.043<br>0.7                   | not applied                    | 0.012<br>0.4                    | 0.065<br>0.7                   | not applied                    |
| Amylase solubilisate   | 0.002<br>0.1                    | 0.018<br>0.3                   | not applied                    | 0.005<br>0.2                    | 0.026<br>0.3                   | not applied                    |
| Pepsin solubilisate  | not applied                     | 0.006<br>0.1                   | not applied                    | not applied                     | 0.008<br>0.1                   | not applied                    |
| Pancreatin solubilisate  | not applied                     | 0.014<br>0.2                   | not applied                    | not applied                     | 0.016<br>0.2                   | not applied                    |
| <b>Sum of solubilised residues</b>                             | <b>0.066</b><br><b>2.5</b>      | <b>0.707</b><br><b>11.9</b>    | <b>0.001</b><br><b>15.6</b>    | <b>0.094</b><br><b>3.5</b>      | <b>1.195</b><br><b>12.3</b>    | <b>0.001</b><br><b>12.1</b>    |
| Final residue  | 0.021<br>0.8                    | 0.113<br>1.9                   | 0.003<br>30.5                  | 0.035<br>1.3                    | 0.122<br>1.3                   | 0.003<br>27.2                  |
| <b>Sum of solubilised radioactive residues + final residue</b> | <b>0.088</b><br><b>3.3</b>      | <b>0.820</b><br><b>13.8</b>    | <b>0.004</b><br><b>46.1</b>    | <b>0.130</b><br><b>4.8</b>      | <b>1.317</b><br><b>13.5</b>    | <b>0.005</b><br><b>39.3</b>    |

*Characterisation, Identification and Quantification of Radioactive Residues in Wheat Matrices*

HPLC-MS and NMR analysis of purified methanol extracts and fractions thereof of wheat forage (phenyl and cyclohexane label) and wheat straw (cyclohexane label) resulted in the identification of the parent compound BAS 684 H and its metabolites M684H005, M684H006, M684H007, M684H008, M684H015, M684H016, M684H054 and M684H055. Metabolites M684H047 and M684H048 were identified by co-chromatography with external sample from a related metabolism study of BAS 684 H in oilseed rape. Details of identified and characterised metabolites in all investigated matrices are given in

Table 7-11 to

Table 7-18. An overall summary of identified and characterised radioactive residues is compiled in Table 7-19.

An overview of the components of the extractable residue is given in Table 7-21. Structures of the metabolites are outlined in Table 7-1. Residues of BAS 684 H in wheat grain, straw and forage have been sufficiently characterised and identified as summarised below.

#### *Forage*

##### *Phenyl-label*

Analysis of the concentrated methanol extract of wheat forage, phenyl label, with quantitative HPLC-MS (method LC13) resulted in a pattern of 31 peaks, of which eight were identified. The most abundant compound was metabolite M684H006 and accounted for 0.749 mg eq/kg (28.5% TRR). Metabolite M684H005 was the second most abundant with 0.259 mg eq/kg (9.8% TRR), followed by metabolites M684H007 with 0.209 mg eq/kg (7.9% TRR) and M684H016 with 0.132 mg eq/kg (5.0% TRR). The compounds M684H047 (0.085 mg eq/kg, 3.2% TRR), BAS 684 H (0.081 mg eq/kg, 3.1 % TRR), M684H015 (0.062 mg eq/kg, 2.4% TRR) and M684H055 (0.061 mg eq/kg, 2.3% TRR) were less abundant on a similar level. The active and the seven metabolites identified were confirmed using HPLC-MS method LC02Z. An aliquot of the methanol extract was concentrated and fortified with reference items: BAS 684 H, M684H005, M684H006, M684H007, M684H015, M684H016, M684H047, M684H048 and M684H055. The fortified aliquot was taken for co-chromatography identification using quantitative and confirmatory HPLC-MS methods (LC13 and LC02Z, respectively) where there was a positive retention time match of metabolites with authentic reference standards. Furthermore, quantification of metabolite M684H048 on the concentrated methanol extract of wheat forage was not feasible, since a corresponding peak could not be detected in the quantitative chromatogram. Consequently, co-chromatography with HPLC-MS method LC02Z was omitted for M684H048. 23 additionally characterised peaks were detected, 19 of which were  $\geq 0.01$  mg eq/kg. The total radioactivity present in the methanol extract of wheat forage (phenyl label) was 2.296 mg eq/kg (87.2% TRR) and 71.3% of that has been conclusively identified; therefore, the identification of 17 individual residues in the range of 0.053-0.021 mg eq/kg is not required in accordance with the OECD 501 Metabolism in Crops Guideline given straightforward identification was not possible. However, the higher level individual residues at 0.070 mg eq/kg (2.7% TRR) and 0.124 mg eq/kg (4.7% TRR) have not been identified despite 8 residues within a similar range of 0.259 – 0.061 mg eq/kg being identified. The applicant justified that the attempts at identification by comparison of retention times and MS data with a range of reference items for postulated metabolites (listed above) did not produce a conclusive structural assignment. Characterisation and identification need to be decided on a case by case basis for this residue based on how much has been identified. Given 71.3% of the methanol extract has been identified, the major components of the residue have been identified, and there is no representative use on cereal forage hence no effect on the dietary burden and overall consumer risk assessment, the degree of characterisation and identification performed is considered acceptable. Overall, 1.638 mg/kg or 56.93% TRR were identified in the methanol extract of wheat forage (phenyl label). The remaining compounds were present at 0.124 mg eq/kg (4.37% TRR) or below, summed up to 0.658 mg eq/kg (25.0% TRR) and were classified as characterised.

The HPLC-MS chromatogram of the purified water extract of wheat forage (phenyl label) shows 31 peaks, of which seven were identified. The most abundant compounds, though on a low level, were metabolites M684H005 (0.022 mg eq/kg; 0.8% TRR) and M684H006 (0.021 mg eq/kg; 0.8% TRR). Minor amounts of metabolites M684H047, M684H016, M684H007, (each at 0.003 mg eq/kg; 0.1% TRR), M684H055 (0.002 mg eq/kg; 0.1% TRR) and M684H015 (0.001 mg eq/kg; <0.1% mg eq/kg) were detected. The seven metabolites identified were confirmed using HPLC-MS method LC02Z. The seven metabolites identified were based on comparison of the retention times and metabolite patterns with those of the HPLC-MS analyses from co-chromatography experiments. The total radioactivity present in the water extract of wheat forage (phenyl label) was 0.119 mg eq/kg (4.5% TRR) and 46.2% of that has been conclusively identified. The 24 individual extractable radioactive residues that have not been identified are present at <0.01 mg eq/kg and do not require further analysis. The 24 unidentified components were detected at levels up to 0.007 mg eq/kg (0.3 % TRR), summed up to 0.064 mg eq/kg (2.4% TRR) and were classified as characterised. Overall, 0.055 mg eq/kg or 2.0% TRR was identified in the water extract of wheat forage (phenyl label).

In the analysis of the concentrated ammonia solubilisate of wheat forage (phenyl label) with HPLC-MS (method LC13) a pattern of 19 peaks were detected. The most abundant peak was identified as metabolite M684H005 and accounted for 0.015 mg eq/kg (0.6% TRR). The one metabolite identified was confirmed using HPLC-MS method LC02Z. The metabolite identified was based on comparison of the retention time and metabolite pattern with those of the HPLC-MS analyses from co-chromatography experiments. The total radioactivity present in the ammonia solubilisate of wheat forage (phenyl label) was 0.031 mg eq/kg (1.2% TRR) and 50% of that has

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been conclusively identified. The 17 individual extractable radioactive residues that have not been identified are present at <0.01 mg eq/kg and do not require further analysis. The 17 unidentified components were detected at levels up to 0.006 mg eq/kg (0.2% TRR) or below, summed up to 0.016 mg eq/kg (0.6% TRR) and were classified as characterised.

Due to the low amounts of radioactive residues, the rinse water and enzyme solubilisates of residual radioactive residue (RRR) of wheat forage (phenyl label), which accounted for a total of 0.033 mg eq/kg or 1.2% TRR, were not analysed by HPLC.

In sum, 1.708 mg eq/kg or 64.9% TRR were identified and an additional 0.770 mg eq/kg or 29.2% TRR were characterised in wheat forage (phenyl label). Overall, 2.478 mg eq/kg or 94.2% TRR were identified and characterised in the ERR and the RRR of wheat forage (phenyl label).

Table 7-11 Summary of Identified and characterised residues in the ERR of Wheat Forage (Phenyl label)

| Labelled radioactive component                    | Extracts                         |       |                                 |       | Sum of extracts |       |
|---|----------------------------------|-------|---------------------------------|-------|-----------------|-------|
|   | Concentrated methanol extract    |       | Purified water extract          |       |                 |       |
|   | mg eq/kg                         | % TRR | mg eq/kg                        | % TRR | mg eq/kg        | % TRR |
| Total radioactive residue (TRR)                   |                                  |       |                                 |       | 2.632           | 100   |
| Identified  |                                  |       |                                 |       |                 |       |
| M684H005  | 0.259                            | 9.8   | 0.022                           | 0.8   | 0.281           | 10.7  |
| M684H006  | 0.749                            | 28.5  | 0.021                           | 0.8   | 0.770           | 29.2  |
| M684H007  | 0.209                            | 7.9   | 0.003                           | 0.1   | 0.212           | 8.1   |
| M684H015  | 0.062                            | 2.4   | 0.001                           | <0.1  | 0.063           | 2.4   |
| M684H016  | 0.132                            | 5.0   | 0.003                           | 0.1   | 0.135           | 5.1   |
| M684H047  | 0.085                            | 3.2   | 0.003                           | 0.1   | 0.088           | 3.3   |
| M684H048  | N.D.                             | N.D.  | N.D.                            | N.D.  | N.D.            | N.D.  |
| M684H055  | 0.061                            | 2.3   | 0.002                           | 0.1   | 0.063           | 2.4   |
| BAS 684 H   | 0.081                            | 3.1   | N.D.                            | N.D.  | 0.081           | 3.1   |
| Total identified by HPLC in ERR                   |                                  |       |                                 |       | 1.693           | 64.3  |
| Characterised                                     |                                  |       |                                 |       |                 |       |
| Number of additionally characterised peaks        | 23<br>(19 peaks ≥ 0.01 mg eq/kg) |       | 24<br>(0 peaks ≥ 0.01 mg eq/kg) |       | -               | -     |
| Maximum of additionally characterised peaks       | 0.124                            | 4.7   | 0.007                           | 0.3   | -               | -     |
| Sum of additionally characterised peaks           | 0.658                            | 25.0  | 0.064                           | 2.4   | 0.722           | 27.4  |
| Total characterised by HPLC in ERR                |                                  |       |                                 |       | 0.722           | 27.4  |
| Total identified and characterised by HPLC in ERR |                                  |       |                                 |       | 2.415           | 91.7  |
| Residual radioactive residue (RRR, calculated)    |                                  |       |                                 |       | 0.1             | 3.8   |
| Total identified and characterised + RRR          |                                  |       |                                 |       | 2.515           | 95.6  |

N.D = Not detected

Table 7-12 Summary of Identified and characterised residues in the RRR of Wheat Forage (Phenyl label)

| Labelled radioactive component                                   | Solubilisates<br>Concentrated ammonia solubilise |       | Sum of solubilisates |            |
|--|--|-------|----------------------|------------|
|  | mg eq/kg   | % TRR | mg eq/kg             | % TRR      |
| <b>Residual radioactive residue, calculated</b>                  |  |       | 0.100                | 3.8        |
| <b>Identified</b>  |  |       |                      |            |
| <b>M684H005</b>  | 0.015  | 0.6   | 0.015                | 0.6        |
| <b>Total identified by HPLC in RRR</b>                           |  |       | <b>0.015</b>         | <b>0.6</b> |
| <b>Characterised</b>   |  |       |                      |            |
| <b>Number of additionally characterised peaks</b>                | 18<br>(0 peaks $\geq$ 0.01 mg eq/kg)             |       | -                    | -          |
| <b>Maximum of additionally characterised peaks</b>               | 0.006  | 0.2   | -                    | -          |
| <b>Sum of additionally characterised peaks</b>                   | 0.016  | 0.6   | 0.016                | 0.6        |
| <b>Total characterised by HPLC in RRR</b>                        |  |       | <b>0.016</b>         | <b>0.6</b> |
| <b>Total identified and characterised by HPLC in RRR</b>         |  |       | <b>0.031</b>         | <b>1.2</b> |
| <b>Rinse water</b>   |  |       | 0.006                | 0.2        |
| <b>Macerozyme solubilise</b>                                     |  |       | 0.019                | 0.7        |
| <b>Tyrosinase solubilise</b>                                     |  |       | 0.006                | 0.2        |
| <b>Amylase solubilise</b>  |  |       | 0.002                | 0.1        |
| <b>Total additionally characterised in RRR</b>                   |  |       | <b>0.033</b>         | <b>1.2</b> |
| <b>Total identified and characterised in RRR</b>                 |  |       | <b>0.063</b>         | <b>2.4</b> |
| <b>Final residue</b>   |  |       | 0.021                | 0.8        |
| <b>Total identified and characterised in RRR + final residue</b> |  |       | <b>0.085</b>         | <b>3.2</b> |

N.D = Not detected

*Cyclohexane-label*

Analysis of the concentrated methanol extract of wheat forage (cyclohexane label) with quantitative HPLC-MS method LC13 resulted in a pattern of 41 peaks, of which eight were identified. Metabolite M684H006 was the most abundant component accounting for 0.781 mg eq/kg (29.2% TRR). The second most abundant component was metabolite M684H005 with amounts of 0.370 mg eq/kg (13.8% TRR). Metabolites M684H016 (0.213 mg eq/kg, 8.0% TRR), M684H007 (0.177 mg eq/kg, 6.6% TRR) and M684H015 (0.113 mg eq/kg, 4.2% TRR) were detected at lower levels. Additionally, metabolites M684H047 (0.063 mg eq/kg, 2.4% TRR), M684H055 (0.058 mg eq/kg, 2.2% TRR) and the parent compound BAS 684 H (0.054 mg eq/kg, 2.0% TRR) were identified in similar amounts. The active and the seven metabolites identified were confirmed using HPLC-MS method LC02Z. An aliquot of the methanol extract was concentrated and fortified with reference items: BAS 684 H, M684H005, M684H006, M684H007, M684H015, M684H016, M684H047, M684H048 and M684H055. The fortified aliquot was taken for co-chromatography identification using quantitative and confirmatory HPLC-MS methods (LC13 and LC02Z, respectively) where there was a positive retention time match of metabolites with authentic reference standards. Furthermore, the parent compound (BAS 684 H) was identified by co-chromatography with concentrated methanol extracts in both chromatographic systems LC13 and LC02Z. Due to its low intensity, the corresponding peak was not detected in the confirmatory HPLC chromatogram. However, since the parent compound was identified in a concentrated aliquot of the sample, the corresponding peak in the HPLC chromatogram used for quantification was designated as BAS 684 H and classified as identified. The total radioactivity present in the methanol extract of wheat forage (cyclohexane label) was 2.562 mg eq/kg (95.7% TRR) and 71.4% of that has been conclusively identified including the main components of the residue; therefore, the identification of 3 individual residues in the range of 0.073-0.053 mg eq/kg is not required in accordance with the OECD 501 Metabolism in Crops Guideline. Additionally, the 9 individual



residues quantified in the range of 0.01-0.002 mg eq/kg do not require further analysis. The remaining 21 extractable radioactive residues within the range of 0.011 – 0.033 mg eq/kg are considered characterised and no further attempts to identify these peaks are required. Overall, 1.829 mg eq/kg or 68.4% TRR was identified in the methanol extract of wheat forage (cyclohexane label). The remaining components were detected up to 0.073 mg eq/kg (2.7% TRR), summed up to 0.732 mg eq/kg (27.3% TRR) and were classified as characterised.

Analysis of the concentrated water extract of wheat forage (cyclohexane label) with quantitative HPLC-MS method LC13 revealed a pattern of 24 peaks, of which five were identified. The most abundant compounds were metabolites M684H005 and M684H006 with amounts of 0.025 mg eq/kg (0.9 % TRR) and 0.015 mg eq/kg (0.6 % TRR), respectively. Metabolites M684H016 (0.005 mg eq/kg, 0.2% TRR), M684H015 and M684H047 (each at 0.003 mg eq/kg and 0.1% TRR) were detected at significantly lower levels. Other components were detected at levels up to 0.007 mg eq/kg (0.2 % TRR), summed up to 0.05 mg eq/kg (1.9% TRR) and were classified as characterised. The eight metabolites identified were confirmed using HPLC-MS method LC02Z. The eight metabolites identified were based on comparison of the retention times and metabolite patterns with those of the HPLC-MS analyses from co-chromatography experiments. The total radioactivity present in the water extract of wheat forage (cyclohexane label) was 0.101 mg eq/kg (3.8% TRR) and 50% of that has been conclusively identified. The 19 individual extractable radioactive residues that have not been identified are present at <0.01 mg eq/kg and do not require further analysis. The 19 unidentified components were detected at levels up to 0.007 mg eq/kg (0.2 % TRR), summed up to 0.05 mg eq/kg (1.9% TRR) and were classified as characterised. Overall, 0.051 mg eq/kg or 1.9% TRR was identified in the water extract of wheat forage (cyclohexane label).

Due to the low amounts of radioactive residues, the ammonia and enzyme solubilisates of the residual radioactive residue (RRR) of wheat forage (cyclohexane label), which accounted for a total of 0.094 mg eq/kg or 3.45% TRR, were not analysed by HPLC.

In sum, 1.881 mg eq/kg or 70.2% TRR were identified and an additional 0.876 mg eq/kg or 32.7% TRR were characterised in wheat forage (cyclohexane label). Overall, 2.757 mg eq/kg or 103.0% TRR were identified and characterised in the ERR and RRR of wheat forage (cyclohexane label).

Table 7-13 Summary of Identified and characterised residues in the ERR and RRR of Wheat Forage (cyclohexane label)

| Labelled radioactive component                     | Extracts                      |       |                            |       | Sum of extracts |       |
|--|-------------------------------|-------|----------------------------|-------|-----------------|-------|
|  | Concentrated methanol extract |       | Concentrated water extract |       |                 |       |
|  | mg eq/kg                      | % TRR | mg eq/kg                   | % TRR | mg eq/kg        | % TRR |
| Total radioactive residue (TRR)                    |                               |       |                            |       | 2.678           | 100   |
| Identified   |                               |       |                            |       |                 |       |
| M684H005   | 0.370                         | 13.8  | 0.025                      | 0.9   | 0.396           | 14.8  |
| M684H006   | 0.781                         | 29.2  | 0.015                      | 0.6   | 0.796           | 29.7  |
| M684H007   | 0.177                         | 6.6   | N.D.                       | N.D.  | 0.177           | 6.6   |
| M684H015   | 0.113                         | 4.2   | 0.003                      | 0.1   | 0.116           | 4.3   |
| M684H016   | 0.213                         | 8.0   | 0.005                      | 0.2   | 0.219           | 8.2   |
| M684H047   | 0.063                         | 2.4   | 0.003                      | 0.1   | 0.066           | 2.5   |
| M684H048   | N.D.                          | N.D.  | N.D.                       | N.D.  | N.D.            | N.D.  |
| M684H055   | 0.058                         | 2.2   | N.D.                       | N.D.  | 0.058           | 2.2   |
| BAS 684 H  | 0.054                         | 2.0   | N.D.                       | N.D.  | 0.054           | 2.0   |
| Characterised                                      |                               |       |                            |       |                 |       |
| Number of additionally characterised peaks         | 33 (26 peaks ≥ 0.01 mg eq/kg) |       | 19                         |       | -               | -     |
| Maximum of additionally characterised peaks        | 0.073                         | 2.7   | 0.007                      | 0.2   | -               | -     |
| Sum of additionally characterised peaks            | 0.732                         | 27.3  | 0.05                       | 1.9   | 0.782           | 29.2  |
| Total characterised by HPLC in ERR                 |                               |       |                            |       | 0.782           | 29.2  |
| Total identified and characterised by HPLC in ERR  |                               |       |                            |       | 2.663           | 99.5  |
| Residual radioactive residue (RRR, calculated)     |                               |       |                            |       | 0.130           | 4.8   |
| Ammonia solubilisate                               |                               |       |                            |       | 0.05            | 1.9   |
| Macerozyme solubilisate                            |                               |       |                            |       | 0.028           | 1.9   |
| Tyrosinase solubilisate                            |                               |       |                            |       | 0.012           | 0.4   |
| Amylase solubilisate                               |                               |       |                            |       | 0.005           | 0.2   |
| Total additionally characterised in RRR            |                               |       |                            |       | 0.094           | 3.5   |
| Total characterised                                |                               |       |                            |       | 0.876           | 32.7  |
| Total identified and characterised                 |                               |       |                            |       | 2.757           | 103.0 |
| Final residue                                      |                               |       |                            |       | 0.035           | 1.3   |
| Total identified and characterised + final residue |                               |       |                            |       | 2.793           | 104.3 |

N.D = Not detected

Straw

*Phenyl-label*

Analysis of the concentrated methanol extract of wheat straw (phenyl label) with quantitative HPLC-MS method LC13 resulted in pattern of 46 peaks, whereof eight were identified. The most abundant compounds were metabolites M684H005 (0.547 mg eq/kg, 9.2% TRR) and M684H006 (0.47 mg eq/kg, 7.8% TRR). Metabolites M684H016 (0.147 mg eq/kg, 2.5% TRR), M684H007 (0.096 mg eq/kg, 1.6% TRR) M684H047 (0.079 mg eq/kg, 1.3% TRR), M684H015 (0.058 mg eq/kg, 1.0% TRR) and M68H055 (0.056 mg eq/kg, 0.9% TRR) were detected at significantly lower level. Likewise, the parent compound was detected in low amounts accounting for 0.026 mg eq/kg (0.4% TRR). The active and the seven metabolites identified were confirmed using HPLC-MS method LC02Z. An aliquot of the methanol extract was concentrated and fortified with reference items: BAS 684 H, M684H005, M684H006, M684H007, M684H015, M684H016, M684H047,

M684H048 and M684H055. The fortified aliquot was taken for co-chromatography identification using quantitative and confirmatory HPLC-MS methods (LC13 and LC02Z, respectively) where there was a positive retention time match of metabolites with authentic reference standards. 38 additionally characterised peaks were detected, 35 of which were  $\geq 0.01$  mg eq/kg. The total radioactivity present in the methanol extract of wheat straw (phenyl label) was 3.054 mg eq/kg (51.3% TRR) and 48.3% of that has been conclusively identified. The identification of 23 individual residues in the range of 0.050 – 0.094 mg/kg was not achieved. Moreover, 7 higher level individual residues, the highest of which were at 0.104 mg eq/kg (1.7% TRR) and at 0.207 mg eq/kg (3.5% TRR), have not been identified despite 8 residues within a similar range of 0.026 – 0.547 mg eq/kg (0.4 – 9.2% TRR) being identified. The applicant justified that attempts at identification by comparison of retention times and MS data with a range of reference items for postulated metabolites (listed above) did not produce a conclusive structural assignment. Characterisation and identification need to be decided on a case by case basis for this residue based on how much has been identified. Given only 48.3% of the extract has been identified, it would have been preferable for further identification to have been performed. However, the dietary burden is significantly below 0.004 mg/kg bw/day and the animal metabolism studies are significantly overdosed ( $> 300$  N) compared to the dietary burden (Vol 1 Section 2.7.5) hence the level of identification does not affect the overall consumer risk assessment for the representative uses. Additionally, the levels of metabolites M684H005 and M684H006 in wheat straw in the residues trials (Vol 3 CA B.7.3.1) are lower than in this metabolism study by a factor of approximately 100, hence the level of the unidentified peaks may be lower in practice. The major components of the residue have been identified and a clear metabolic pathway has been elucidated. Therefore the extent of identification is not considered a major deficiency. Additionally, the 3 extractable radioactive residues quantified in the range of 0.007 – 0.009 mg eq/kg do not require further analysis. The remaining 23 extractable residues in the range of 0.011 – 0.048 mg/kg are considered characterised and no further attempts to identify these peaks are required.

A number of 32 peaks were detected upon analysis of the concentrated water extract of wheat straw (phenyl label) with quantitative HPLC method LC13, of which seven were identified. The most abundant compound was metabolite M684H005 accounting for 0.306 mg eq/kg (5.1% TRR). Metabolite M684H006 was present in similar amounts of 0.253 mg eq/kg (4.2% TRR), whereas other identified metabolites M684H016 (0.068 mg eq/kg, 1.1% TRR), M684H007 (0.066 mg eq/kg, 1.1% TRR), M684H055 (0.052 mg eq/kg, 0.9% TRR), M684H047 (0.047 mg eq/kg, 0.8% TRR) and M684H015 (0.022 mg eq/kg, 0.4% TRR) were detected at lower levels. The seven metabolites identified were confirmed using HPLC-MS method LC02Z. The seven metabolites identified were based on comparison of the retention times and metabolite patterns with those of the HPLC-MS analyses from co-chromatography experiments. 26 additionally characterised peaks were detected, all of which were  $\geq 0.01$  mg eq/kg. The total radioactive residues present in the water extract of wheat straw (phenyl label) was 1.925 mg eq/kg (32.3% TRR) and 42.3% of that has been conclusively identified. The identification of 4 individual residues in the range of 0.059 – 0.078 mg eq/kg (1.0 – 1.3% TRR) was not achieved. Moreover, the higher level individual residue at 0.289 mg eq/kg (4.8% TRR) has not been identified despite 7 residues within a similar range of 0.022 - 0.306 mg eq/kg being identified. The applicant justified that attempts at identification by comparison of retention times and MS data with a range of reference items for postulated metabolites (listed above) did not produce a conclusive structural assignment. Characterisation and identification need to be decided on a case by case basis for this residue based on how much has been identified. Given only 42.3% of the extract was identified, it would have been preferable for further identification to have been performed. However, the dietary burden is significantly below 0.004 mg/kg bw/day and the animal metabolism studies are significantly overdosed ( $> 300$  N) compared to the dietary burden (Vol 1 Section 2.7.5) hence the level of identification does not affect the overall consumer risk assessment for the representative uses. Additionally, the levels of metabolites M684H005 and M684H006 in wheat straw in the residues trials (Vol 3 CA B.7.3.1) are lower than in this metabolism study by a factor of approximately 100, hence the level of the unidentified peaks may be lower in practice. The major components of the residue have been identified and a clear metabolic pathway has been elucidated. Therefore the extent of identification is not considered a major deficiency. 19 extractable radioactive residues within the range of 0.014 – 0.047 mg eq/kg (0.25 – 0.8% TRR) are considered characterised and no further attempts to identify these peaks are required. Overall, 0.814 mg eq/kg (13.7% TRR) was identified in the water extract of wheat straw (phenyl label).

In the concentrated ammonia solubilisate of wheat straw (phenyl label), 23 peaks were detected upon analysis with quantitative HPLC method LC13. The main compound was identified as metabolite M684H005 and accounted for 0.160 mg eq/kg (2.7% TRR). M684H005 identification was confirmed using HPLC-MS method LC02Z. The metabolite identified was based on comparison of the retention times and metabolite patterns with those of the HPLC-MS analyses from co-chromatography experiments. 22 additionally characterised peaks were detected, 8 of which were  $\geq 0.01$  mg eq/kg. The total radioactive residues present in the ammonia solubilisate of wheat straw (phenyl label) was 0.408 mg eq/kg (6.9% TRR) and 39.2% of that has been conclusively

identified. The 17 individual residues quantified in the range of 0.01-0.003 mg eq/kg do not require further analysis. The 5 extractable radioactive residues within the range of 0.015 – 0.058 mg eq/kg (0.3 – 1.0% TRR) are considered characterised and no further attempts to identify these peaks are required. Overall, 0.160 mg eq/kg (2.7% TRR) were identified in the ammonia solubilise of wheat straw (phenyl label). Other compounds accounted for a total of 0.247 mg eq/kg (4.8% TRR), were present at 0.058 mg eq/kg (1.0% TRR) or below and were classified as characterised.

Metabolite M684H005 (0.015 mg eq/kg, 0.3% TRR) was also the most abundant among 27 compounds in the concentrated rinse water of wheat straw (phenyl label), which was analysed with quantitative HPLC method LC13. Another peak at 0.007 mg eq/kg (0.1% TRR) was designated as OH-metabolite and classified characterised. OH-metabolite was assigned based on comparison with retention times of reference items, peaks at approx. 113 min in quantitative HPLC-MS (LC13) and the corresponding peaks at approx. 68 min in confirmatory HPLC-MS chromatograms (LC02Z). M684H005 identification was confirmed using HPLC-MS method LC02Z. The metabolite identified was based on comparison of the retention times and metabolite patterns with those of the HPLC-MS analyses from co-chromatography experiments. 25 additionally characterised peaks were detected, none of which were  $\geq 0.01$  mg eq/kg. The total radioactive residues present in concentrated rinse water of wheat straw (phenyl label) are 0.044 mg eq/kg (0.7% TRR) and 34.1% of that has been conclusively identified. The 25 individual radioactive residues within the range of  $<0.001$  – 0.003 mg eq/kg do not require further analysis. Overall, 0.022 mg eq/kg (0.4% TRR) were identified in concentrated rinse water of wheat straw (phenyl label).

Analysis of the concentrated macerozyme solubilise of wheat straw (phenyl label) with quantitative HPLC-MS method LC13 resulted in a pattern of 19 peaks. The main compound accounted for 0.040 mg eq/kg (0.7% TRR), was designated OH-metabolite and was classified as characterised. M684H005 identification was confirmed using HPLC-MS method LC02Z. The metabolite identified was based on comparison of the retention times and metabolite patterns with those of the HPLC-MS analyses from co-chromatography experiments. 18 additionally characterised peaks were detected, none of which were  $\geq 0.01$  mg eq/kg. The 18 individual radioactive residues were quantified in the range of  $<0.001$  – 0.009 mg eq/kg are classified as characterised and do not require further analysis.

Due to the low amounts of radioactive residues, the other enzymes solubilisates of the residual radioactive residue (RRR) of wheat straw (phenyl label), which accounted for a total of 0.082 mg eq/kg or 1.4% TRR, were not analysed by HPLC. In sum, 2.464 mg eq/kg or 41.4% TRR were identified and an additional 3.151 mg eq/kg or 52.9% TRR were characterised in wheat straw (phenyl label). Overall, 5.615 mg eq/kg or 94.3% TRR were identified and characterised in the ERR and RRR of wheat straw (phenyl label).

Table 7-14 Summary of identified and characterised residues in the ERR of wheat straw (phenyl label)

| Labelled radioactive component                    | Extracts                           |       |                                    |       | Sum of extracts |       |
|---|------------------------------------|-------|------------------------------------|-------|-----------------|-------|
|   | Concentrated methanol extract      |       | Purified water extract             |       |                 |       |
|   | mg eq/kg                           | % TRR | mg eq/kg                           | % TRR | mg eq/kg        | % TRR |
| Total radioactive residue (TRR)                   |                                    |       |                                    |       | 5.954           | 100   |
| Identified  |                                    |       |                                    |       |                 |       |
| M684H005  | 0.547                              | 9.2   | 0.306                              | 5.1   | 0.852           | 14.3  |
| M684H006  | 0.47                               | 7.8   | 0.253                              | 4.2   | 0.720           | 12.1  |
| M684H007  | 0.096                              | 1.6   | 0.066                              | 1.1   | 0.162           | 2.7   |
| M684H015  | 0.058                              | 1.0   | 0.022                              | 0.4   | 0.08            | 1.3   |
| M684H016  | 0.147                              | 2.5   | 0.068                              | 1.1   | 0.214           | 3.6   |
| M684H047  | 0.079                              | 1.3   | 0.047                              | 0.8   | 0.16            | 2.1   |
| M684H048  | N.D.                               | N.D.  | N.D.                               | N.D.  | N.D.            | N.D.  |
| M684H055  | 0.056                              | 0.9   | 0.052                              | 0.9   | 0.108           | 1.8   |
| BAS 684 H   | 0.026                              | 0.4   | N.D.                               | N.D.  | 0.026           | 0.4   |
| Total identified by HPLC in ERR                   |                                    |       |                                    |       | 2.288           | 38.4  |
| Characterised                                     |                                    |       |                                    |       |                 |       |
| Number of additionally characterised peaks        | 38 (35 peaks $\geq$ 0.01 mg eq/kg) |       | 26 (26 peaks $\geq$ 0.01 mg eq/kg) |       | -               | -     |
| Maximum of additionally characterised peaks       | 0.207                              | 3.5   | 0.289                              | 4.8   | -               | -     |
| Sum of additionally characterised peaks           | 1.579                              | 26.5  | 1.112                              | 18.7  | 2.691           | 45.2  |
| Total characterised by HPLC in ERR                |                                    |       |                                    |       | 2.691           | 45.2  |
| Total identified and characterised by HPLC in ERR |                                    |       |                                    |       | 4.979           | 83.6  |
| Residual radioactive residue (RRR, calculated)    |                                    |       |                                    |       | 0.853           | 14.3  |
| Total identified and characterised + RRR          |                                    |       |                                    |       | 5.832           | 98.0  |

N.D. = Not detected

Table 7-15 Summary of Identified and characterised residues in the RRR of Wheat straw (Phenyl label)

| Labelled radioactive component                            | Solubilisates                     |       |                          |       |                                      |       | Sum of solubilisates |       |
|---|-----------------------------------|-------|--------------------------|-------|--------------------------------------|-------|----------------------|-------|
|   | Concentrated ammonia solubilisate |       | Concentrated rinse water |       | Concentrated macerozyme solubilisate |       |                      |       |
|   | mg eq/kg                          | % TRR | mg eq/kg                 | % TRR | mg eq/kg                             | % TRR | mg eq/kg             | % TRR |
| Residual radioactive residue (RRR, calculated)            |                                   |       |                          |       |                                      |       | 0.853                | 14.94 |
| Identified  |                                   |       |                          |       |                                      |       |                      |       |
| M684H005  | 0.160                             | 2.7   | 0.015                    | 0.3   | N.D.                                 | N.D.  | 0.175                | 2.9   |
| Total identified by HPLC in RRR                           |                                   |       |                          |       |                                      |       | 0.175                | 2.9   |
| Characterised   |                                   |       |                          |       |                                      |       |                      |       |
| OH-metabolite   | N.D.                              | N.D.  | 0.007                    | 0.1   | 0.04                                 | 0.7   | 0.048                | 0.8   |
| Number of additionally characterised peaks                | 22 (8 peaks ≥ 0.01 mg eq/kg)      |       | 25                       |       | 18                                   |       | -                    | -     |
| Maximum of additionally characterised peaks               | 0.058                             | 1.0   | 0.003                    | 0.1   | 0.009                                | 0.2   | -                    | -     |
| Sum of additionally characterised peaks                   | 0.247                             | 4.2   | 0.022                    | 0.4   | 0.062                                | 1.0   | 0.331                | 5.6   |
| Total characterised by HPLC in RRR                        |                                   |       |                          |       |                                      |       | 0.379                | 6.4   |
| Total identified and characterised by HPLC in RRR         |                                   |       |                          |       |                                      |       | 0.554                | 9.3   |
| Tyrosinase solubilisate                                   |                                   |       |                          |       |                                      |       | 0.043                | 0.7   |
| Amylase solubilisate                                      |                                   |       |                          |       |                                      |       | 0.018                | 0.3   |
| Pepsin solubilisate                                       |                                   |       |                          |       |                                      |       | 0.006                | 0.1   |
| Pancreatin solubilisate                                   |                                   |       |                          |       |                                      |       | 0.014                | 0.2   |
| Total additionally characterised in RRR                   |                                   |       |                          |       |                                      |       | 0.082                | 1.4   |
| Total identified and characterised in RRR                 |                                   |       |                          |       |                                      |       | 0.636                | 10.7  |
| Final residue   |                                   |       |                          |       |                                      |       | 0.113                | 1.9   |
| Total identified and characterised in RRR + final residue |                                   |       |                          |       |                                      |       | 0.749                | 12.6  |

N.D = Not detected

*Cyclohexane-label*

A pattern of 70 peaks was found upon analysis of the concentrated methanol extract of wheat straw (cyclohexane label) with quantitative HPLC-MS method LC13, eight of them were identified. The most abundant components were metabolites M684H006 (1.516 mg eq/kg, 15.6% TRR) and M684H005 (0.700 mg eq/kg, 7.2 % TRR), followed by M684H007 (0.362 mg eq/kg, 3.7% TRR), M684H016 (0.321 mg eq/kg, 3.3% TRR), M684H015 (0.279 mg eq/kg, 2.9% TRR) and M684H047 (0.226 mg eq/kg, 2.3% TRR) at lower levels. Furthermore, metabolites M684H055 and M684H048 were identified, accounting for 0.127 mg eq/kg (1.3% TRR) and 0.022 mg eq/kg (0.2% TRR), respectively. Two peaks totalled 0.113 mg eq/kg (1.2% TRR) and was designated as OH-metabolite and were classified as characterised. The eight metabolites identified were confirmed using HPLC-MS method LC02Z. The eight metabolites identified were based on comparison of the retention times and metabolite patterns with those of the HPLC-MS analyses from co-chromatography experiments. 60 additionally characterised peaks were detected, 57 of which were  $\geq$  0.01 mg eq/kg. The total radioactivity present in the methanol extract of wheat straw (cyclohexane label) was 6.535 mg eq/kg (67.1% TRR) and 54.4% of that has been conclusively identified. The identification of 21 individual residues in the range of 0.050 – 0.095 mg eq/kg was not achieved. Moreover, the higher-level individual residues at 0.100 mg eq/kg (1.0% TRR), 0.111 mg eq/kg (1.1% TRR), 0.125 mg eq/kg (1.3% TRR), and 0.179 mg eq/kg (1.8% TRR) have not been identified despite 8 residues within a similar range of 0.022 – 1.516 mg eq/kg (0.022 – 15.6%



TRR) being identified. The applicant justified that attempts at identification by comparison of retention times and MS data with a range of reference items for postulated metabolites (listed above) did not produce a conclusive structural assignment. Characterisation and identification need to be decided on a case by case basis for this residue based on how much has been identified. Given only 54.4% of the extract has been identified, it would have been preferable for further identification to have been performed. However, the dietary burden is significantly below 0.004 mg/kg bw/day and the animal metabolism studies are significantly overdosed (> 300 N) compared to the dietary burden (Vol 1 Section 2.7.5) hence the level of identification does not affect the overall consumer risk assessment for the representative uses. Additionally, the levels of metabolites M684H005 and M684H006 in wheat straw in the residues trials (Vol 3 CA B.7.3.1) are lower than in this metabolism study by a factor of approximately 100, hence the level of the unidentified peaks may be lower in practice. The major components of the residue have been identified and a clear metabolic pathway has been elucidated. Therefore the extent of identification is not considered a major deficiency. Additionally, the 6 extractable radioactive residues quantified in the range of 0.004 – 0.01 mg eq/kg do not require further analysis. The remaining 28 extractable residues in the range of 0.012 – 0.045 mg eq/kg are considered characterised and no further attempts to identify these peaks are required. Overall, a total of 6.535 mg eq/kg (67.1% TRR) residues were present in the concentrated methanol extract, where a total of 3.553 mg eq/kg (36.5% TRR) were classed as identified and 2.868 mg eq/kg (29.5%) were classed as characterised.

The chromatogram of the concentrated water extract of wheat straw (cyclohexane label) from analysis with quantitative HPLC method LC13 shows a pattern of 22 peaks. Six peaks were identified. Again, the predominant compounds were metabolites M684H005 and M684H006, present at 0.420 mg eq/kg (4.3% TRR) and 0.282 mg eq/kg (2.9% TRR), respectively. Metabolite M684H047 was detected in lower amounts (0.206 mg eq/kg, 2.3% TRR). Additionally, M684H007 (0.061 mg eq/kg, 0.6% TRR), M684H016 (0.058 mg eq/kg, 0.6% TRR) and M684H015 (0.037 mg eq/kg, 0.4% TRR) were identified. Other components were detected at 0.263 mg eq/kg (2.7% TRR) or below, summed up to 1.203 mg eq/kg or 12.4% TRR and were classified as characterised. The six metabolites identified were confirmed using HPLC-MS method LC02Z. The six metabolites identified were based on comparison of the retention times and metabolite patterns with those of the HPLC-MS analyses from co-chromatography experiments. 16 additionally characterised peaks were detected, 15 of which were  $\geq 0.01$  mg eq/kg. The total radioactivity present in the water extract of wheat straw (cyclohexane label) was 2.268 mg eq/kg (23.3% TRR) and 46.9% of that has been conclusively identified. The identification of 8 individual residues in the range of 0.05 – 0.089 mg eq/kg (0.5 – 0.9% TRR) was not achieved. Moreover, the higher-level individual residues at 0.107 mg eq/kg (1.1% TRR), 0.182 mg eq/kg (1.9% TRR) and 0.263 mg eq/kg (2.7% TRR) have not been identified despite 6 residues within a similar range 0.037 – 0.420 mg eq/kg (0.4 – 4.3% TRR) being identified. The applicant justified that attempts at identification by comparison of retention times and MS data with a range of reference items for postulated metabolites (listed above) did not produce a conclusive structural assignment. The applicant reported that poor peak resolution and co-elution of peaks, particularly those at 0.182 mg/kg and 0.263 mg/kg meant identification would not have been straightforward. Characterisation and identification need to be decided on a case by case basis for this residue based on how much has been identified. Given only 46.9% of the extract was identified, it would have been preferable for further identification work to have been performed. However, the dietary burden is significantly below 0.004 mg/kg bw/day and the animal metabolism studies are significantly overdosed (> 300 N) compared to the dietary burden (Vol 1 Section 2.7.5) hence the level of identification does not affect the overall consumer risk assessment for the representative uses. Additionally, the levels of metabolites M684H005 and M684H006 in wheat straw in the residues trials (Vol 3 CA B.7.3.1) are lower than in this metabolism study by a factor of approximately 100, hence the level of the unidentified peaks may be lower in practice. The major components of the residue have been identified and a clear metabolic pathway has been elucidated. Therefore the extent of identification is not considered a major deficiency. Additionally, the 1 extractable radioactive residue quantified at 0.005 mg eq/kg does not require further identification. The remaining 4 residues at in the range of 0.012 – 0.038 mg eq/kg (0.1 – 0.4% TRR) are considered characterised and no further attempts to identify these peaks are required. Overall, a total of 2.268 mg eq/kg (23.3% TRR) residues were present, where a total of 1.064 mg eq/kg (10.9% TRR) were classed as identified and 1.203 mg eq/kg (12.4%) were classed as characterised.

The analysis of the concentration ammonia solubilisate of wheat straw (cyclohexane label) with quantitative HPLC method LC13 revealed a pattern of 29 peaks. The predominant compound was identified as metabolite M684H005 present at 0.320 mg eq/kg (3.3% TRR). Metabolite M684H005 was confirmed using HPLC-MS method LC02Z. M684H005 identification was based on comparison of the retention times and metabolite patterns with those of the HPLC-MS analyses from co-chromatography experiments. The total radioactivity present in the ammonia solubilisate of wheat straw (cyclohexane label) was 0.724 mg eq/kg (7.4% TRR) and only 44.2% of that has been conclusively identified. Therefore, whilst it would have been preferable for the peak at 0.074 mg eq/kg (0.8% TRR) to be identified, it represents a very low % TRR so this is not considered a major

deficiency. Additionally, 19 extractable radioactive residues quantified in the range 0.001 – 0.01 mg eq/kg do not require further analysis. The remaining 7 peaks in the range of 0.016 – 0.041 mg eq/kg (0.2 – 0.4% TRR) are considered characterised and no further attempts to identify these peaks are required. Overall, a total of 0.724 mg eq/kg (7.4% TRR) residues were present, where 0.320 mg eq/kg (3.3% TRR) of residues were identified and 0.404 mg eq/kg (4.2% TRR) were classified as characterised.

The concentrated macerozyme solubilise of wheat straw (cyclohexane label) shows a pattern of 19 peaks in the chromatogram obtained with quantitative HPLC method LC13. The predominant compound was accounted for 0.061 mg eq/kg (0.6% TRR) and was designated as OH-metabolite and was classified as characterised. The total radioactivity present in macerozyme solubilise of wheat straw (cyclohexane label) was 0.105 mg eq/kg (1.1% TRR) and 58.1% of that has been conclusively identified. All 18 extractable radioactive residues were quantified within the range of <0.001 – 0.01 mg eq/kg does not require further identification. Overall, a total of 0.105 mg eq/kg (1.1% TRR) residues were present, where 0.061 mg eq/kg (0.6% TRR) of residues were identified and 0.044 mg eq/kg (0.4% TRR) were classified as characterised.

The tyrosinase solubilise of wheat straw (cyclohexane label), which accounts for 0.065 mg eq/kg (0.7% TRR), was not analysed by HPLC due to low amounts of radioactivity after purification procedure. However, the SPE purification step indicates, that the radioactivity contained in the tyrosinase solubilise is distributed among several fractions. Likewise, the other enzyme solubilisates of the residual radioactive residue (RRR) of wheat straw (cyclohexane label), which accounted for 0.026 mg eq/kg (0.3% TRR) or below, were not analysed by HPLC due to low amounts of radioactivity. The final residue after the enzyme incubations is not considered bioavailable, since it was not released upon treatment with amylase, artificial gastric juice and artificial intestinal fluid, respectively. In sum, 4.938 mg eq/kg or 50.7% TRR were identified and an additional 4.809 mg eq/kg or 49.4% TRR were characterised in the ERR and RRR of wheat straw (cyclohexane label). Overall, 9.747 mg eq/kg or 100.2% TRR were identified and characterised in the ERR and RRR of wheat straw (cyclohexane label).



Table 7-16 Summary of Identified and characterised residues on the ERR of wheat straw (Cyclohexane label)

| Labelled radioactive components                   | Extracts                      |       |                               |       | Sum of extracts |       |
|---|-------------------------------|-------|-------------------------------|-------|-----------------|-------|
|   | Concentrated methanol extract |       | Purified water extract        |       |                 |       |
|   | mg eq/kg                      | % TRR | mg eq/kg                      | % TRR | mg eq/kg        | % TRR |
| Total radioactive residue (TRR)                   |                               |       |                               |       | 9.732           | 100   |
| Identified  |                               |       |                               |       |                 |       |
| M684H005  | 0.700                         | 7.2   | 0.420                         | 4.3   | 1.120           | 11.5  |
| M684H006  | 1.516                         | 15.6  | 0.282                         | 2.9   | 1.798           | 18.5  |
| M684H007  | 0.362                         | 3.7   | 0.061                         | 0.6   | 0.423           | 4.3   |
| M684H015  | 0.279                         | 2.9   | 0.037                         | 0.4   | 0.317           | 3.3   |
| M684H016  | 0.321                         | 3.3   | 0.058                         | 0.6   | 0.379           | 3.9   |
| M684H047  | 0.226                         | 2.3   | 0.206                         | 2.1   | 0.432           | 4.4   |
| M684H048  | 0.022                         | 0.2   | N.D.                          | N.D.  | 0.022           | 0.2   |
| M684H055  | 0.127                         | 1.3   | N.D.                          | N.D.  | 0.127           | 1.3   |
| BAS 684 H   | N.D.                          | N.D.  | N.D.                          | N.D.  | N.D.            | N.D.  |
| Total identified by HPLC in ERR                   |                               |       |                               |       | 4.619           | 47.5  |
| Characterised                                     |                               |       |                               |       |                 |       |
| OH-metabolite                                     | 0.113                         | 1.2   | n.d.                          | n.d.  | 0.113           | 1.2   |
| Number of additionally characterised peaks        | 60 (57 peaks ≥ 0.01 mg eq/kg) |       | 16 (15 peaks ≥ 0.01 mg eq/kg) |       | -               | -     |
| Maximum of additionally characterised peaks       | 0.179                         | 1.8   | 0.263                         | 2.7   | -               | -     |
| Sum of additionally characterised peaks           | 2.868                         | 29.5  | 1.203                         | 12.4  | 4.071           | 41.8  |
| Total characterised by HPLC in ERR                |                               |       |                               |       | 4.184           | 43.0  |
| Total identified and characterised by HPLC in ERR |                               |       |                               |       | 8.802           | 90.4  |
| Residual radioactive residue (RRR, calculated)    |                               |       |                               |       | 1.379           | 14.2  |
| Total identified and characterised + RRR          |                               |       |                               |       | 10.181          | 104.6 |

N.D = Not detected

Table 7-17 Summary of identified and characterised residues in the RRR of wheat straw (cyclohexane label)

| Labelled radioactive components                    | Solubilisates                 |       |                             |       | Sum of solubilisates |       |
|--|-------------------------------|-------|-----------------------------|-------|----------------------|-------|
|  | Ammonia solubilisate          |       | Macerozyme                  |       |                      |       |
|  | mg eq/kg                      | % TRR | mg eq/kg                    | % TRR | mg eq/kg             | % TRR |
| Residual radioactive residue (RRR, calculated)     |                               |       |                             |       | 1.379                | 14.10 |
| Identified   |                               |       |                             |       |                      |       |
| M684H005   | 0.320                         | 3.3   | N.D.                        | N.D.  | 0.320                | 3.3   |
| Total identified by HPLC in ERR                    |                               |       |                             |       | 0.320                | 3.3   |
| Characterised                                      |                               |       |                             |       |                      |       |
| OH-metabolite                                      | N.D.                          | N.D.  | 0.061                       | 0.6   | 0.061                | 0.6   |
| Number of additionally characterised peaks         | 28 (15 peaks ≥ 0.01 mg eq/kg) |       | 18 (1 peak ≥ 0.01 mg eq/kg) |       | -                    | -     |
| Maximum of additionally characterised peaks        | 0.074                         | 0.8   | 0.012                       | 0.1   | -                    | -     |
| Sum of additionally characterised peaks            | 0.404                         | 4.2   | 0.044                       | 0.4   | 0.448                | 4.6   |
| Total characterised by HPLC in RRR                 |                               |       |                             |       | 0.509                | 5.2   |
| Total identified and characterised by HPLC in RRR  |                               |       |                             |       | 0.829                | 8.5   |
| Tyrosinase solubilisate                            |                               |       |                             |       | 0.065                | 0.7   |
| Amylase solubilisate                               |                               |       |                             |       | 0.026                | 0.3   |
| Pepsin solubilisate                                |                               |       |                             |       | 0.008                | 0.1   |
| Pancreatin solubilisate                            |                               |       |                             |       | 0.016                | 0.2   |
| Total additionally characterised in RRR            |                               |       |                             |       | 0.116                | 1.2   |
| Total identified and characterised                 |                               |       |                             |       | 0.945                | 9.7   |
| Final residue                                      |                               |       |                             |       | 0.122                | 1.3   |
| Total identified and characterised + final residue |                               |       |                             |       | 1.066                | 11.0  |

N.D = Not detected

*Grain*

As only very low levels of radioactive residues were recovered in wheat grain of both labels, the extracts and solubilisates were not analysed by HPLC. In total, 0.007 mg eq/kg or 69.5% TRR (phenyl label) and 0.009 mg eq/kg or 72.8% TRR (cyclohexane label) were characterised.

Table 7-18 Summary of Characterised Residues in the ERR and RRR of Wheat grains (phenyl label)

| Designation   | Sum of extracts/solubilisate |              |
|---|------------------------------|--------------|
|   | mg eq/kg                     | % TRR        |
| <b>Total radioactive residue (TRR)</b>                    | 0.009                        | 100          |
| <b>Methanol extract</b>                                   | 0.002                        | 22.7         |
| <b>Water extract</b>                                      | 0.003                        | 31.2         |
| <b>Total characterised in ERR</b>                         | <b>0.005</b>                 | <b>53.9</b>  |
| <b>Residual radioactive residue (RRR, calculated)</b>     | 0.004                        | 46.1         |
| <b>Ammonia solubilisate</b>                               | 0.001                        | 15.6         |
| <b>Total characterised in RRR</b>                         | <b>0.001</b>                 | <b>15.6</b>  |
| <b>Total characterised in ERR and RRR</b>                 | <b>0.007</b>                 | <b>69.5</b>  |
| <b>Final residue</b>                                      | 0.003                        | 30.5         |
| <b>Total characterised in ERR and RRR + final residue</b> | <b>0.009</b>                 | <b>100.0</b> |

Table 7-19 Summary of Characterised Residues in the ERR and RRR of Wheat grains (Cyclohexane label)

| Designation   | Sum of extracts/solubilisate |              |
|---|------------------------------|--------------|
|   | mg eq/kg                     | % TRR        |
| <b>Total radioactive residue (TRR)</b>                    | 0.012                        | 100          |
| <b>Methanol extract</b>                                   | 0.003                        | 27.6         |
| <b>Water extract</b>                                      | 0.004                        | 33.1         |
| <b>Total characterised in ERR</b>                         | <b>0.007</b>                 | <b>60.7</b>  |
| <b>Residual radioactive residue (RRR, calculated)</b>     | 0.005                        | 39.3         |
| <b>Ammonia solubilisate</b>                               | 0.001                        | 12.1         |
| <b>Total characterised in RRR</b>                         | <b>0.001</b>                 | <b>12.1</b>  |
| <b>Total characterised in ERR and RRR</b>                 | <b>0.009</b>                 | <b>72.8</b>  |
| <b>Final residue</b>                                      | 0.003                        | 27.2         |
| <b>Total characterised in ERR and RRR + final residue</b> | <b>0.012</b>                 | <b>100.0</b> |

Table 7-20 Summary of metabolites identified in wheat matrices

| Designation   | Wheat forage              |         | Wheat straw  |         |
|---|---------------------------|---------|--------------|---------|
|   | [mg eq/kg]                | [% TRR] | [mg eq/kg]   | [% TRR] |
| <b>Phenyl label</b>   |                           |         |              |         |
| M684H005 <sup>1</sup>                                       | 0.296                     | 11.2    | 1.028        | 17.3    |
| M684H006  | 0.770                     | 29.2    | 0.720        | 12.1    |
| M684H007  | 0.212                     | 8.1     | 0.162        | 2.7     |
| M684H015  | 0.063                     | 2.4     | 0.080        | 1.3     |
| M684H016  | 0.135                     | 5.1     | 0.214        | 3.6     |
| M684H047  | 0.088                     | 3.3     | 0.126        | 2.1     |
| M684H048  | not detected <sup>2</sup> |         | not detected |         |
| M684H055  | 0.063                     | 2.4     | 0.108        | 1.8     |
| BAS 684 H   | 0.081                     | 3.1     | 0.026        | 0.4     |
| Total identified  | 1.708                     | 64.9    | 2.464        | 41.4    |
| Total characterised from ERR                                | 0.722                     | 27.4    | 2.691        | 45.2    |
| Total characterised from RRR                                | 0.048                     | 1.8     | 0.461        | 7.7     |
| Total identified and characterised                          | 2.478                     | 94.2    | 5.615        | 94.3    |
| Final residue   | 0.021                     | 0.8     | 0.113        | 1.9     |
| Grand total of identified and characterised + final residue | 2.499                     | 95.0    | 5.728        | 96.2    |
| <b>Cyclohexane label</b>                                    |                           |         |              |         |
| M684H005 <sup>1</sup>                                       | 0.396                     | 14.8    | 1.440        | 14.8    |
| M684H006  | 0.796                     | 29.7    | 1.798        | 18.5    |
| M684H007  | 0.177                     | 6.6     | 0.423        | 4.3     |
| M684H015  | 0.116                     | 4.3     | 0.317        | 3.3     |
| M684H016  | 0.219                     | 8.2     | 0.379        | 3.9     |
| M684H047  | 0.066                     | 2.5     | 0.432        | 4.4     |
| M684H048  | not detected              |         | 0.022        | 0.2     |
| M684H055  | 0.058                     | 2.2     | 0.127        | 1.3     |
| BAS 684 H   | 0.054                     | 2.0     | not detected |         |
| Total identified  | 1.881                     | 70.3    | 4.938        | 50.7    |
| Total characterised from ERR                                | 0.782                     | 29.2    | 4.184        | 43.0    |
| Total characterised from RRR                                | 0.094                     | 3.5     | 0.625        | 6.4     |
| Total identified and characterised                          | 2.757                     | 103.0   | 9.747        | 100.2   |
| Final residue   | 0.035                     | 1.3     | 0.122        | 1.3     |
| Grand total of identified and characterised + final residue | 2.793                     | 104.3   | 9.869        | 101.4   |

1 Including amounts, which were detected in the solubilisates and rinse water

2 M684H048 was identified in a concentrated sample. However, M684H048 could not be assigned to a certain peak in the respective chromatogram used for quantification.

#### *Cleavage experiments*

Cleavage experiments were conducted with the methanol extract of wheat forage (phenyl label) and with individual fractions of the methanol extracts of wheat straw (cyclohexane label) to investigate the stability of conjugated metabolites to support the development of the residue analytical method.

#### *Wheat forage (Phenyl-label)*

Upon treatment of the methanol extract with hydrochloric acid or ammonia, metabolite M684H006 was converted into metabolite M684H005 due to saponification of the malonic acid glucosyl ester. After hydrochloric treatment or ammonia treatment of the methanol extract of wheat forage (phenyl label) metabolites M684H006 and M684H007 decreased, whilst metabolite M684H005 increased. The metabolites were identified by co-elution with reference standards using HPLC-MS method LC02Z. Following treatment of methanol extract with  $\beta$ -glucosidase results in depletion of metabolite M684H005 to aglycone M684H002, whereas, no significant change in metabolites M684H006 and M684H007 was observed. The four metabolites were identified by co-elution with reference standards using HPLC-MS method LC02Z. Upon treatment of the methanol extracts with consecutive ammonia and  $\beta$ -glucosidase results in complete depletion of the main fractions and formation aglycone M684H002 due to the consecutive cleavage of the malonic acid ester and the glucosidic bond. The metabolites were identified by co-elution with reference standards using HPLC-MS method LC02Z. Following rumen fluid treatment metabolites M684H005, M684H006 and M684H007 deplete to metabolite M684H002. The metabolites were identified by co-elution with reference standards using HPLC-MS method LC02Z.

*Wheat straw (Cyclohexane-label)*

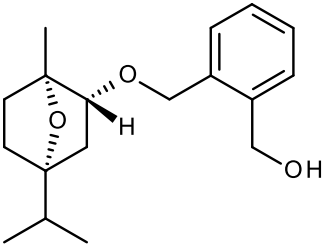
Upon treatment of wheat straw methanol extract (cyclohexane label) with ammonia treatment (experiment of fraction 23 min), saponification of metabolite M684H047 to metabolite M684H48 was observed. The metabolites were identified by co-elution with reference standards using HPLC-MS method LC02Z. Treatment of wheat straw methanol extract (cyclohexane label) with rumen fluids (experiment of fraction 23 min) results in a pattern of peaks. Results from a related metabolism study in oilseed rape, indicate that the peak corresponds to a twofold hydroxylated aglycone M684H044 after rumen fluid treatment. Prior to rumen fluid treatment, metabolite M684H047 was identified based on comparison of retention times with external standard.

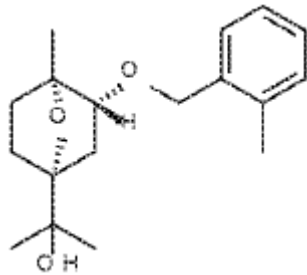
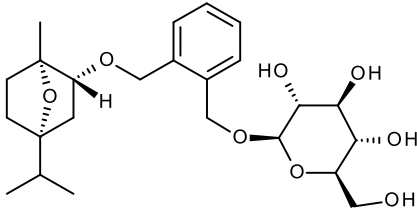
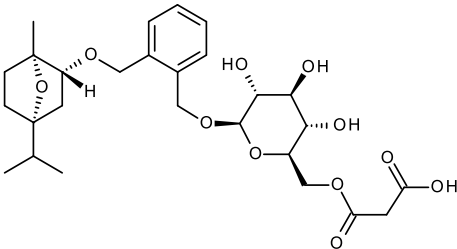
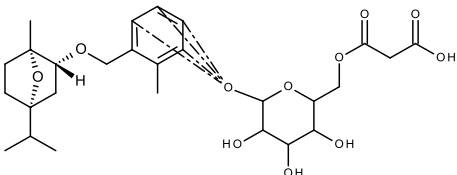
In fraction 33 min, metabolite M684H005 was converted to the aglycone M684H002 (after treatment with rumen fluid and ammonia) from metabolite M684H005. Metabolites M684H002 and M684H005 were identified by co-elution with reference standards; furthermore, metabolite M684H005 was identified by NMR spectroscopy.

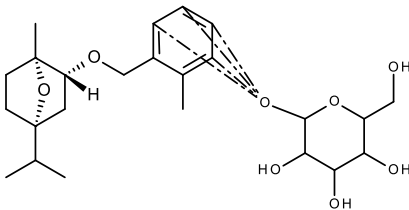
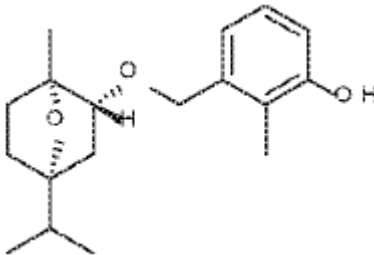
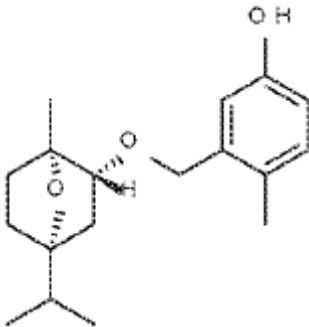
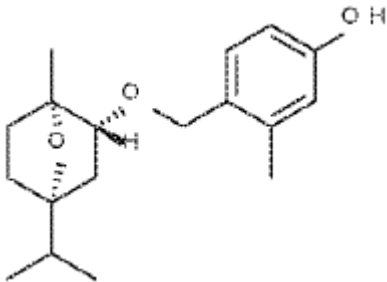
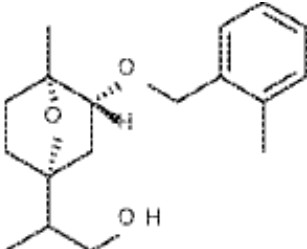
In fraction 53 min, treatment with rumen fluid resulted in formation of peaks from M684H007, which were identified as aglycones M684H004; M684H017; M684H018; M684H019; M684H024 and metabolite/aglycone M684H002. Metabolites were identified by co-elution of references standards using HPLC-MS method LC02Z.

In summary the step-wise degradation of malonyl glucosidic components has been proposed below (see Figure 7-3). In a first step, the malonyl moiety is cleaved in presence of acid or base. Subsequent exposure of the glucosidic components towards  $\beta$ -glucosidase results in the formation of the corresponding aglycones. Furthermore, the direct degradation of both malonyl glucosidic and malonyl components to the respective aglycones in presence of rumen fluids has been discussed above, suggests that the conjugated metabolites are converted into the respective aglycones by passing the gastrointestinal track of ruminant animals.

Table 7-21 Summary of identified metabolites

| Designation | Structure   | Fraction(s) the metabolite(s) are observed in  |
|-------------|---|--|
| M684H002    |  | <p>Wheat forage (Phenyl label)<br/>-consecutive Ammonia and <math>\beta</math>-glucosidase treatment<br/>-Rumen Fluid Treatment</p> <p>Fraction 33 of the Wheat Straw Methanol Extract (Cyclohexane label) – Rumen Fluid Treatment</p> <p>Fraction 47 of the Wheat Straw Methanol extraction (Cyclohexane label) – Rumen Fluid Treatment</p> <p>Fraction 53 of the Wheat Straw Methanol extraction</p> |

|          |  |   |
|----------|--|---|
|          |  | (Cyclohexane label) – Rumen Fluid Treatment   |
| M684H004 |     | Fraction 53 of the Wheat Straw Methanol extraction<br>(Cyclohexane label) – Rumen Fluid Treatment   |
| M684H005 |     | Wheat forage (Phenyl label)<br>- Hydrochloric Acid Treatment,<br>- Ammonia Treatment and $\beta$ -glucosidase<br>- and consecutive Ammonia and $\beta$ -glucosidase treatment<br>- Rumen Fluid Treatment<br><br>Fraction 33 of the Wheat Straw Methanol Extract (Cyclohexane label) – Rumen Fluid Treatment<br><br>Fraction 47 of the Wheat Straw Methanol extraction (Cyclohexane label) – Ammonia Treatment<br><br>Fraction 47 of the Wheat Straw Methanol extraction (Cyclohexane label) – Rumen Fluid Treatment |
| M684H006 |  | Wheat forage (Phenyl label)<br>- Hydrochloric Acid Treatment,<br>- Ammonia Treatment and $\beta$ -glucosidase<br>- and consecutive Ammonia and $\beta$ -glucosidase treatment<br>- Rumen Fluid Treatment<br><br>Fraction 47 of the Wheat Straw Methanol extraction (Cyclohexane label) – Ammonia Treatment<br><br>Fraction 47 of the Wheat Straw Methanol extraction (Cyclohexane label) – Rumen Fluid Treatment  |
| M684H007 |  | Wheat forage (Phenyl label) - - Hydrochloric Acid treatment<br>- Ammonia Treatment and $\beta$ -glucosidase<br>- and consecutive Ammonia and $\beta$ -glucosidase treatment<br>- Rumen Fluid Treatment<br><br>Fraction 53 of the Wheat Straw  |

|          |   |  |
|----------|---|--|
|          |   | <p>Methanol extraction<br/>(Cyclohexane label) – Ammonia<br/>Treatment</p> <p>Fraction 53 of the Wheat Straw<br/>Methanol extraction<br/>(Cyclohexane label) – Rumen<br/>Fluid Treatment</p> |
| M684H008 |    | <p>Fraction 53 of the Wheat Straw<br/>Methanol extraction<br/>(Cyclohexane label) – Ammonia<br/>Treatment</p>  |
| M684H017 |    | <p>Fraction 53 of the Wheat Straw<br/>Methanol extraction<br/>(Cyclohexane label) – Rumen<br/>Fluid Treatment</p>  |
| M684H018 |   | <p>Fraction 53 of the Wheat Straw<br/>Methanol extraction<br/>(Cyclohexane label) – Rumen<br/>Fluid Treatment</p>  |
| M684H019 |  | <p>Fraction 53 of the Wheat Straw<br/>Methanol extraction<br/>(Cyclohexane label) – Rumen<br/>Fluid Treatment</p>  |
| M684H024 |  | <p>Fraction 53 of the Wheat Straw<br/>Methanol extraction<br/>(Cyclohexane label) – Rumen<br/>Fluid Treatment</p>  |
| M684H044 |   | <p>Fraction 23 of the Wheat Straw<br/>Methanol Extract (cyclohexane)</p>   |

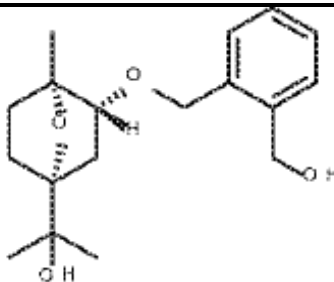
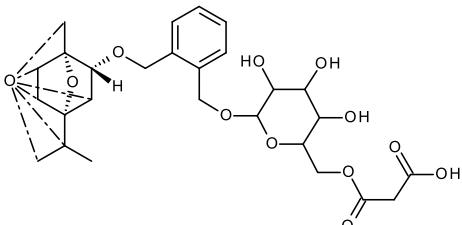
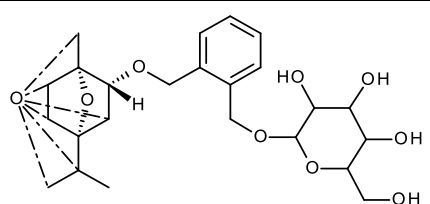
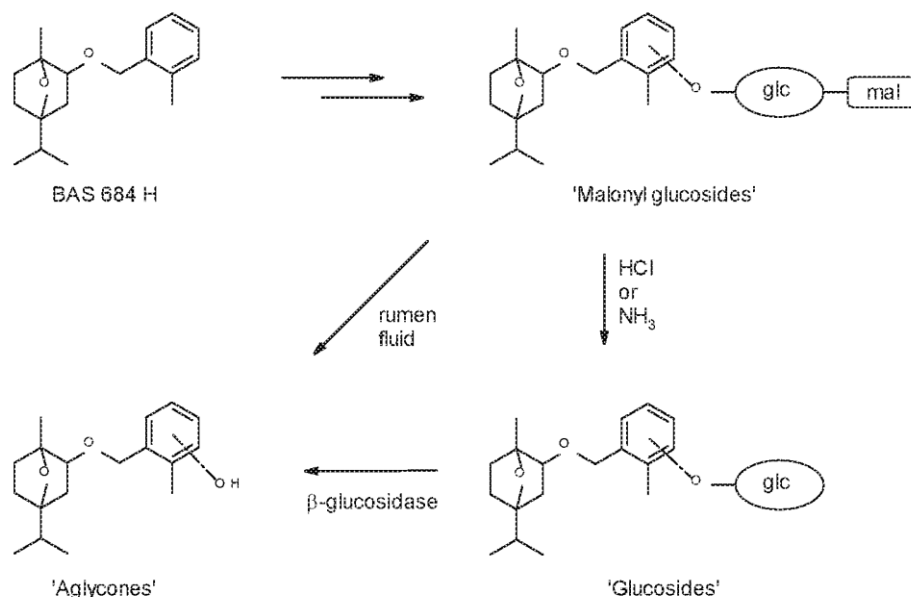
|          |  |   |
|----------|--|---|
|          |   | label) – Rumen Fluid Treatment  |
| M684H047 |  | Fraction 23 of the Wheat Straw Methanol Extract (cyclohexane label) – Ammonia Treatment - Rumen Fluid Treatment |
| M684H048 |   | Fraction 23 of the Wheat Straw Methanol Extract (cyclohexane label) – Ammonia Treatment                         |

Figure 7-3 Cleavage experiments with individual fractions of extracts of wheat forage and straw



Note: cineol-substituted metabolites are omitted for the sake of clarity. Abbreviations: glc: glucosyl moiety; mal: malonyl moiety.

#### Chiral analysis

Chiral analysis was performed to investigate whether one enantiomer of BAS 684 H was preferably metabolised in wheat matrices (forage, straw and grain). BAS 684 H was not detected at sufficient levels (<0.01 mg eq/kg)



in all wheat matrices for both  $^{14}\text{C}$ -labels, therefore, its main metabolite M684H005 was investigated in representative samples of wheat forage and straw (both cyclohexane label) by HPLC-MS.

The methanol extracts of wheat forage and straw were fractionated using HPLC-MS method LC03. The analysis of these fractions with chiral HPLC-MS method LC014 resulted in a pattern of two peaks corresponding to stereoisomeric ratios listed below in Table 7-22.

**Table 7-22 Determination of the diastereomeric ratio of M684H005 in wheat matrices**

| Matrix                   | Diastereomer 1 [%AR] | Diastereomer 2 [%AR] | SE [%] |
|--------------------------|----------------------|----------------------|--------|
| <b>Cyclohexane-label</b> |                      |                      |        |
| <b>forage</b>            | 36                   | 64                   | -28    |
| <b>straw</b>             | 34                   | 66                   | -32    |

SE = stereoisomers excess,  $SE = [(A1\% \text{ AR} - A2\% \text{ AR}) / (A1\% \text{ AR} + A2\% \text{ AR})]$

The analyses with chiral HPLC method of the isolated fractions demonstrate the stereoisomer excess (SE) are similar in both matrices (-28 and -32 in forage and straw respectively). The stereoisomeric ratio of parent BAS 684 H as applied was 51:49 ((-):(+)), whereas the stereoisomeric ratio of M684H005 in wheat forage and straw is 35:65 (diastereomer 1: diastereomer 2), which represents a significant change in stereoisomeric ratio upon metabolism in wheat.

The two diastereomers of M684H005 were not available as reference items to allow definite assignment of each peak to a diastereomer. However, given the column and solvents used were similar to that for enantiomeric analysis of parent, the order of elution may suggest that the first eluting peak, diastereomer 1 originates from the (-)-enantiomer of parent, and diastereomer 2 from the (+)-enantiomer of parent. The inability to definitively assign a diastereomer to each peak and the significant change in stereoisomeric ratio are not considered to affect the consumer risk assessment given the toxicological evaluation of the two diastereomers concluded they are of equivalent toxicity (Vol 1 Section 2.12.3).

Wheat forage and straw (phenyl-label) were not analysed by the chiral HPLC-MS method despite sufficient levels of M684H005 (0.547 mg eq/kg and 0.259 mg eq/kg for straw and forage respectively) given M684H005 is not a label-specific metabolite.

#### *Storage stability*

Wheat forage, straw and grain were extracted up to 369 days after sampling and the extracts were analysed up to 384 days after extraction. The period from sampling to analysis was up to 609 days.

*Matrix stability:* Wheat forage and straw were extracted with methanol and water 173 days and 731 days after harvest and analysed by HPLC. The chromatograms of the initial methanol extract before and after storage show similar peak patterns with changes in peak intensities, with the level of metabolite M684H006 slightly increased.

*Extract stability:* The methanol and water extracts of wheat straw (phenyl label) and wheat forage (cyclohexane label) were analysed after extraction (2–66 days) and after storage of the extracts for 554–590 days. The chromatograms showed changes in the levels of metabolites, particularly some conversion of M684H006 to M684H005, but similar overall metabolic patterns.

Given M684H005 and M684H006 are observed before and after matrix and extract storage, and the overall metabolic patterns are similar, the matrix and extract storage intervals in the study are considered acceptable.

Therefore it is concluded that sample integrity was maintained for the storage intervals in the study.

### *Proposed metabolic pathway*

Metabolism was investigated in foliar treated wheat using phenyl- and cyclohexane- labelled BAS 684 H. When the results from both labels are considered together the data demonstrate consistent metabolic pathway in wheat forage and straw. The proposed metabolic pathway is outlined in Figure 7-4.

No cleavage of the molecule was observed though metabolism of BAS 684 H in wheat. The metabolic routes include hydroxylation of the parent compound at various positions and subsequent conjugation of these hydroxyl groups with glycoside and malonyl glycoside. Furthermore, BAS 684 H was only detected in trace amounts by MS and accounted for 0.4% TRR (0.026 mg eq/kg) in wheat straw (phenyl-label only) and for  $\geq 2.0\%$  TRR ( $\geq 0.054$  mg eq/kg) in wheat forage (for both radio labels).

The metabolites M684H005 and M684H006 represent the two major parts of radioactive residues in the directly exposed plant parts forage and straw ( $\geq 29.4\%$  TRR ( $\geq 0.166$  mg eq/kg) combined). These two primary metabolites in wheat (M684H005 and M684H006) result from hydroxylation of the parent compound BAS 684H at the methyl group of the phenyl moiety and subsequent conjugation with glucoside and malonyl glucoside, respectively. Hydroxylation and subsequent conjugation on other positions of the phenyl moiety results in the metabolites: M684H015, M684H016 and M684H007, which are present at lower levels in both wheat straw and forage. Metabolite M684H008 was not identified but represents the generic structure of the phenyl ring conjugates of glucosides and is included in the metabolic pathway for the sake of completeness.

In contrast, in wheat grain, which was not present during the time of application, BAS 684 H and its metabolites were not detected.

The parent compound was also hydroxylated and conjugated at the cineol moiety, resulting in metabolite M684H055 (in both wheat straw and forage).

Multiple hydroxylation at both the phenyl and cineol moiety and subsequent conjugation with glucoside and malonyl glucoside result in the formation of metabolites M684H048 and M684H047, respectively. M684H047 was detected in wheat straw for both phenyl- and cyclohexane-label (2.1 and 4.4% TRR (0.126 mg eq/kg and 0.432 mg eq/kg) respectively) and in wheat forage for both phenyl- and cyclohexane-label (3.3 and 2.5% TRR (0.088 mg eq/kg and 0.066 mg eq/kg) respectively). Whereas, M684H048 was found at low amounts (0.2% TRR (0.022 mg eq/kg)) in wheat straw only for the cyclohexane-label only.

Metabolite M684H054, a phenyl ring conjugate of acetylglycoside, most likely results from abiotic degradation of a corresponding malonyl glucoside through a decarboxylation reaction. However, since it was not detected in the solvent extracts and solubilisates used for quantification, M684H054 is not included in the metabolic pathway.

Since the aglycones after deglucosilation were not identified in the solvent extracts and solubilisates of wheat matrices, they are likewise not included in the metabolic pathway.

### *Conclusion*

The present study describes the metabolism of BAS 684 H (BAS 684 H) in wheat after single foliar spray application at a maximum rate of 500 g a.s./ha. Immature wheat plants (wheat forage) were collected at growth stage BBCH 59 (11 DAT for the phenyl label and 13 DAT for the cyclohexane label). Mature wheat plants were harvested at growth stage BBCH 89 (56 DAT) and separated into wheat straw and wheat grains.

The TRRs of wheat grain were very low ( $\leq 0.012$  mg eq/kg, both labels), the TRRs of wheat forage were 2.632 - 2.678 mg eq/kg (both labels) whilst the TRRs for wheat straw accounted for 5.954 mg eq/kg (phenyl label) and 8.271 - 9.732 mg eq/kg (cyclohexane label) (Table 7-8). Within the same matrix, the amount of radioactive residues was comparable for both labels.

Wheat matrices were extracted with methanol and water. The overall extractability was high for wheat forage and straw ranging from 85.7% TRR to 96.2% TRR (2.531 – 8.353 mg eq/kg) and was low for wheat grain ( $\leq 60.7\%$  TRR ( $\leq 0.007$  mg eq/kg)). For wheat forage and straw, radioactive residues were mainly extracted with methanol. For wheat grain, radioactive residues were extracted in similar small amounts with methanol and water.

The residues after solvent extraction were further solubilised by ammonia and enzyme incubations. For all matrices, ammonia solubilisation released the highest portions of radioactive residues. Further procedures solubilised radioactive residues ranging from 0.1% TRR to 15.6% TRR (0.001 – 0.772 mg eq/kg). For wheat forage and straw, the final residues were each below or equal to 1.9% TRR or 0.122 mg eq/kg and are not considered to be bioavailable (

Table 7-11 to

Table 7-17). For wheat grain, the final residues were each below or equal to 0.003 mg eq/kg (30.5 % TRR) (

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Table 7-18 and Table 7-19).

Structure elucidation was mainly based on HPLC-MS and NMR analysis of purified methanol extracts and fraction thereof of wheat forage (phenyl and cyclohexane label) and wheat straw (cyclohexane label). Peaks were assigned by co-chromatography with external samples or by comparison of the retention time and the metabolites pattern. This resulted in the identification of the parent compound BAS 684 H and its metabolites M684H005, M684H006, M684H007, M684H008, M684H015, M684H016, M684H054 and M684H055. Metabolites M684H047 and M684H048 were identified by co-chromatography with external sample from a related metabolism study of BAS 684 H in oilseed rape.

The most abundant components in wheat forage and straw were metabolites M684H005 ranging between 11.2-17.3% TRR (0.296 mg eq/kg – 1.440 mg eq/kg) for both labels and metabolite M684H006 ranging between 12.1 – 29.7 % TRR (0.720 mg eq/kg – 1.798 mg eq/kg) for both labels (

Table 7-11 to

Table 7-17). The parent compound was detected at 3.1% TRR (0.081 mg eq/kg) or below in wheat forage and straw in both labels (with the exception of wheat straw, cyclohexane label where the parent compound was not identified). Other metabolites accounted for up to 8.2% TRR (0.432 mg eq/kg) (

Table 7-11 to



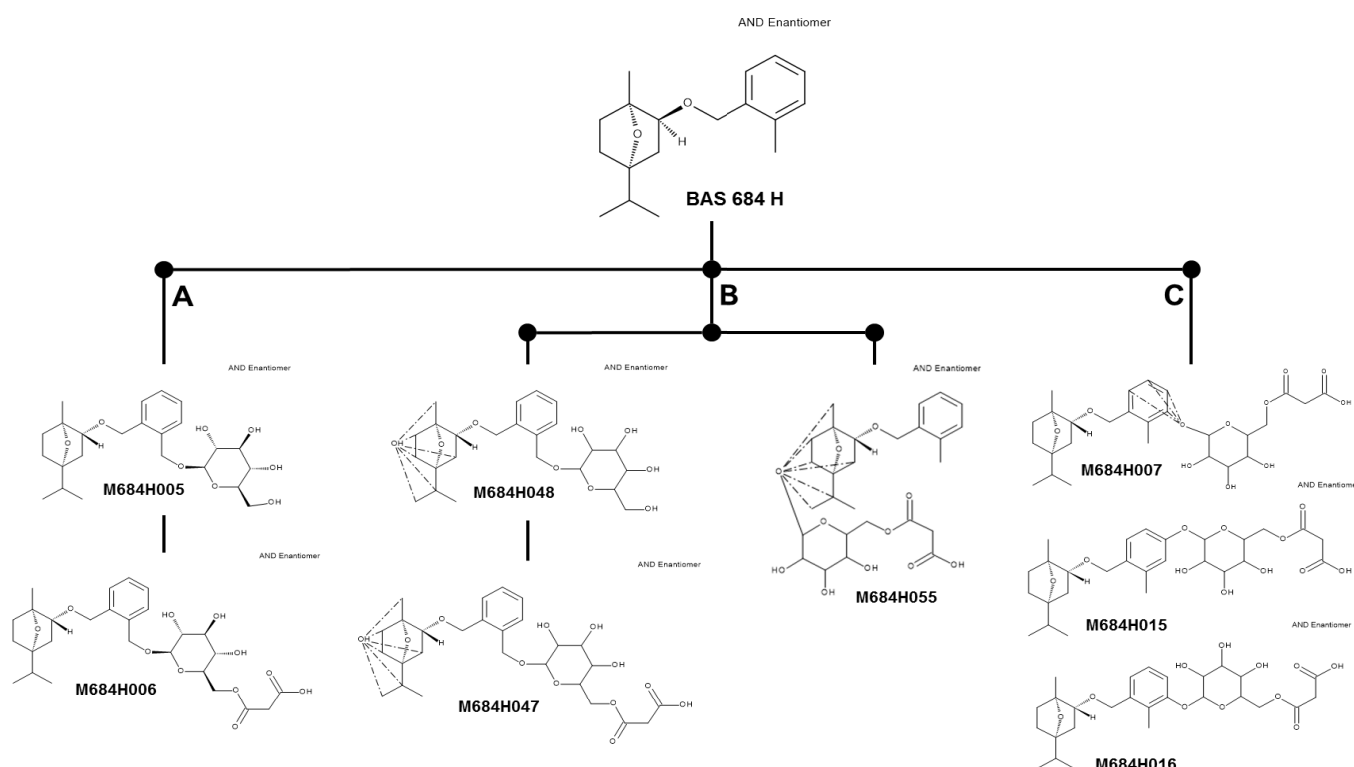
Table 7-17).

Up to 37 peaks  $\geq 0.01$  mg/kg in the extracts of wheat forage and straw were not identified, including up to 7 unidentified peaks  $\geq 0.05$  mg/kg at a maximum of 0.289 mg eq/kg or 4.8% TRR. Attempts were made to identify these peaks including comparison of retention times and MS data with a range of reference items for postulated metabolites. For some extracts, poor peak resolution and co-elution of peaks hindered identification. Whilst further identification would have been preferable, there are no representative uses on cereal forage; for cereal straw, the dietary burden is significantly below 0.004 mg/kg bw/day and the animal metabolism studies are significantly overdosed ( $> 300$  N) compared to the dietary burden (Vol 1 Section 2.7.5). Therefore the extent of identification does not affect the overall consumer risk assessment for the representative uses. Additionally, the levels of metabolites M684H005 and M684H006 in wheat straw in the residues trials (Vol 3 CA B.7.3.1) are lower than in the metabolism study by a factor of approximately 100, hence the levels of the unidentified components may be lower in practice. The major components of the residue have been identified and a clear metabolic pathway has been elucidated with all likely metabolites excluded by comparison to reference standards. Therefore the extent of identification is not considered a major deficiency for the representative uses. If there are future uses on cereals, including forage uses, which significantly increase the dietary burden, consideration should be made of whether these conclusions remain valid.

The metabolic transformation steps of BAS 684 H in wheat are hydroxylation of the parent compound at various positions and subsequent conjugation of hydroxyl groups with glycoside and malonyl glycoside.

No cleavage of the molecule was observed through metabolism of BAS 684 H in wheat.

Figure 7-4 Proposed pathway of BAS 684 H in spring wheat



#### B.7.2.1.2. Oilseed rape

**Report:** CA 6.2.1/002  
Rabe U., Forieri I., 2018a  
Metabolism of  $^{14}\text{C}$ -BAS 684 H in oilseed rape  
2017/1110861

**Guidelines:** EPA 860.1000, EPA 860.1300: Nature of the Residue in Plants Livestock, PMRA Residue Chemistry Guidelines Section 97.2 Nature of the Residue - Plants - Livestock (Canada), EEC 7028/VI/95 rev. 3 Appendix A (EU): Metabolism and distribution in plants, JMAFF 59 NohSan No 4200, Test No. 501: Metabolism in crops

GLP: yes

### Materials and methods

#### Materials

##### 1. C-label BAS 684 H (CAS No. 87818-31-3)

|                              |  |
|------------------------------|--|
| <b>Description:</b>          | Phenyl-U- <sup>14</sup> C (spec. activity of a.s. 17.1 MBq/mg) |
| <b>Lot/Batch #:</b>          | 1147-2001  |
| <b>Radiochemical Purity:</b> | 98.9%  |
| <b>Chemical Purity:</b>      | 97.0%  |

##### 2. C-label BAS 684 H (CAS No. 87818-31-3)

|                              |                                |
|------------------------------|--------------------------------|
| <b>Description:</b>          | Cyclohexane-4- <sup>14</sup> C |
| <b>Lot/Batch #:</b>          | 1146-1001                      |
| <b>Radiochemical Purity:</b> | 99.4%                          |
| <b>Chemical Purity:</b>      | 99.3%                          |

##### 3. C-label BAS 684 H (CAS No. 87818-31-3)

|                         |                         |
|-------------------------|-------------------------|
| <b>Description:</b>     | Benzyl- <sup>13</sup> C |
| <b>Lot/Batch #:</b>     | 1159-1012               |
| <b>Chemical Purity:</b> | <b>99.6%</b>            |

##### 4. BAS 684 H (CAS No. 87818-31-3)

|                         |                      |
|-------------------------|----------------------|
| <b>Description:</b>     | Unlabelled BAS 684 H |
| <b>Lot/Batch #:</b>     | L87-84               |
| <b>Chemical Purity:</b> | <b>99.0%</b>         |

#### Methods

A metabolism study on oilseed rape (variety *Brassica napus L.*) was investigated using BAS 684 H radiolabelled in the cyclohexane ring (cyclohexane-label) or in the phenyl ring (phenyl-label). The study was carried out in 2015-2019 at the Agricultural Research Centre of BASF SE in Limburgerhof, Germany indoors. Plants were grown in a climatic chamber, which simulated natural climatic conditions of a typical oilseed rape growing area. The experiments with both labels were carried out within the same time period.

Oilseed rape seeds were sown into boxes filled with sandy loam. After 26-28 days plants were reducing to 15 plants per box. For both labels, the crops were treated once with BAS 684 H, at growth stage BBCH 18 (timings in line with the proposed post-emergence GAP). The test item was applied as EC (emulsifiable concentrate) formulation at a total nominal application rate of 250.0 g a.s./ha. Straw, hulls and seeds samples of both labels were generated 90 DAT at growth stage BBCH 89 for both labels. A summary of the applications in the study are given in Table 7-23.

**Preparation 1:** For the preparation of the application formulation of the phenyl label: phenyl-U-<sup>14</sup>C (dissolved in toluene), benzyl-<sup>13</sup>C and unlabelled BAS 684 H were mixed to obtain a ratio of approximately 1:1:1.

**Preparation 2:** For the preparation of the application formulation of the cyclohexane label, cyclohexane-4-<sup>14</sup>C (dissolved in toluene) and unlabelled BAS 684 H were mixed in an approximate ratio of 1:1.

The solvents were evaporated and stored in a freezer. On the day of application, the mixtures were taken up in water and blank formulation assisted by ultrasonication. The purity of the application solutions was confirmed using HPLC and the isotopic pattern as well as the identity was determined and verified by HPLC-MS analysis.

The structural formulae of the labelled BAS 684 H molecules are given in Figure 7.2-2.

Table 7-23 Study design: plant uptake part (oilseed rape)

| Label                               | <sup>14</sup> C-Phenyl-label<br>(with <sup>13</sup> C-Benzyl-label) |    | <sup>14</sup> C-Cyclohexane-label |    |
|-------------------------------------|---|----|-----------------------------------|----|
| intended use rate [g a.s./ha]       | 250   |    | 250                               |    |
| Actual application rate [g a.s./ha] | 242.29  |    | 251.48                            |    |
| application number                  | 1   |    | 1                                 |    |
| application growth stage            | BBCH18  |    | BBCH18                            |    |
| sampled matrices                    | Straw, hulls, seeds   |    | Straw, hulls, seeds               |    |
| sampling [DALA] <sup>1)</sup>       | Straw   | 90 | Straw                             | 90 |
|                                     | Hulls   | 90 | Hulls                             | 90 |
|                                     | Seeds   | 90 | Seeds                             | 90 |

1) days after last application

Samples of fully ripe oilseed rape (BBCH 89) were taken 90 days after application of both labels. The pods were opened with a thresher and separated into seeds and hulls. Leaves and stems were chopped and designated as oilseed rape straw. All samples were weighed and stored in a freezer ( $\leq -18^{\circ}\text{C}$ ). Rape straw, hulls and seeds were extracted up to 605 days after sampling and the extracts were analysed up to 626 days after extraction. The period from sampling to analysis was up to 645 days.

#### Description of analytical procedures

Homogenised solid plant samples were weighed and combusted by means of an automatic sample oxidiser. The limit of quantitation in mg eq/kg was calculated from the twofold background radioactivity level (dpm/g matrix) divided by the corresponding specific radioactivity. For the quantitation of radioactive residues in liquid samples a liquid scintillation counter (LSC) was used. In order to determine the background radioactivity samples of untreated rape plants were combusted under the same conditions

Homogenisation/solvent extraction (ERR): The samples (oilseed rape straws, hulls and seeds) were homogenised with a mill along with dry ice. After sublimation of the dry ice (overnight in a freezer), the samples were weighed and stored in a freezer.

The homogenised samples of rape straw and rape hulls were extracted sequentially three times with cyclohexane (only oilseed rape seeds), three times with methanol and two times with water. Each extraction was performed applying a homogeniser.

After each extraction step, the solid material was separated from the extract by centrifugation, followed by filtration (filter paper). The resulting filtrates were separately pooled and made up to defined volumes. The residues after solvent extractions were transferred to a vessel at room temperature. Aliquots of the extracts were radio-assayed. For extracts containing sufficient amounts of radioactive residues, further aliquots were analysed by HPLC.

Solubilisation of the RRR: The RRR of oilseed rape matrices (both labels) were subjected to sequential solubilisation procedures. All incubations were carried out in buffered enzyme solutions and were stopped by addition of acetonitrile. After each incubation step, the solubilise was separated from the residue by centrifugation and filtration.

The RRR after solvent extraction was extraction twice with 1% ammonia and once with water (designated as ammonia solubilise). Then, the dried residues were taken up in 1% ammonia solution and shaken at  $37^{\circ}\text{C}$  for 2-24h. The resulting residues after ammonia treatment were suspended in 0.1 M acetate buffer and incubated with macerozyme and cellulase at  $37^{\circ}\text{C}$  for 72 h. The residues after macerozyme/ cellulase incubation were taken up in 1/15 M phosphate buffer and incubated with tyrosinase/ laccase at  $37^{\circ}\text{C}$  for 72 h to solubilise lignin. For solubilisation of polysaccharides, the residues after incubation with tyrosinase/ laccase were taken up with 1/15M phosphate buffer and incubated with  $\alpha$ -amylase/  $\beta$ -amylase/ amyloglycosidase at  $37^{\circ}\text{C}$  for 72h. The residues after  $\alpha$ -amylase/  $\beta$ -amylase/ amyloglycosidase at treatment was dissolved in an artificial gastric juice containing pepsin and incubated at  $37^{\circ}\text{C}$  overnight. Finally, the residue after pepsin treatment was incubated in an artificial intestine fluid containing pancreatin at  $37^{\circ}\text{C}$  overnight. Aliquots of the solubilisates were radio-assayed. For solubilisates containing sufficient amounts of radioactive residues, further aliquots were analysed by HPLC-MS.

Components of the residue were identified by HPLC-MS (HPLC methods LC07 and LC02, details below), NMR, as well as by co-chromatography and comparison of retention times by HPLC methods LC07 and LC02.

HPLC method LC07: A Phenomenex Luna Synergi Polar RP column (250 x 4.6 mm, 4 µm) was used with a Phenomenex Polar RP pre-column. A gradient elution was used (mobile phase A: ammonium formate (20 mM, pH 6.0; mobile phase B: acetonitrile).

HPLC method LC02: A YMC Pro C18 RS column (250 x 4.6 mm, 5 µm) was used with a Phenomenex C18 pre-column. A gradient elution was used (mobile phase A: water:formic acid (1000:1); mobile phase B: acetonitrile:formic acid (1000:1).

Table 7-24 How identification of metabolites was achieved

| Metabolite | Initial identification   |
|------------|--|
| BAS 684 H  | HPLC-MS in methanol extract of rape straw  |
| M684H006   | HPLC-MS of fractions of the water phase of the methanol extract of rape straw (phenyl label)   |
| M684H008   | HPLC-MS/MS of purified ethyl acetate phase of the methanol extract of rape straw (phenyl label)  |
| M684H046   | HPLC-MS/MS of purified ethyl acetate phase of the methanol extract of rape straw (phenyl label)  |
| M684H047   | HPLC-MS of fractions of the water phase of the methanol extract of rape straw (phenyl label)   |
| M684H048   | HPLC-MS/MS of fractions of the water phase of the methanol extract of rape straw (phenyl label)  |
| M684H051   | HPLC-MS/MS of fractions of the water phase of the methanol extract of rape straw (phenyl label)  |
| M684H005   | Co-chromatography with external sample from metabolism study of BAS 684 H in wheat (Section B.7.2.1.1, CA 6.2.1/001) by two sufficiently dissimilar techniques (LC07 and LC02) |
| M684H007   |  |
| M684H015   |  |
| M684H016   |  |
| M684H055   |  |

Either HPLC-MS, a technique capable of positive structural identification, or HPLC co-chromatography using two sufficiently dissimilar techniques has been used to identify metabolites. Following initial identification through the techniques above, metabolites were identified in all other matrices by co-chromatography using two sufficiently dissimilar techniques (HPLC methods LC07 and LC02) with reference standards and isolated fractions of identified compounds. Therefore the techniques used to identify the metabolites are considered sufficient.

Chiral analysis: For enantiomer specific analysis, a subsample of the ethyl acetate phase obtained from the methanol extract of oilseed rape straw (phenyl label) was fractionated by HPLC to isolate the metabolite M684H005. An aliquot of the collected fraction was analysed by a chiral HPLC method.

Isolation of radioactive residues: An aliquot of the oilseed rape straw methanol extract (phenyl label) was partitioned three times against ethyl acetate. The ethyl acetate phase was loaded onto a preconditioned solid phase extraction (SPE) column and with water / acetonitrile mixtures and acetonitrile. The SPE eluate acetonitrile / water (60 / 40, V / V) was concentrated and analysed by HPLC-MS. An aliquot of the water phase obtained from partition was concentrated and fractionated by HPLC. For fractions were collected separately and subjected to HPLC-MS analysis.

Cleavage experiments: Cleavage experiments were conducted with individual fractions of the water phase of the methanol extract of rape straw (phenyl label). The purpose of these experiments was to investigate the stability of conjugated metabolites to support the development of the residue analytical method. An aliquot of the water phase was concentrated and then fractionated with HPLC method LC05. The aliquots of isolated fractions from the water phase of the methanol extract of oilseed rape straw (phenyl-label) were evaporated to dryness and subjected to sequential solubilisation with ammonia and β-glycosidase (conditions summarised below in Table 7-25). All final solubilisates were subject to HPLC-MS method LC02 (confirmatory technique).

An additional aliquot following the treatment with ammonia and  $\beta$ -glucosidase the solubilisate was partitioned three times against ethyl acetate and the obtained ethyl acetate phases were analysed by HPLC-MS method LC10.

Table 7-25 Cleavage experiments with isolated fractions

| Method   | Conditions   |
|--|--|
| Hydrolysis of ester bonds<br>Ammonia treatment | Suspension in 1mL acetonitrile and 2mL 25% ammonia for 2 hours at room temperature.  |
| Hydrolysis of $\beta$ -glucosidase             | Suspension in acetate buffer (0.1M, ~pH 5), incubation with $\beta$ -glucosidase for 24 hours at 37°C and 180 rpm. The incubation was stopped by addition of acetonitrile. |

Table 7-26 How identification of aglycones was achieved after deglucosilation

| Aglycone after deglucosilation | Initial identification  |
|--------------------------------|---|
| M684H002                       | HPLC-MS in ammonia and $\beta$ -glucosidase-treated concentrated water phase of rape straw methanol extract |
| M684H017                       | HPLC-MS in ammonia and $\beta$ -glucosidase-treated concentrated water phase of rape straw methanol extract |
| M684H039                       | HPLC-MS in ammonia and $\beta$ -glucosidase-treated concentrated water phase of rape straw methanol extract |
| M684H044                       | HPLC-MS in ammonia and $\beta$ -glucosidase-treated concentrated water phase of rape straw methanol extract |
| M684H046                       | HPLC-MS in ammonia and $\beta$ -glucosidase-treated concentrated water phase of rape straw methanol extract |

## Results and discussion

### Total radioactive residue

The total radioactive residue (TRR) was measured directly via combustion (LSC) and calculated by summarising the extractable radioactive residue (ERR) and the residual radioactive residue (RRR) after solvent extraction. There are no significant differences between the TRR measured and TRR calculated for each label and matrix.

The calculated total radioactive residues (TRR) with the Phenyl-label were highest in straw (DALA 90) at 3.730 mg eq/kg, lower in hulls at 0.552 mg eq/kg, and lowest in seeds with 0.100 mg eq/kg.

A similar distribution was seen with the cyclohexane-label (TRR highest in straw: 3.417 mg eq/kg, lower in hulls at 0.577 mg eq/kg and seeds at 0.083 mg eq/kg). Within the same matrix, the amount of radioactive residues was comparable for both labels. A summary of the TRRs are presented in Table 7-27.

Table 7-27 Total radioactive residue after foliar spray application of BAS 684 H

| Matrix [BBCH]            | DALA <sup>1)</sup> | TRR measured (LSC) <sup>2)</sup> [mg eq/kg] | TRR calculated <sup>3)</sup> [mg eq/kg] |
|--------------------------|--------------------|---|---|
| <b>Phenyl label</b>      |                    |   |   |
| straw [89]               | 90                 | 3.804                                       | 3.730                                   |
| hulls [89]               | 90                 | 0.560                                       | 0.552                                   |
| seeds [89]               | 90                 | 0.106                                       | 0.100                                   |
| <b>Cyclohexane label</b> |                    |   |   |
| straw [89]               | 90                 | 3.792                                       | 3.417                                   |
| hulls [89]               | 90                 | 0.601                                       | 0.577                                   |
| seeds [89]               | 90                 | 0.083                                       | 0.083                                   |

1) days after last application, 2) TRR measured directly via combustion LSC, 3) TRR calculated as the sum of ERR(extractable radioactive residue) and RRR (residual radioactive residue)after extraction of the residues

#### Extractability of radioactive residues

The extractability of  $^{14}\text{C}$  residues from rape straw, rape hulls and rape seeds are summarised in Table 7-28. The extractability ranged from 619.4% TRR to 89.5% TRR. High extractability of  $^{14}\text{C}$  residue was seen in straw (>89% TRR for total extract for both labels) and hulls (>70% TRR for total extract for both labels). The majority of the radioactivity was extracted with methanol (16-73% TRR) while subsequent water extraction resulted in additional extraction of 14 - 46% TRR. Solvent extraction left an RRR (residual radioactive residue) in straw of 10.9% TRR (phenyl-label, 0.407 mg eq/kg) and 10.5% TRR (cyclohexane label, 0.359 mg eq/kg), while the RRR in seeds amounted to 37.0% TRR (0.037 mg eq/kg, phenyl-label) and 38.6% TRR (0.032 mg eq/kg, cyclohexane-label). For oilseed rape seeds, the major amount was extracted with cyclohexane ( $\leq 32.2\%$  TRR) and smaller amounts were extracted with methanol and water. The RRR was therefore further investigated by enzyme treatment as discussed below.

Table 7-28 Extractability of radioactive residues of BAS 684 H in oilseed rape samples

| Matrix            | DALA <sup>1)</sup> | TRR<br>calculated <sup>2)</sup> | distribution of radioactive residues |             |                                 |             |                                       |             |                         |             |                   |          |
|-------------------|--------------------|---------------------------------|--------------------------------------|-------------|---------------------------------|-------------|---------------------------------------|-------------|-------------------------|-------------|-------------------|----------|
|                   |                    |                                 | methanol<br>extracts <sup>3)</sup>   |             | water<br>extracts <sup>3)</sup> |             | cyclohexane<br>extracts <sup>3)</sup> |             | Total ERR <sup>4)</sup> |             | RRR <sup>5)</sup> |          |
|                   |                    | mg eq/kg                        | % TRR                                | mg<br>eq/kg | % TRR                           | mg<br>eq/kg | % TRR                                 | mg<br>eq/kg | % TRR                   | mg<br>eq/kg | % TRR             | mg eq/kg |
| Phenyl-label      |                    |                                 |                                      |             |                                 |             |                                       |             |                         |             |                   |          |
| Straw             | 11                 | 3.730                           | 72.7                                 | 2.713       | 16.4                            | 0.610       | N.D.                                  | N.D.        | 89.1                    | 3.323       | 10.9              | 0.407    |
| Hulls             | 56                 | 0.552                           | 35.7                                 | 0.197       | 35.9                            | 0.198       | N.D.                                  | N.D.        | 71.6                    | 0.395       | 28.4              | 0.157    |
| Seeds             | 56                 | 0.100                           | 16.2                                 | 0.016       | 14.6                            | 0.015       | 32.2                                  | 0.032       | 63.0                    | 0.063       | 37.0              | 0.037    |
| Cyclohexane-label |                    |                                 |                                      |             |                                 |             |                                       |             |                         |             |                   |          |
| Straw             | 13                 | 3.417                           | 73.5                                 | 2.512       | 16.0                            | 0.546       | N.D.                                  | N.D.        | 89.5                    | 3.058       | 10.5              | 0.359    |
| Hulls             | 56                 | 0.577                           | 35.3                                 | 0.204       | 45.9                            | 0.265       | N.D.                                  | N.D.        | 81.3                    | 0.469       | 18.7              | 0.108    |
| Seeds             | 56                 | 0.083                           | 17.1                                 | 0.014       | 15.6                            | 0.013       | 28.7                                  | 0.024       | 61.4                    | 0.051       | 38.6              | 0.032    |

<sup>1)</sup> days after last application, <sup>2)</sup> TRR calculated as the sum of ERR + RRR was set to 100% TRR, <sup>3)</sup> pool of combined repetitive extracts, <sup>4)</sup> Extractable radioactive residue was calculated as sum of methanol and water extract, <sup>5)</sup> Residual radioactive residues (after solvent extraction)

#### Solubilisation of radioactive residues

The residues after solvent extraction were further solubilised with ammonia and enzymes (macerozyme, tyrosinase, amylase/ amyloglycosidase). Thereafter, the residues were further incubated with an artificial gastric juice/ pepsin and artificial intestinal fluid/ pancreatin to assess the bioavailability of the residues. The results are summarised in Table 7-29.

For oilseed rape straw and hulls (both labels), the highest portions of radioactive residues were solubilised by ammonia treatment amounting to up to 9.7% TRR. For all other solubilisation procedures, small amounts were release ranging from 0.1% TRR to 4.3% TRR. The final residues were each below or equal to 5.4% TRR.

For oilseed rape seeds (both labels), the highest portions of radioactive residues were solubilised by macerozyme treatment accounting for 10.1% TRR (0.010 mg eq/kg, phenyl-label) and 9.6% TRR (0.008 mg eq/kg, cyclohexane-label). For the phenyl label, high portions, in comparison, were additionally released by ammonia and amylase/ amyloglycosidase incubation (8.8% TRR and 2.8% TRR, respectively). For all other solubilisation procedures, small amounts were released ranging from 0.1% TRR to 2.4% TRR.



Table 7-29 Summary of solubilised components in oilseed rape

| Designation  | Matrix                            |                                   |                                   |                                   |                                   |                                   |
|--|-----------------------------------|-----------------------------------|-----------------------------------|-----------------------------------|-----------------------------------|-----------------------------------|
|  | Phenyl label                      |                                   |                                   | Cyclohexane label                 |                                   |                                   |
|  | Straw<br>[mg<br>eq/kg]<br>[% TRR] | Hulls<br>[mg<br>eq/kg]<br>[% TRR] | Seeds<br>[mg<br>eq/kg]<br>[% TRR] | Straw<br>[mg<br>eq/kg]<br>[% TRR] | Hulls<br>[mg<br>eq/kg]<br>[% TRR] | Seeds<br>[mg<br>eq/kg]<br>[% TRR] |
| <i>Residue after solvent<br/>extraction</i>                            | 0.407<br>10.9                     | 0.157<br>28.4                     | 0.037<br>37.0                     | 0.359<br>10.5                     | 0.108<br>18.7                     | 0.032<br>38.6                     |
| Ammonia solubilisate   | 0.202<br>5.4                      | 0.054<br>9.7                      | 0.009<br>8.8                      | 0.207<br>6.0                      | 0.033<br>5.8                      | 0.003<br>4.0                      |
| Macerozyme solubilisate  | 0.052<br>1.4                      | 0.024<br>4.3                      | 0.010<br>10.1                     | 0.033<br>1.0                      | 0.015<br>2.7                      | 0.008<br>9.6                      |
| Tyrosinase solubilisate  | 0.017<br>0.5                      | 0.003<br>0.6                      | < 0.001<br>0.3                    | 0.015<br>0.4                      | 0.003<br>0.5                      | 0.001<br>1.1                      |
| Amylase / amyloglycosidase<br>solubilisate                             | 0.009<br>0.3                      | 0.010<br>1.8                      | 0.003<br>2.8                      | 0.004<br>0.1                      | 0.004<br>0.7                      | 0.002<br>2.2                      |
| Pepsin solubilisate  | 0.003<br>0.1                      | 0.001<br>0.3                      | < 0.001<br>0.5                    | 0.003<br>0.1                      | 0.001<br>0.2                      | 0.002<br>2.4                      |
| Pancreatin solubilisate  | 0.007<br>0.2                      | 0.003<br>0.5                      | < 0.001<br>0.4                    | 0.005<br>0.1                      | 0.003<br>0.6                      | 0.001<br>0.9                      |
| <b>Sum of released residue</b>   | <b>0.290</b><br><b>7.8</b>        | <b>0.094</b><br><b>17.1</b>       | <b>0.023</b><br><b>22.8</b>       | <b>0.266</b><br><b>7.8</b>        | <b>0.060</b><br><b>10.5</b>       | <b>0.017</b><br><b>20.2</b>       |
| Final residue  | 0.061<br>1.6                      | 0.030<br>5.4                      | 0.009<br>9.0                      | 0.038<br>1.1                      | 0.025<br>4.3                      | 0.010<br>11.5                     |
| <b>Sum of solubilised<br/>radioactive<br/>residues + final residue</b> | <b>0.351</b><br><b>9.4</b>        | <b>0.124</b><br><b>22.4</b>       | <b>0.032</b><br><b>31.8</b>       | <b>0.304</b><br><b>8.9</b>        | <b>0.085</b><br><b>14.8</b>       | <b>0.026</b><br><b>31.7</b>       |

#### *Characterisation, Identification and Quantification of Radioactive Residues in Oilseed Matrices*

The results are summarised in Table 7-30 to Table 7-41. Structure elucidation was based on HPLC-MS and MS/MS analysis of the purified ethyl acetate phase and fractions of the water phase both obtained from the methanol extract of oilseed rape straw (phenyl label), which resulted in the identification of the metabolites M684H006, M684H008, M684H046, M684H047, M684H048 and M684H051. Metabolites M684H005, M684H007, M684H015, M684H016 and M684H055 were assigned by co-chromatography experiments with external samples from a related metabolism study of BAS 684 H in wheat. The parent compound was identified by co-chromatography with a reference item thereof. An overview of the components of the extractable residue is given below in Table 7-42. Structures of the metabolites are outlined in Table 7-1.

#### *Oilseed rape straw*

##### *Phenyl-label*

For the phenyl label, analysis of the concentrated methanol extract of rape straw with quantitative HPLC-MS method LC07 resulted in a pattern of 32 peaks, of which 12 were identified. The most abundant compound was metabolite M684H006 and accounted for 0.375 mg eq/kg (10.1% TRR). Metabolite M684H005 was the second most abundant compound with 0.331 mg eq/kg (8.9% TRR), followed by metabolites M684H008 with 0.167 mg eq/kg (4.5% TRR), M684H048 with 0.151 mg eq/kg (4.0% TRR), M684H016 with 0.126 mg eq/kg (3.4% TRR), M684H046 with 0.111 mg eq/kg (3.0% TRR), M684H047 with 0.102 mg eq/kg (2.7% TRR), M684H007 with 0.095 mg eq/kg (2.5% TRR) and M684H051 with 0.097 mg eq/kg (2.6% TRR). The metabolites M684H055 (0.060 mg eq/kg; 1.6% TRR) and M684H015 (0.054 mg eq/kg; 1.4% TRR) and the parent compound BAS 684 H (0.043 mg eq/kg) were less abundant on a similar level. The early eluting peaks were designated as polar compounds and classified as characterised (in sum: 0.257 mg eq/kg; 6.9% TRR) was also classified as characterised.

The active and the eleven metabolites identified were confirmed using HPLC-MS method LC02. An aliquot of the methanol extract was concentrated and fortified with reference items: BAS 684 H, M684H005, M684H006,

M684H015, M684H016, M684H047, M684H048, M684H051, M684H007 and M684H055. The fortified aliquot was taken for co-chromatography identification using quantitative and confirmatory HPLC-MS methods (LC07 and LC02, respectively) where there was a positive retention time match of metabolites with authentic reference standards. Furthermore, the SPE eluate of the ethyl acetate phase of the methanol extract of rape straw was fortified with the reference item of BAS 684 H and analysed with HPLC-MS/MS method LC07 and LC02. Metabolite M684H008 and M684H046 were identified by LC-MS (applying parameters of HPLC method LC07) in the purified ethyl acetate phase of the methanol extract of rape straw (phenyl label), therefore, they were assigned to the corresponding peaks in the quantitative chromatograms (LC07) of the other extracts and solubilisates (phenyl and cyclohexane label).

The total radioactivity present in the methanol extract of rape straw (phenyl label) was 2.698 mg eq/kg (72.3% TRR) and 63.5% of that has been conclusively identified. 17 additionally characterised peaks were detected, all of which were  $\geq 0.01$  mg eq/kg. The 13 individual residues in the range of 0.013 - 0.042 mg eq/kg (0.3-1.1% TRR) are considered characterised and no further attempts to identify these peaks are required. However, the higher-level individual residues at 0.056 mg eq/kg (1.5% TRR), 0.083 mg eq/kg (2.2% TRR), 0.091 mg eq/kg (2.4% TRR) and 0.096 mg eq/kg (2.6% TRR) have not been identified despite 8 residues quantified within a similar range of 0.043 – 0.375 mg eq/kg (1.2 – 10.1% TRR). The applicant justified that attempts at identification by comparison of retention times and MS data with reference items (listed above) did not produce a conclusive structural assignment. The applicant reported that 3 of the 4 higher-level peaks mentioned above were poorly resolved by HPLC, with issues of co-elution and matrix load in the extracts which made identification by MS difficult. Characterisation and identification need to be decided on a case by case basis for this residue based on how much has been identified. Whilst only 63.5% of the extract was identified, oilseed rape straw is neither a food nor feed commodity so there is no effect on the dietary burden and overall consumer risk assessment, hence the degree of characterisation and identification performed is considered acceptable. Overall, 1.82 mg eq/kg or 48.8% TRR was identified in the methanol extract of rape straw (phenyl label). The remaining components were detected up to 0.096 mg eq/kg (2.6% TRR), summed up to 0.665 mg eq/kg (17.8% TRR) and were classified as characterised.

The chromatogram (LC07) of the concentrated water extract of rape straw (phenyl label) shows eleven peaks, of which seven were identified. The most abundant compounds, though on a low level, were metabolites M684H005 (0.062 mg eq/kg, 1.7% TRR) and M684H006 (0.064 mg eq/kg, 1.7% TRR) and M684H047 (0.063 mg eq/kg; 1.7% TRR). Minor amounts of metabolites M684H048 (0.026 mg eq/kg; 0.8% TRR), M684H016 (0.044 mg eq/kg; 1.2% TRR), M684H007 (0.027 mg eq/kg, 10.9% TRR) and M684H008 (0.035 mg eq/kg; 0.9% TRR) were detected. The early eluting peaks were designated as polar components and classified as characterised (in sum: 0.191 mg eq/kg, 5.1% TRR).

The seven metabolites identified were confirmed using HPLC-MS method LC02. An aliquot of the concentrated water was fortified with reference items: BAS 684 H, M684H005, M684H006, M684H015, M684H016, M684H047, M684H048, M684H051, M684H007 and M684H055. The fortified aliquot was taken for co-chromatography identification using quantitative and confirmatory HPLC-MS methods (LC07 and LC02, respectively) where there was a positive retention time match of metabolites with authentic reference standards.

The total radioactivity present in the concentrated water extract of rape straw (phenyl label) was 0.605 mg eq/kg (16.2% TRR) and 53.6% of that has been conclusively identified. 2 additionally characterised peaks were detected, both of which were  $\geq 0.01$  mg eq/kg. Two individual peaks were quantified at 0.046 mg eq/kg (1.2% TRR) and 0.043 mg eq/kg (1.2% TRR) and were classified as characterised but not identified despite 7 metabolites quantified within a similar range of 0.026 – 0.064 mg eq/kg (0.8% TRR – 1.7% TRR). The applicant justified that attempts at identification by comparison of retention times with a range of reference items for postulated metabolites (listed above) did not produce a conclusive structural assignment. Given identification is not required for residues between 0.01-0.05 mg eq/kg unless it is straightforward and that oilseed rape straw is neither a food nor feed commodity so there is no effect on the dietary burden and overall consumer risk assessment, the degree of characterisation and identification performed is considered acceptable. Overall, 0.321 mg eq/kg (8.6% TRR) was identified in the concentrated water extract of rape straw (phenyl label). The remaining compounds were detected up to 0.046 mg eq/kg (1.2% TRR), summed up to 0.09 mg eq/kg (2.4% TRR) and were classified as characterised.

In the analysis of the concentrated ammonia solubilisate of rape straw (phenyl label) with quantitative HPLC method LC07 a pattern of seven peaks was detected, of which two were identified. The most abundant peak was identified as metabolite M684H005 and accounted for 0.094 mg eq/kg (2.5% TRR), followed by M684H008 (0.039 mg eq/kg; 1.1% TRR).



Metabolite M684H005 was confirmed using HPLC-MS method LC02. An aliquot of the concentrated ammonia solubilisate was fortified with reference items: BAS 684 H, M684H005, M684H006, M684H015, M684H016, M684H047, M684H048, M684H051, M684H007 and M684H055. The fortified aliquot was taken for co-chromatography identification using quantitative and confirmatory HPLC-MS methods (LC07 and LC02, respectively) where there was a positive retention time match of metabolites with authentic reference standards.

The total radioactivity present in the concentrated ammonia solubilisate of rape straw (phenyl label) was 0.186 mg eq/kg (5.0% TRR) and 71.5% of that has been conclusively identified. The five remaining individual peaks were quantified within a range of 0.004 - 0.017 mg eq/kg (0.1 - 0.4% TRR), 3 of which were > 0.01 mg eq/kg. They are considered characterised and no further attempts to identify these peaks are required. Overall, a total of 0.186 mg eq/kg (5.0% TRR) residues were present, where a total of 0.133 mg eq/kg (3.6% TRR) were classified as identified and 0.052 mg eq/kg (1.4% TRR) were classified as characterised.

The chromatogram (LC07) of the concentrated macerozyme solubilisate of rape straw (phenyl label) shows a pattern of 6 peaks. The polar peaks probably consist of carbohydrates, were designated as polar components and classified as characterised (in sum: 0.027 mg eq/kg; 0.7% TRR). The peaks designated as OH-metabolites accounted together for 0.017 mg eq/kg (0.4% TRR) and were classified as characterised.

Polar components and OH-metabolites were confirmed using HPLC-MS method LC02. The metabolite identification was based on comparison of retention time and metabolite pattern with those of the HPLC-MS analyses from co-chromatography.

The total radioactivity present in the concentrated macerozyme solubilisate of rape straw (phenyl label) was 0.052 mg eq/kg (1.4% TRR). Four peaks were identified as polar components and OH-metabolites. The remaining individual peaks were <0.01 mg eq/kg and are classified as characterised and no further attempts to identify these peaks are required. Overall, a total of 0.052 mg eq/kg (1.4% TRR) were present, where a total of 0.044 mg eq/kg (1.3% TRR) were identified as polar components and OH-metabolites and 0.008 mg eq/kg (0.2% TRR) were classified as characterised.

The chromatogram (LC07) of the concentrated tyrosinase solubilisate of rape straw (phenyl label) shows a pattern of three peaks, of which two were designated as OH-metabolite (0.003 mg eq/kg; 0.1% TRR) and classified as characterised. The remaining two peaks were at <0.01 mg eq/kg and are classified as characterised and no further attempt to identify the peaks are required. The total radioactivity present in the concentrated tyrosinase solubilisate of rape straw (phenyl label) was 0.016 mg eq/kg (0.4% TRR). The OH-metabolite was confirmed using HPLC-MS method LC02. The metabolite identification was based on comparison of retention time and metabolite pattern with those of the HPLC-MS analyses from co-chromatography.

The chromatogram (LC07) of the concentrated amylase solubilisate of rape straw (phenyl label) shows a pattern of three peaks, of which two were designated as OH-metabolite, classified as characterised at 0.004 mg eq/kg and 0.001 mg eq/kg (0.1% TRR and <0.1% TRR). The total radioactivity present in the concentrated amylase solubilisate of rape straw (phenyl label) was 0.009 mg eq/kg (0.2% TRR). The remaining peak was at <0.01 mg eq/kg and is classified as characterised and no further attempt to identify the peak is required.

Taken together, 3.575 mg eq/kg (95.8% TRR) were identified and characterised in the ERR and RRR of rape straw (phenyl label).

Table 7-30 Summary of identified and characterised residues in the ERR of rape straw (phenyl label)

| Designation                                       | Extracts                      |       |                             |       | Sum of extracts |       |
|---|-------------------------------|-------|-----------------------------|-------|-----------------|-------|
|   | Concentrated methanol extract |       | Concentrated water extract  |       |                 |       |
|   | mg eq/kg                      | % TRR | mg eq/kg                    | % TRR | mg eq/kg        | % TRR |
| Total radioactive residue (TRR)                   |                               |       |                             |       | 3.730           | 100   |
| Identified  |                               |       |                             |       |                 |       |
| M684H005  | 0.331                         | 8.9   | 0.062                       | 1.7   | 0.393           | 10.5  |
| M684H006  | 0.375                         | 10.1  | 0.064                       | 1.7   | 0.439           | 11.8  |
| M684H007  | 0.095                         | 2.5   | 0.027                       | 0.7   | 0.122           | 3.3   |
| M684H008  | 0.167                         | 4.5   | 0.035                       | 0.9   | 0.202           | 5.4   |
| M684H015  | 0.054                         | 1.4   | N.D.                        | N.D.  | 0.054           | 1.4   |
| M684H016  | 0.126                         | 3.4   | 0.044                       | 1.2   | 0.17            | 4.5   |
| M684H046  | 0.111                         | 3.0   | N.D.                        | N.D.  | 0.111           | 3.0   |
| M684H047  | 0.102                         | 2.7   | 0.063                       | 1.7   | 0.164           | 4.4   |
| M684H048  | 0.151                         | 4.0   | 0.026                       | 0.8   | 0.180           | 4.8   |
| M684H051  | 0.097                         | 2.6   | N.D.                        | N.D.  | 0.097           | 2.6   |
| M684H055  | 0.06                          | 1.6   | N.D.                        | N.D.  | 0.06            | 1.6   |
| BAS 684 H   | 0.043                         | 1.2   | N.D.                        | N.D.  | 0.043           | 1.2   |
| Total identified by HPLC in ERR                   |                               |       |                             |       | 2.035           | 54.6  |
| Characterised                                     |                               |       |                             |       |                 |       |
| Polar compounds                                   | 0.257                         | 6.9   | 0.191                       | 5.1   | 0.448           | 12.0  |
| OH-metabolite                                     | 0.065                         | 1.8   | N.D.                        | N.D.  | 0.065           | 1.8   |
| Number of additionally characterised peaks        | 17 (17 peaks ≥ 0.01 mg eq/kg) |       | 2 (2 peaks ≥ 0.01 mg eq/kg) |       | -               | -     |
| Maximum of additionally characterised peaks       | 0.096                         | 2.6   | 0.046                       | 1.2   | -               | -     |
| Sum of additionally characterised peaks           | 0.75                          | 17.8  | 0.09                        | 2.4   | 0.755           | 20.2  |
| Total characterised by HPLC                       |                               |       |                             |       | 1.268           | 34.0  |
| Total identified and characterised by HPLC in ERR |                               |       |                             |       | 3.303           | 88.6  |
| Residual radioactive residue (RRR, calculated)    |                               |       |                             |       | 0.407           | 10.9  |
| Total identified and characterised + RRR          |                               |       |                             |       | 3.711           | 99.5  |

Not detected = N.D.

Table 7-31 Summary of Identified and characterised residues in the RRR of rape straw (phenyl label)

| Designation  | Solubilisates               |       |            |       |            |       |          |       | Sum of solubilisates |       |
|--|-----------------------------|-------|------------|-------|------------|-------|----------|-------|----------------------|-------|
|  | Ammonia                     |       | Macerozyme |       | Tyrosinase |       | Amylase  |       |                      |       |
|  | mg eq/kg                    | % TRR | mg eq/kg   | % TRR | mg eq/kg   | % TRR | mg eq/kg | % TRR | mg eq/kg             | % TRR |
| Residual radioactive residue (RRR)                 |                             |       |            |       |            |       |          |       | 0.407                | 10.9  |
| Identified   |                             |       |            |       |            |       |          |       |                      |       |
| M684H005   | 0.094                       | 2.5   | N.D.       | N.D.  | N.D.       | N.D.  | N.D.     | N.D.  | 0.094                | 2.5   |
| M684H008   | 0.039                       | 1.1   | N.D.       | N.D.  | N.D.       | N.D.  | N.D.     | N.D.  | 0.039                | 1.1   |
| Total identified                                   |                             |       |            |       |            |       |          |       | 0.134                | 3.6   |
| Characterised                                      |                             |       |            |       |            |       |          |       |                      |       |
| Polar comp.  | N.D.                        | N.D.  | 0.027      | 0.7   | N.D.       | N.D.  | N.D.     | N.D.  | 0.027                | 0.7   |
| OH-metabolites                                     | N.D.                        | N.D.  | 0.017      | 0.4   | 0.003      | 0.1   | 0.006    | 0.2   | 0.025                | 0.7   |
| Number of additionally characterised peaks         | 5 (3 peaks ≥ 0.01 mg eq/kg) |       | 2          |       | 2          |       | 1        |       | -                    | -     |
| Maximum of additionally characterised peaks        | 0.017                       | 0.4   | 0.006      | 0.2   | 0.008      | 0.21  | 0.003    | 0.1   | -                    | -     |
| Sum of additionally characterised peaks            | 0.052                       | 1.37  | 0.008      | 0.21  | 0.012      | 0.32  | 0.003    | 0.1   | 0.076                | 2.0   |
| Total characterised by HPLC                        |                             |       |            |       |            |       |          |       | 0.128                | 3.4   |
| Pepsin solubilisate                                |                             |       |            |       |            |       |          |       | 0.003                | 0.1   |
| Pancreatin solubilisate                            |                             |       |            |       |            |       |          |       | 0.007                | 0.2   |
| Total characterised                                |                             |       |            |       |            |       |          |       | 0.138                | 3.7   |
| Total identified and characterised                 |                             |       |            |       |            |       |          |       | 0.272                | 7.3   |
| Final residue                                      |                             |       |            |       |            |       |          |       | 0.061                | 1.6   |
| Total identified and characterised + final residue |                             |       |            |       |            |       |          |       | 0.333                | 8.9   |

N.D. = Not detected

Table 7-32 Summary of Identified and characterised residues in Rape straw (Phenyl label)

| Designation  | Extracts                      |       |                        |       | Sum of extracts |       |
|--|-------------------------------|-------|------------------------|-------|-----------------|-------|
|  | Concentrated methanol extract |       | Purified water extract |       |                 |       |
|  | mg eq/kg                      | % TRR | mg eq/kg               | % TRR | mg eq/kg        | % TRR |
| Total radioactive residue (TRR)                    |                               |       |                        |       | 3.730           | 100   |
| Identified   |                               |       |                        |       |                 |       |
| M684H005   | 0.393                         | 10.5  | 0.094                  | 2.5   | 0.487           | 13.0  |
| M684H006   | 0.439                         | 11.8  | N.D.                   | N.D.  | 0.439           | 11.8  |
| M684H007   | 0.122                         | 3.3   | N.D.                   | N.D.  | 0.122           | 3.3   |
| M684H008   | 0.202                         | 5.4   | 0.039                  | 1.1   | 0.241           | 6.5   |
| M684H015   | 0.054                         | 1.4   | N.D.                   | N.D.  | 0.054           | 1.4   |
| M684H016   | 0.170                         | 4.5   | N.D.                   | N.D.  | 0.170           | 4.5   |
| M684H046   | 0.111                         | 3.0   | N.D.                   | N.D.  | 0.111           | 3.0   |
| M684H047   | 0.164                         | 4.4   | N.D.                   | N.D.  | 0.164           | 4.4   |
| M684H048   | 0.180                         | 4.8   | N.D.                   | N.D.  | 0.180           | 4.8   |
| M684H051   | 0.097                         | 2.6   | N.D.                   | N.D.  | 0.097           | 2.6   |
| M684H055   | 0.06                          | 1.6   | N.D.                   | N.D.  | 0.060           | 1.6   |
| BAS 684 H  | 0.043                         | 1.2   | N.D.                   | N.D.  | 0.043           | 1.2   |
| Total identified                                   |                               |       |                        |       | 2.169           | 58.1  |
| Characterised                                      |                               |       |                        |       |                 |       |
| Additionally characterised by HPLC                 | 0.755                         | 20.2  | 0.076                  | 2.0   | 0.831           | 22.3  |
| Polar components                                   | 0.448                         | 12.0  | 0.027                  | 10.9  | 0.475           | 12.7  |
| OH-metabolites                                     | 0.065                         | 1.8   | 0.025                  | 0.7   | 0.091           | 2.4   |
| Pepsin solubilisate                                | N.A.                          | -     | 0.003                  | 0.1   | 0.003           | 0.1   |
| Pancreatin solubilisate                            | N.A.                          | -     | 0.007                  | 0.2   | 0.007           | 0.2   |
| Total characterised                                | 1.268                         | 34.0  | 0.138                  | 3.7   | 1.407           | 37.7  |
| Sum  |                               |       |                        |       |                 |       |
| Total identified and characterised                 |                               |       |                        |       | 3.575           | 95.8  |
| Final residue                                      |                               |       |                        |       | 0.061           | 1.6   |
| Total identified and characterised + final residue |                               |       |                        |       | 3.636           | 97.5  |

N.D. = Not detected

*Cyclohexane – label*

For the cyclohexane label, analysis of the concentrated methanol extract of rape straw with HPLC method resulted in a pattern of 18 peaks, of which five were identified. The most abundant compound was metabolite M684H005 (0.633 mg eq/kg; 18.5% TRR), followed by M684H008 (0.196 mg eq/kg; 5.7% TRR), M684H048 (0.181 mg eq/kg; 5.3% TRR), M684H051 (0.096 mg eq/kg; 2.8% TRR) and M684H046 (0.086 mg eq/kg; 2.5% TRR). The early eluting peak was designated as polar components (in sum: 0.038 mg eq/kg; 1.1 % TRR) and classified as characterised. The peak corresponding to OH-metabolite (0.022 mg eq/kg; 0.6% TRR) was also classified as characterised.

Three of the five metabolites identified (M684H051, M684H048, and M684H005) were confirmed using HPLC-MS method LC02. An aliquot of the methanol extract was concentrated and fortified with reference items: BAS 684 H, M684H005, M684H006, M684H015, M684H016, M684H047, M684H048, M684H051, M684H007 and M684H055. The fortified aliquot was taken for co-chromatography identification using

quantitative and confirmatory HPLC-MS methods (LC07 and LC02, respectively) where there was a positive retention time match of metabolites with authentic reference standards.

The total radioactivity present in the methanol extract of rape straw (cyclohexane label) was 2.297 mg eq/kg (67.2% TRR) and 64.5% of that has been conclusively identified. 11 additionally characterised peaks were detected, all of which were  $\geq 0.01$  mg eq/kg. Two individual residues at 0.049 mg eq/kg and 0.032 mg eq/kg (1.4 and 0.9% TRR, respectively) are considered characterised and no further attempts to identify these peaks are required. However, the higher-level individual residues at 0.108 mg eq/kg (3.2% TRR), 0.128 mg eq/kg (3.7% TRR), 0.164 mg eq/kg (4.8% TRR) and 0.234 mg eq/kg (6.9 % TRR) have not been identified despite 5 residues within a similar range of 0.086 – 0.633 mg eq/kg (2.5 – 18.5% TRR) being identified. The applicant justified that attempts at identification by comparison of retention times with a range of reference items for postulated metabolites (listed above) with two dissimilar HPLC systems did not produce a conclusive structural assignment and no further attempts to identify the residues were made. Characterisation and identification need to be decided on a case by case basis for this residue based on how much has been identified; therefore whilst only 64.5% of the extract has been identified, given that oilseed rape straw is neither a food nor feed commodity so there is no effect on the dietary burden and overall consumer risk assessment, the degree of characterisation and identification performed is considered acceptable. Overall, 1.192 mg eq/kg or 43.4% TRR was identified in the methanol extract of rape straw (cyclohexane label). The remaining components were detected up to 0.234 mg eq/kg (6.8% TRR), summed up to 1.046 mg eq/kg (30.6% TRR) and were classified as characterised.

The chromatogram of the concentrated water extract of rape straw (cyclohexane label) resulted in a pattern of 12 peaks, of which five were identified. The most abundant compound was M684H005 (0.079 mg eq/kg; 2.3% TRR), followed by M684H047 (0.072 mg eq/kg; 2.1% TRR), M684H048 (0.053 mg eq/kg; 1.6% TRR), M684H008 (0.040 mg eq/kg; 1.2% TRR) and M684H006 (0.046 mg eq/kg, 1.4% TRR). The early eluting peak was designated as polar components (in sum: 0.029 mg eq/kg; 0.8% TRR) and classified as characterised.

The polar components and four metabolites (M684H048, M684H047, M684H005 and M684H006) were confirmed using HPLC-MS method LC02. An aliquot of the concentrated water extract of the rape straw (cyclohexane label) was concentrated and fortified with reference items: M684H051, M684H048, M684H006, M684H016, M684H005, M684H015, M684H007 and M684H055. The concentrated solvent extracts were taken for co-chromatography analyses with quantitative HPLC-MS (LC07) and confirmatory HPLC-MS (LC02), where there was a positive retention time match of metabolites with authentic reference standards.

The total radioactivity present in the water extract of rape straw (cyclohexane label) was 0.528 mg eq/kg (15.5% TRR) and 55% of that has been conclusively identified. 6 additionally characterised peaks were detected, all of which were  $\geq 0.01$  mg eq/kg. Three metabolites at 0.036 mg eq/kg (1.1% TRR), 0.031 mg eq/kg (0.9% TRR) and 0.014 mg eq/kg (0.4% TRR) are considered characterised and no further attempts to identify these peaks are required. However, the higher-level individual residues at 0.040 mg eq/kg (1.2% TRR), 0.042 mg eq/kg (1.2% TRR) and 0.044 mg eq/kg (1.3% TRR) have not been identified despite five residues within a similar range of 0.04 – 0.079 mg eq/kg (1.2 – 2.3% TRR) being identified. The applicant justified that attempts at identification by comparison of retention times with a range of reference items for postulated metabolites (listed above) did not produce a conclusive structural assignment. Given identification is not required for residues between 0.01-0.05 mg eq/kg unless it is straightforward, and that oilseed rape straw is neither a food nor feed commodity so there is no effect on the dietary burden and overall consumer risk assessment, the degree of characterisation and identification performed is considered acceptable. Overall, 0.290 mg eq/kg or 8.5% TRR was identified in the water extract of rape straw (cyclohexane label). The remaining components were detected up to 0.044 mg eq/kg (1.3% TRR), summed up to 0.209 mg eq/kg (6.1% TRR) and were classified as characterised.

The analysis of the concentrated ammonia solubilise of rape straw (cyclohexane label) resulted in a pattern of 15 peaks, of which five were identified. The most abundant component was M684H005 with 0.043 mg eq/kg (1.3% TRR), followed by M684H008 (0.032 mg eq/kg; 0.9% TRR), M684H046 (0.030 mg eq/kg; 0.9% TRR), M684H048 (0.009 mg eq/kg; 0.3% TRR) and M684H051 (0.007 mg eq/kg; 0.2% TRR).

The polar components and three metabolites (M684H048, M684H005 and M684H051) were confirmed using HPLC-MS method LC02. An aliquot of the concentrated ammonia solubilise of the rape straw (cyclohexane label) was concentrated and fortified with reference items: M684H051, M684H048, M684H006, M684H016, M684H005, M684H015, M684H007 and M684H055. The concentrated solvent extracts were taken for co-chromatography analyses with quantitative HPLC-MS (LC07) and confirmatory HPLC-MS (LC02), where there was a positive retention time match of metabolites with authentic reference standards.

The total radioactivity present in the ammonia solubilisate of rape straw (cyclohexane label) was 0.192 mg eq/kg (5.6 % TRR) and 63.0% of that has been conclusively identified. 10 additionally characterised peaks were detected. 9 individual radioactive residues were quantified at levels <0.01 mg eq/kg are considered characterised and no further attempts to identify these peaks are required. However, the higher-level individual residue at 0.03 mg eq/kg (0.9% TRR) has not been identified despite five residues within a similar range 0.007 – 0.043 mg eq/kg (0.2 – 1.3% TRR) being identified. The applicant justified that attempts at identification by comparison of retention times with a range of reference items for postulated metabolites (listed above) did not produce a conclusive structural assignment. Given identification is not required for residues between 0.01-0.05 mg eq/kg unless it is straightforward, and that oilseed rape straw is neither a food nor feed commodity so there is no effect on the dietary burden and overall consumer risk assessment, the degree of characterisation and identification performed is considered acceptable. Overall, 0.122 mg eq/kg (3.6% TRR) was identified in the ammonia solubilisate of rape straw (cyclohexane label). The remaining components were detected up to 0.03 mg eq/kg (0.9% TRR), summed up to 0.07 mg eq/kg (2.0% TRR) and were classified as characterised.

Taken together, 3.069 mg eq/kg (89.8% TRR) were identified and characterised in the ERR and RRR of rape straw (cyclohexane label).

Table 7-33 Summary of identified and characterised residues in the ERR of rape straw (cyclohexane label)

| Designation                                       | Extracts                      |       |                             |       | Sum of extracts |       |
|---|-------------------------------|-------|-----------------------------|-------|-----------------|-------|
|   | Concentrated methanol extract |       | Concentrated water extract  |       |                 |       |
|   | mg eq/kg                      | % TRR | mg eq/kg                    | % TRR | mg eq/kg        | % TRR |
| Total radioactive residue (TRR)                   |                               |       |                             |       | 3.417           | 100   |
| Identified  |                               |       |                             |       |                 |       |
| M684H005  | 0.633                         | 18.5  | 0.079                       | 2.3   | 0.712           | 20.8  |
| M684H006  | N.D.                          | N.D.  | 0.046                       | 1.4   | 0.046           | 1.4   |
| M684H008  | 0.196                         | 5.7   | 0.04                        | 1.2   | 0.236           | 6.9   |
| M684H046  | 0.086                         | 2.5   | N.D.                        | N.D.  | 0.086           | 2.5   |
| M684H047  | N.D.                          | N.D.  | 0.072                       | 2.1   | 0.072           | 2.1   |
| M684H048  | 0.181                         | 5.3   | 0.053                       | 1.6   | 0.234           | 6.9   |
| M684H051  | 0.096                         | 2.8   | N.D.                        | N.D.  | 0.096           | 2.8   |
| Total identified                                  |                               |       |                             |       | 1.482           | 43.4  |
| Characterised                                     |                               |       |                             |       |                 |       |
| Polar compounds                                   | 0.038                         | 1.1   | 0.029                       | 0.8   | 0.067           | 2.0   |
| OH-metabolite                                     | 0.022                         | 0.6   | N.D.                        | N.D.  | 0.022           | 0.6   |
| Number of additionally characterised peaks        | 11 (11 peaks ≥ 0.01 mg eq/kg) |       | 6 (6 peaks ≥ 0.01 mg eq/kg) |       | -               | -     |
| Maximum of additionally characterised peaks       | 0.234                         | 6.8   | 0.044                       | 1.3   | -               | -     |
| Sum of additionally characterised peaks           | 1.046                         | 30.6  | 0.209                       | 6.1   | 1.254           | 36.7  |
| Total characterised by HPLC                       |                               |       |                             |       | 1.343           | 39.3  |
| Total identified and characterised by HPLC in ERR |                               |       |                             |       | 2.825           | 82.7  |
| Residual radioactive residue (RRR, calculated)    |                               |       |                             |       | 0.359           | 10.5  |
| Total identified and characterised + RRR          |                               |       |                             |       | 3.184           | 93.2  |

N.D. = Not detected

Table 7-34 Summary of identified and characterised residues in the RRR of rape straw (cyclohexane label)

| Designation   | Solubilisates<br>Concentrated ammonia solubilisate |       | Sum of solubilisates |       |
|---|--|-------|----------------------|-------|
|   | mg eq/kg   | % TRR | mg eq/kg             | % TRR |
| Residual radioactive residue, calculated                  |  |       | 0.359                | 10.5  |
| M684H005  | 0.043  | 1.3   | 0.043                | 1.3   |
| M684H008  | 0.032  | 0.9   | 0.032                | 0.9   |
| M684H046  | 0.03   | 0.9   | 0.03                 | 0.9   |
| M684H048  | 0.009  | 0.3   | 0.009                | 0.3   |
| M684H051  | 0.007  | 0.2   | 0.007                | 0.2   |
| Total identified  |  |       | 0.122                | 3.6   |
| Characterised   |  |       |                      |       |
| Number of additionally characterised peaks                | 10 (1 peak $\geq$ 0.01 mg eq/kg)                   |       | -                    | -     |
| Maximum of additionally characterised peaks               | 0.03   | 0.9   | -                    | -     |
| Sum of additionally characterised peaks                   | 0.07   | 2.0   | 0.07                 | 2.0   |
| Total characterised by HPLC in RRR                        |  |       | 0.07                 | 2.0   |
| Macerozyme solubilisate                                   |  |       | 0.026                | 0.8   |
| Tyrosinase solubilisate                                   |  |       | 0.014                | 0.4   |
| Amylase solubilisate                                      |  |       | 0.004                | 0.1   |
| Pepsin solubilisate                                       |  |       | 0.003                | 0.1   |
| Pancreatin solubilisate                                   |  |       | 0.005                | 0.1   |
| Total characterised                                       |  |       | 0.122                | 3.6   |
| Total identified and characterised                        |  |       | 0.244                | 7.1   |
| Final residue   |  |       | 0.038                | 1.1   |
| Total identified and characterised in RRR + final residue |  |       | 0.281                | 8.2   |

N.D. = Not detected

*Rape hulls**Phenyl-label*

For the phenyl label, the analysis of the concentrated methanol extract of rape hulls resulted in a pattern of 24 peaks, of which five were identified. The most abundant compound was M684H006 with 0.022 mg eq/kg (3.9% TRR), followed by M684H016 (0.014 mg eq/kg, 2.5 % TRR), M684H005 (0.009 mg eq/kg; 1.6% TRR), M684H008 (0.005 mg eq/kg; 1.0% TRR) and the parent compound BAS 684 H (0.003 mg eq/kg; 0.5% TRR). The early eluting peaks were designated as polar compounds and classified as characterised (0.021 mg eq/kg, 3.7% TRR).

The active (BAS 684 H), its 3 metabolites (M684H005, M684H006 and M684H016) and polar components were confirmed using HPLC-MS method LC02. An aliquot of the methanol extract was concentrated and fortified with reference items: BAS 684 H, M684H005, M684H006, M684H015, M684H016, M684H047, M684H048, M684H051, M684H007 and M684H055. The fortified aliquot was taken for co-chromatography identification using quantitative and confirmatory HPLC-MS methods (LC07 and LC02, respectively) where there was a positive retention time match of metabolites with authentic reference standards.

The total radioactivity present in concentrated methanol extract of rape hulls (phenyl label) was 0.193 mg eq/kg (34.9% TRR) and 38.3% of that has been conclusively identified. 17 additionally characterised peaks were detected, 5 of which were  $\geq$  0.01 mg eq/kg. The 14 individual residues in the range of 0.002 – 0.014 mg eq/kg



(0.5 - 2.6% TRR) are considered characterised and no further attempts to identify these peaks are required. Overall, 0.053 mg eq/kg (9.6% TRR) was identified in the methanol extract of rape hulls (phenyl label). The remaining components were detected up to 0.014 mg eq/kg (2.6% TRR), summed up to 0.12 mg eq/kg (21.7% TRR) and were classified as characterised.

The analysis of the concentrated water extract of rape hulls (phenyl label) with HPLC method LC07 resulted in a pattern of 16 peaks, of which two were identified. The most abundant compound was M684H006 (0.024 mg eq/kg; 4.3% TRR) followed by M684H016 (0.017 mg eq/kg; 3.1% TRR). The early eluting peaks were designated as polar compounds and classified as characterised (0.039 mg eq/kg, 7.06% TRR).

Polar components and 2 metabolites (M684H006 and M684H016) were confirmed using HPLC-MS method LC02. An aliquot of the methanol extract was concentrated and fortified with reference items: BAS 684 H, M684H005, M684H006, M684H015, M684H016, M684H047, M684H048, M684H051, M684H007 and M684H055. The fortified aliquot was taken for co-chromatography identification using quantitative and confirmatory HPLC-MS methods (LC07 and LC02, respectively) where there was a positive retention time match of metabolites with authentic reference standards.

The total radioactivity present in concentrated water extract of rape hulls (phenyl label) was 0.207 mg eq/kg (37.5% TRR) and 38.7% of that has been conclusively identified. 12 additionally characterised peaks were detected, 6 of which were  $\geq 0.01$  mg eq/kg. The 9 individual residues in the range of 0.003 – 0.011 mg eq/kg (0.6 – 2.0% TRR) are considered characterised and no further attempts to identify these peaks are required. However, the higher-level individual residues at 0.017 mg eq/kg (3.1% TRR), 0.018 mg eq/kg (3.2% TRR) and 0.024 mg eq/kg (4.3% TRR) have not been identified despite three residues within a similar range of 0.017 – 0.024 mg eq/kg (3.1 – 4.4% TRR) being identified. The applicant justified that attempts at identification by comparison of retention times with a range of reference items for postulated metabolites (listed above) did not produce a conclusive structural assignment. Given identification is not required for residues between 0.01-0.05 mg eq/kg unless it is straightforward, and that oilseed rape hulls are neither a food nor feed commodity so there is no effect on the dietary burden and overall consumer risk assessment, the degree of characterisation and identification performed is considered acceptable. Overall, 0.041 mg eq/kg (7.4% TRR) was identified in concentrated water extract of rape hulls (phenyl label). The remaining components were detected up to 0.024 mg eq/kg (4.4% TRR) and summed up to 0.128 mg eq/kg (23.1% TRR) and were classified as characterised.

The analysis of the concentrated ammonia solubilise of rape hulls (phenyl label) with HPLC method LC07 resulted in a pattern of 11 peaks, of which two were identified. The most abundant compound was M684H008 (0.004 mg eq/kg; 0.7% TRR) followed by M684H005 (0.002 mg eq/kg; 0.4% TRR). The early eluting peaks were designated as polar compounds and classified as characterised (0.018 mg eq/kg; 3.3% TRR).

Two metabolites (M684H005 and M684H008) were confirmed using HPLC-MS method LC02. An aliquot of the methanol extract was concentrated and fortified with reference items: BAS 684 H, M684H005, M684H006, M684H015, M684H016, M684H047, M684H048, M684H051, M684H007 and M684H055. The fortified aliquot was taken for co-chromatography identification using quantitative and confirmatory HPLC-MS methods (LC07 and LC02, respectively) where there was a positive retention time match of metabolites with authentic reference standards.

The total radioactivity present in ammonia solubilise of rape hulls (phenyl label) was 0.047 mg eq/kg (8.5% TRR) and 51.1% of that has been conclusively identified. The 7 individual residues in the range of 0.001 – 0.006 mg eq/kg (0.2 – 1.1% TRR) are considered characterised and no further attempts to identify these peaks are required. Overall, 0.006 mg eq/kg (1.1% TRR) was identified in ammonia solubilise of rape hulls (phenyl label). The remaining components were detected up to 0.006 mg eq/kg (1.1% TRR) and summed up to 0.023 mg eq/kg (4.2% TRR) and were classified as characterised.

Taken together, 0.487 mg eq/kg (86.2 % TRR) were identified and characterised in the ERR and RRR of rape hulls (phenyl label).



Table 7-35 Summary of identified and characterised residues in the ERR of rape hulls (phenyl label)

| Designation                                       | Extracts                      |       |                              |       | Sum of extracts |       |
|---|-------------------------------|-------|------------------------------|-------|-----------------|-------|
|   | Concentrated methanol extract |       | Concentrated water extract   |       |                 |       |
|   | mg eq/kg                      | % TRR | mg eq/kg                     | % TRR | mg eq/kg        | % TRR |
| Total radioactive residue (TRR)                   |                               |       |                              |       | 0.552           | 100   |
| Identified  |                               |       |                              |       |                 |       |
| M684H005  | 0.009                         | 1.6   | N.D.                         | N.D.  | 0.009           | 1.6   |
| M684H006  | 0.022                         | 3.9   | 0.024                        | 4.3   | 0.046           | 8.3   |
| M684H008  | 0.005                         | 1.0   | N.D.                         | N.D.  | 0.005           | 1.0   |
| M684H016  | 0.014                         | 2.5   | 0.017                        | 3.1   | 0.031           | 5.6   |
| BAS 684 H   | 0.003                         | 0.5   | N.D.                         | N.D.  | 0.003           | 0.5   |
| Total identified by HPLC in ERR                   |                               |       |                              |       | 0.094           | 17.0  |
| Characterised                                     |                               |       |                              |       |                 |       |
| Polar compounds                                   | 0.021                         | 3.7   | 0.039                        | 7.0   | 0.059           | 10.7  |
| Number of additionally characterised peaks        | 17 (5 peaks ≥ 0.01 mg eq/kg)  |       | 12 (6 peaks ≥ 0.01 mg eq/kg) |       | -               | -     |
| Maximum of additionally characterised peaks       | 0.014                         | 2.6   | 0.024                        | 4.4   | -               | -     |
| Sum of additionally characterised peaks           | 0.12                          | 21.7  | 0.128                        | 23.1  | 0.247           | 44.8  |
| Total characterised by HPLC                       |                               |       |                              |       | 0.306           | 55.5  |
| Total identified and characterised by HPLC in ERR |                               |       |                              |       | 0.400           | 72.5  |
| Residual radioactive residue (RRR, calculated)    |                               |       |                              |       | 0.157           | 28.4  |
| Total identified and characterised + RRR          |                               |       |                              |       | 0.557           | 100.8 |

Not detected = N.D.

Table 7-36 Summary of identified and characterised residues in the RRR of rape hulls (phenyl label)

| Designation  | Solubilisates                   |       | Sum of solubilisates |             |
|--|---------------------------------|-------|----------------------|-------------|
|  | Concentrated ammonia solubilise |       | mg eq/kg             | % TRR       |
|  | mg eq/kg                        | % TRR |                      |             |
| <b>Residual radioactive residue, calculated</b>                  |                                 |       | <b>0.157</b>         | <b>28.4</b> |
| <b>Identified</b>  |                                 |       |                      |             |
| <b>M684H005</b>  | 0.002                           | 0.4   | 0.002                | 0.4         |
| <b>M684H008</b>  | 0.004                           | 0.7   | 0.004                | 0.7         |
| <b>Total identified by HPLC in RRR</b>                           |                                 |       | <b>0.006</b>         | <b>1.07</b> |
| <b>Characterised</b>   |                                 |       |                      |             |
| <b>Polar components</b>  | 0.018                           | 3.3   | 0.018                | 3.3         |
| <b>Number of additionally characterised peaks</b>                | 7                               |       | -                    | -           |
| <b>Maximum of additionally characterised peaks</b>               | 0.006                           | 1.1   | -                    | -           |
| <b>Sum of additionally characterised peaks</b>                   | 0.023                           | 4.2   | 0.023                | 4.2         |
| <b>Total characterised by HPLC in RRR</b>                        |                                 |       | <b>0.041</b>         | <b>7.5</b>  |
| <b>Macerozyme solubilise</b>                                     |                                 |       | 0.023                | 4.2         |
| <b>Amylase solubilise</b>  |                                 |       | 0.01                 | 1.8         |
| <b>Tyrosinase solubilise</b>                                     |                                 |       | 0.003                | 0.6         |
| <b>Pepsin solubilise</b>   |                                 |       | 0.001                | 0.3         |
| <b>Pancreatin solubilise</b>                                     |                                 |       | 0.003                | 0.5         |
| <b>Total additionally characterised in RRR</b>                   |                                 |       | <b>0.082</b>         | <b>14.8</b> |
| <b>Total identified and characterised in RRR</b>                 |                                 |       | <b>0.087</b>         | <b>15.8</b> |
| <b>Final residue</b>   |                                 |       | 0.03                 | 5.4         |
| <b>Total identified and characterised in RRR + final residue</b> |                                 |       | <b>0.117</b>         | <b>21.2</b> |

Not detected = N.D.

#### *Cyclohexane-label*

For the cyclohexane label, the analysis of the concentrated methanol extract of rape hulls resulted in pattern of 32 peaks, of which seven were identified. The most abundant compound was M684H016 with 0.020 mg eq/kg (3.6% TRR), followed by M684H007 (0.012 mg eq/kg; 2.0% TRR). The other compounds were present at lower levels and accounted for up to 0.006 mg eq/kg (1.0% TRR). The early eluting peak was designated as polar compounds and classified as characterised (0.002 mg eq/kg; 0.4% TRR).

Polar components and 7 metabolites identified (M684H047, M684H005, M684H006, M684H015, M684H016, M684H007 and M684H055) were confirmed using HPLC-MS method LC02. An aliquot of the methanol extract was concentrated and fortified with reference items: BAS 684 H, M684H005, M684H006, M684H015, M684H016, M684H047, M684H048, M684H051, M684H007 and M684H055. The fortified aliquot was taken for co-chromatography identification using quantitative and confirmatory HPLC-MS methods (LC07 and LC02, respectively) where there was a positive retention time match of metabolites with authentic reference standards.

The total radioactivity present in the methanol extract of rape hulls (cyclohexane label) was 0.217 mg eq/kg (37.6% TRR) and 26.7% of that has been conclusively identified. The 22 individual residues in the range of 0.001 – 0.010 mg eq/kg (0.2 – 1.7% TRR) are considered characterised and no further attempts to identify these peaks are required. However, the higher-level individual residues at 0.022 mg eq/kg (3.9% TRR) and 0.032 mg eq/kg (5.6% TRR) have not been identified despite 7 residues within a similar range of 0.004 – 0.020 mg eq/kg (0.7 – 3.6% TRR) being identified. The applicant justified that attempts at identification by comparison of retention times with a range of reference items for postulated metabolites (listed above) did not produce a conclusive structural assignment. Given identification is not required for residues between 0.01-0.05 mg eq/kg unless it is straightforward, and that oilseed rape hulls are neither a food nor feed commodity so there is no effect on the dietary burden and overall consumer risk assessment, the degree of characterisation and identification performed is considered acceptable. Overall, 0.056 mg eq/kg (9.7% TRR) was identified in the

methanol extract of rape hulls (cyclohexane label). The remaining components were detected up to 0.032 mg eq/kg (5.32% TRR), summed up to 0.159 mg eq/kg (26.46% TRR) and were classified as characterised.

The analysis of the concentrated water extract of rape hulls (cyclohexane label) with HPLC method LC07 resulted in a pattern of 23 peaks, of which five were identified. Metabolites M684H048 and M684H006 were detected at the same level (0.014 mg eq/kg; 2.5% TRR), the other compounds accounted for up to 0.011 mg eq/kg (1.9% TRR). The early eluting peak was designated as polar compounds and classified as characterised (0.007 mg eq/kg; 1.2% TRR). The peak corresponding to OH-metabolite accounted for 0.008 mg eq/kg (1.3% TRR) and was classified as characterised.

Polar components, OH-metabolite and 8 metabolites identified (M684H051, M684H048, M684H047, M684H006, M684H015, M684H016, M684H007 and M684H055) were confirmed using HPLC-MS method LC02. An aliquot of the water extract was concentrated and fortified with reference items: BAS 684 H, M684H005, M684H006, M684H015, M684H016, M684H047, M684H048, M684H051, M684H007 and M684H055. The fortified aliquot was taken for co-chromatography identification using quantitative and confirmatory HPLC-MS methods (LC07 and LC02, respectively) where there was a positive retention time match of metabolites with authentic reference standards.

The total radioactivity present in the concentrated water extract of rape hulls (cyclohexane label) was 0.252 mg eq/kg (41.93% TRR) and 28.17% of that has been conclusively identified. The ten individual residues in the range of 0.006 – 0.01 mg eq/kg (1.0 – 1.66% TRR) are considered characterised and no further attempts to identify these peaks are required. However, the higher-level individual residues at 0.011 mg eq/kg (1.83% TRR), at 0.013 mg eq/kg (2.16% TRR), at 0.017 mg eq/kg (2.83% TRR) and at 0.025 mg eq/kg (4.16% TRR) have not been identified despite 5 residues at 0.007 – 0.014 mg eq/kg (1.16 – 2.33% TRR) being identified. The applicant justified that attempts at identification by comparison of retention times with a range of reference items for postulated metabolites (listed above) did not produce a conclusive structural assignment. Given identification is not required for residues between 0.01-0.05 mg eq/kg unless it is straightforward, and that oilseed rape hulls are neither a food nor feed commodity so there is no effect on the dietary burden and overall consumer risk assessment, the degree of characterisation and identification performed is considered acceptable. Overall, 0.056 mg eq/kg (9.7% TRR) was identified in the concentrated water extract of rape hulls (cyclohexane label). The remaining components were detected up to 0.025 mg eq/kg (4.4% TRR), summed up to 0.181 mg eq/kg (31.4% TRR) and were classified as characterised.

The analysis of the ammonia solubilisate with HPLC method LC07 resulted in a pattern of 43 peaks, of which two were identified as metabolite M684H006 (0.001 mg eq/kg; 0.1% TRR) and M684H047 (<0.001 mg eq/kg; <0.1% TRR), respectively. The early eluting peaks were designated as polar compounds and classified as characterised (0.002 mg eq/kg; 0.34% TRR). The peak corresponding to OH-metabolite accounted for <0.001 mg eq/kg (<0.1% TRR) and was classified as characterised.

OH-metabolite and 2 metabolites identified (M684H006 and M684H047) were confirmed using HPLC-MS method LC02. An aliquot of the ammonia solubilisate was concentrated and fortified with reference items: BAS 684 H, M684H005, M684H006, M684H015, M684H016, M684H047, M684H048, M684H051, M684H007 and M684H055. The fortified aliquot was taken for co-chromatography identification using quantitative and confirmatory HPLC-MS methods (LC07 and LC02, respectively) where there was a positive retention time match of metabolites with authentic reference standards.

The total radioactivity present in the ammonia solubilisate of rape hulls (cyclohexane label) was 0.034 mg eq/kg (5.9% TRR) and 17.7% of that has been conclusively identified. The thirty-eight individual residues in the range of <0.001 – 0.003 mg eq/kg (<0.1 – 0.50% TRR) are considered characterised and no further attempts to identify these peaks are required. Overall, 0.002 mg eq/kg (0.2% TRR) was identified in the ammonia solubilisate of rape hulls (cyclohexane label). The remaining components were detected up to 0.003 mg eq/kg (0.5% TRR), summed up to 0.033 mg eq/kg (5.7% TRR) and were classified as characterised.

Taken together, 0.524 mg eq/kg (90.8% TRR) were identified and characterised in the ERR and RRR of rape hulls (cyclohexane label).

Table 7-37 Summary of identified and characterised residues in the ERR of rape hulls (cyclohexane label)

| Designation                                       | Extracts                      |       |                               |       | Sum of extracts |       |
|---|-------------------------------|-------|-------------------------------|-------|-----------------|-------|
|   | Concentrated methanol extract |       | Concentrated water extract    |       |                 |       |
|   | mg eq/kg                      | % TRR | mg eq/kg                      | % TRR | mg eq/kg        | % TRR |
| Total radioactive residue (TRR)                   |                               |       |                               |       | 0.577           | 100   |
| Identified  |                               |       |                               |       |                 |       |
| M684H005  | 0.004                         | 0.7   | N.D.                          | N.D.  | 0.004           | 0.7   |
| M684H006  | 0.004                         | 0.6   | 0.014                         | 2.5   | 0.018           | 3.1   |
| M684H007  | 0.012                         | 2.0   | N.D.                          | N.D.  | 0.012           | 2.0   |
| M684H015  | 0.004                         | 0.7   | N.D.                          | N.D.  | 0.004           | 0.7   |
| M684H016  | 0.02                          | 3.6   | 0.011                         | 1.9   | 0.031           | 5.4   |
| M684H047  | 0.006                         | 1.0   | 0.007                         | 1.2   | 0.013           | 2.2   |
| M684H048  | N.D.                          | N.D.  | 0.014                         | 2.5   | 0.014           | 2.5   |
| M684H051  | N.D.                          | N.D.  | 0.01                          | 1.7   | 0.01            | 1.7   |
| M684H055  | 0.006                         | 1.0   | N.D.                          | N.D.  | 0.006           | 1.0   |
| Total identified                                  |                               |       |                               |       | 0.111           | 19.3  |
| Characterised                                     |                               |       |                               |       |                 |       |
| Polar compounds                                   | 0.002                         | 0.4   | 0.007                         | 1.2   | 0.009           | 1.6   |
| OH-metabolite                                     | N.D.                          | N.D.  | 0.008                         | 1.3   | 0.008           | 1.3   |
| Number of additionally characterised peaks        | 24 (4 peaks ≥ 0.01 mg eq/kg)  |       | 16 (14 peaks ≥ 0.01 mg eq/kg) |       | -               | -     |
| Maximum of additionally characterised peaks       | 0.032                         | 5.6   | 0.025                         | 4.4   | -               | -     |
| Sum of additionally characterised peaks           | 0.159                         | 27.6  | 0.181                         | 31.4  | 0.34            | 59.0  |
| Total characterised by HPLC                       |                               |       |                               |       | 0.357           | 61.9  |
| Total identified and characterised by HPLC in ERR |                               |       |                               |       | 0.469           | 81.2  |
| Residual radioactive residue (RRR, calculated)    |                               |       |                               |       | 0.108           | 18.7  |
| Total identified and characterised + RRR          |                               |       |                               |       | 0.577           | 100.0 |

Not detected = N.D.

Table 7-38 Summary of identified and characterised residues in the RRR of rape hulls (cyclohexane label)

| Designation  | Solubilisates<br>Concentrated ammonia<br>solubilisate |       | Sum of<br>solubilisates |              |
|--|---|-------|-------------------------|--------------|
|  | mg eq/kg  | % TRR | mg eq/kg                | % TRR        |
| <b>Residual radioactive residue</b>                              |   |       | 0.108                   | 18.7         |
| <b>Identified</b>  |   |       |                         |              |
| <b>M684H006</b>  | 0.001   | 0.1   | 0.001                   | 0.1          |
| <b>M684H047</b>  | <0.001  | 0.1   | <0.001                  | 0.1          |
| <b>Total identified</b>  |   |       | <b>0.001</b>            | <b>0.166</b> |
| <b>Characterised</b>   |   |       |                         |              |
| <b>Polar comp.</b>   | 0.002   | 0.4   | 0.002                   | 0.4          |
| <b>OH-metabolites</b>  | <0.001  | <0.1  | <0.001                  | <0.01        |
| <b>Number of additionally characterised peaks</b>                | 38  |       | -                       | -            |
| <b>Maximum of additionally characterised peaks</b>               | 0.003   | 0.5   | -                       | -            |
| <b>Sum of additionally characterised peaks</b>                   | 0.031   | 5.3   | 0.031                   | 5.3          |
| <b>Total characterised by HPLC</b>                               |   |       | <b>0.033</b>            | <b>5.7</b>   |
| <b>Macerozyme solubilisate</b>                                   |   |       | 0.01                    | 1.7          |
| <b>Amylase solubilisate</b>                                      |   |       | 0.004                   | 0.7          |
| <b>Tyrosinase solubilisate</b>                                   |   |       | 0.003                   | 0.5          |
| <b>Pepsin solubilisate</b>                                       |   |       | 0.001                   | 0.2          |
| <b>Pancreatin solubilisate</b>                                   |   |       | 0.003                   | 0.6          |
| <b>Total characterised</b>                                       |   |       | <b>0.054</b>            | <b>9.4</b>   |
| <b>Total identified and characterised in RRR</b>                 |   |       | <b>0.055</b>            | <b>9.5</b>   |
| <b>Final residue</b>   |   |       | 0.025                   | 4.3          |
| <b>Total identified and characterised in RRR + final residue</b> |   |       | <b>0.08</b>             | <b>13.9</b>  |

Not detected = N.D.

*Rape Seeds**Phenyl-label*

For the phenyl label, analysis of the concentrated cyclohexane extract of rape seeds with HPLC-MS method LC07 shows four peaks, of which one was tentatively assigned to the parent compound BAS 684 H (0.014 mg eq/kg, 13.7% TRR).

BAS 684 H was confirmed using HPLC-MS method LC02. In the chromatograms of the cyclohexane extract of rape seeds with HPLC-MS method LC07 the peak at 119.9 min was tentatively assigned to the parent compound BAS 684 H, based on comparison of the retention with that of the HPLC-MS analysis of the methanol extract of rape straw (phenyl label).

The total radioactivity present in the concentrated cyclohexane extract of rape seeds (phenyl label) was 0.032 mg eq/kg (32.2% TRR) and 43.8% of that has been conclusively identified. The 3 individual residues in the range of 0.002-0.007 mg eq/kg (2.4-7.4% TRR) are considered characterised and no further attempts to identify these peaks are required. Overall, 0.014 mg eq/kg (13.7% TRR) was identified in the concentrated cyclohexane extract of rape seeds (phenyl label). The remaining components were detected up to 0.008 mg eq/kg (8.4% TRR), summed up to 0.018 mg eq/kg (18.2% TRR) and were classified as characterised.

The chromatogram of the concentrated methanol extract of rape seeds (phenyl label) with HPLC-MS method LC07 shows five peaks. The early eluting peaks were designated as polar components (in sum: 0.012 mg eq/kg; 12.4% TRR) and classified as characterised. Polar components were confirmed using HPLC-MS method LC02. Early eluting components ( $t_r \leq 9$  min) in HPLC-MS chromatograms (LC07) were designated as polar components and classified as characterised.

The total radioactivity present in the concentrated methanol extract of rape seeds (phenyl label) was 0.015 mg eq/kg (15.3% TRR) and 80% of that has been characterised as polar components. The early eluting peaks were

designated as polar components (in sum: 0.012 mg eq/kg; 12.4% TRR) and classified as characterised. The 3 individual polar components were in the range of 0.001-0.010 mg eq/kg (1.3 – 9.8 % TRR) and are considered characterised and no further attempts to identify these peaks are required. The remaining components detected up to 0.002 mg eq/kg (1.5% TRR), summed up to 0.003 mg eq/kg (2.9% TRR) were classified as characterised.

The chromatogram of the concentrated water extract of rape seeds (phenyl label) with HPLC-MS method LC07 shows five peaks, which were all eluting  $\leq 9$  min and were therefore designated as polar components (in sum: 0.014 mg eq/kg; 14.1% TRR) and classified as characterised. The 5 individual polar components were in the range of 0.001-0.008 mg eq/kg (0.8 – 7.9 % TRR) and are considered characterised and no further attempts to identify these peaks are required. The total radioactivity present in the concentrated water extract of rape seeds (phenyl label) was 0.014 mg eq/kg (14.1% TRR).

Altogether, 0.084 mg eq/kg (84.1% TRR) was identified and characterised in the ERR and RRR of rape seeds (phenyl label).

Table 7-39 Summary of identified and characterised residues in rape seeds (phenyl label)

| Designation   | Extracts            |       |                  |       |               |       | Sum of extracts |       |
|---|---------------------|-------|------------------|-------|---------------|-------|-----------------|-------|
|   | Cyclohexane extract |       | Methanol extract |       | Water extract |       |                 |       |
|   | mg eq/kg            | % TRR | mg eq/kg         | % TRR | mg eq/kg      | % TRR | mg eq/kg        | % TRR |
| Total radioactive residues                                |                     |       |                  |       |               |       | 0.100           | 100   |
| Identified  |                     |       |                  |       |               |       |                 |       |
| BAS 684 H   | 0.014               | 13.7  | N.D.             | N.D.  | N.D.          | N.D.  | 0.014           | 13.7  |
| Total identified  |                     |       |                  |       |               |       | 0.014           | 13.7  |
| Characterised   |                     |       |                  |       |               |       |                 |       |
| Polar comp.   | N.D.                | N.D.  | 0.012            | 12.4  | 0.014         | 14.1  | 0.026           | 26.5  |
| Number of additionally characterised peaks                | 3                   |       | 2                |       | 0             |       | -               | -     |
| Maximum of additionally characterised peaks               | 0.008               | 8.4   | 0.002            | 1.5   | -             | -     | -               | -     |
| Sum of additionally characterised peaks                   | 0.018               | 18.2  | 0.003            | 2.9   | -             | -     | 0.021           | 21.0  |
| Total characterised by HPLC                               |                     |       |                  |       |               |       | 0.047           | 47.5  |
| Ammonia solubilisate                                      |                     |       |                  |       |               |       | 0.009           | 8.8   |
| Macerozyme solubilisate                                   |                     |       |                  |       |               |       | 0.01            | 10.1  |
| Amylase solubilisate                                      |                     |       |                  |       |               |       | 0.003           | 2.8   |
| Tyrosinase solubilisate                                   |                     |       |                  |       |               |       | <0.001          | 0.3   |
| Pepsin solubilisate                                       |                     |       |                  |       |               |       | <0.001          | 0.5   |
| Pancreatin solubilisate                                   |                     |       |                  |       |               |       | <0.001          | 0.4   |
| Total characterised                                       |                     |       |                  |       |               |       | 0.07            | 70.3  |
| Total identified and characterised                        |                     |       |                  |       |               |       | 0.084           | 84.1  |
| RRR   |                     |       |                  |       |               |       | 0.009           | 9.0   |
| Total identified and characterised in RRR + final residue |                     |       |                  |       |               |       | 0.093           | 93.1  |

Not detected = N.D.

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*Cyclohexane-label*

For the cyclohexane label, the chromatogram of the cyclohexane extract of rape seeds with HPLC-MS LC07 shows 17 peaks, of which one was tentatively assigned to the parent compound BAS 684 H (0.004 mg eq/kg; 4.3% TRR).

The confirmatory method used was HPLC-MS method LC02; however, no radioactive residues were detected.

The total radioactivity present in the concentrated cyclohexane extract of rape seeds (cyclohexane label) was 0.024 mg eq/kg (28.7% TRR) and 16.7% of that has been conclusively identified. The 16 individual residues in the range of <0.001 – 0.003 mg eq/kg (0.3 – 3.9% TRR) are considered characterised and no further attempts to identify these peaks are required. Overall, 0.024 mg eq/kg (28.7% TRR) was identified in the concentrated cyclohexane extract of rape seeds (cyclohexane label). The remaining components were detected up to 0.003 mg eq/kg (3.9% TRR), summed up to 0.02 mg eq/kg (24.4% TRR) and were classified as characterised.

The analysis of the concentrated methanol of rape seeds (cyclohexane label) led to a pattern with three peaks. The early eluting peaks were designated as polar components and classified and characterised (in sum: 0.011 mg eq/kg; 13.4% TRR).

The confirmatory method used was HPLC-MS method LC02; however, no radioactive residues were detected only polar components. Early eluting components ( $t_r \leq 9$  min) in HPLC chromatograms (LC07) were designated as polar components and classified as characterised.

The total radioactivity present in the concentrated methanol extract of rape seeds (cyclohexane label) was 0.012 mg eq/kg (14.5% TRR) and 91.7% of that has been characterised as 2 polar components (each 0.003 mg eq/kg (4.1 % TRR) and 0.008 mg eq/kg (9.3 % TRR). The one remaining individual residue at 0.001 mg eq/kg (1.1% TRR) is considered characterised and no further attempts to identify these peaks are required.

The analysis of the concentrated water extract of rape seeds (cyclohexane label) led to a pattern with three early eluting peaks, which were designated as polar components and classified as characterised (in sum: 0.007 mg eq/kg; 8.6% TRR).

The total radioactivity present in the concentrated water extract of rape seeds (cyclohexane label) was 0.011 mg eq/kg (13.24% TRR) and all radioactive components were identified as polar components. The confirmatory method used was HPLC-MS method LC02; however, no radioactive residues were detected.

Altogether, in the ERR and RRR of rape seeds (cyclohexane label) 0.064 mg eq/kg (76.9% TRR) were identified and characterised.

Table 7-40 Summary of identified and characterised residues in rape seeds (cyclohexane label)

| Designation   | Extracts            |       |                  |       |               |       | Sum of extracts |       |
|---|---------------------|-------|------------------|-------|---------------|-------|-----------------|-------|
|   | Cyclohexane extract |       | Methanol extract |       | Water extract |       |                 |       |
|   | mg eq/kg            | % TRR | mg eq/kg         | % TRR | mg eq/kg      | % TRR | mg eq/kg        | % TRR |
| Total radioactive residues                                |                     |       |                  |       |               |       | 0.083           | 100   |
| Identified  |                     |       |                  |       |               |       |                 |       |
| BAS 684 H   | 0.004               | 4.3   | N.D.             | N.D.  | N.D.          | N.D.  | 0.004           | 4.3   |
| Total identified  |                     |       |                  |       |               |       | 0.004           | 4.3   |
| Characterised   |                     |       |                  |       |               |       |                 |       |
| Polar comp.   | N.D.                | N.D.  | 0.011            | 13.4  | 0.011         | 13.4  | 0.022           | 26.8  |
| Number of additionally characterised peaks                | 16                  |       | 1                |       | 0             |       | -               | -     |
| Maximum of additionally characterised peaks               | 0.003               | 3.9   | 0.001            | 1.1   | -             | -     | -               | -     |
| Sum of additionally characterised peaks                   | 0.02                | 24.4  | 0.001            | 1.1   | -             | -     | 0.021           | 25.5  |
| Total characterised by HPLC                               |                     |       |                  |       |               |       | 0.044           | 52.3  |
| Ammonia solubilisate                                      |                     |       |                  |       |               |       | 0.003           | 4.0   |
| Macerozyme solubilisate                                   |                     |       |                  |       |               |       | 0.008           | 9.6   |
| Amylase solubilisate                                      |                     |       |                  |       |               |       | 0.002           | 2.2   |
| Tyrosinase solubilisate                                   |                     |       |                  |       |               |       | 0.001           | 1.1   |
| Pepsin solubilisate                                       |                     |       |                  |       |               |       | 0.002           | 2.4   |
| Pancreatin soulubilisate                                  |                     |       |                  |       |               |       | 0.001           | 0.9   |
| Total characterised                                       |                     |       |                  |       |               |       | 0.061           | 72.5  |
| Total identified and characterised                        |                     |       |                  |       |               |       | 0.064           | 76.9  |
| RRR   |                     |       |                  |       |               |       | 0.01            | 11.5  |
| Total identified and characterised in RRR + final residue |                     |       |                  |       |               |       | 0.074           | 88.3  |

Not detected = N.D.



Table 7-41 Summary of identified/characterised components in oilseed rape matrices

| Designation   | Straw        |         | Hulls        |         | Seeds        |         |
|---|--------------|---------|--------------|---------|--------------|---------|
|   | [mg eq/kg]   | [% TRR] | [mg eq/kg]   | [% TRR] | [mg eq/kg]   | [% TRR] |
| <b>Phenyl label</b>   |              |         |              |         |              |         |
| M684H005  | 0.487        | 13.0    | 0.011        | 1.9     | not detected |         |
| M684H006  | 0.439        | 11.8    | 0.046        | 8.3     | not detected |         |
| M684H007  | 0.122        | 3.3     | not detected |         | not detected |         |
| M684H008  | 0.241        | 6.5     | 0.009        | 1.6     | not detected |         |
| M684H015  | 0.054        | 1.4     | not detected |         | not detected |         |
| M684H016  | 0.170        | 4.5     | 0.031        | 5.6     | not detected |         |
| M684H046  | 0.111        | 3.0     | not detected |         | not detected |         |
| M684H047  | 0.164        | 4.4     | not detected |         | not detected |         |
| M684H048  | 0.180        | 4.8     | not detected |         | not detected |         |
| M684H051  | 0.097        | 2.6     | not detected |         | not detected |         |
| M684H055  | 0.060        | 1.6     | not detected |         | not detected |         |
| BAS 684 H   | 0.043        | 1.2     | 0.003        | 0.5     | 0.014        | 13.7    |
| Total identified  | 2.169        | 58.1    | 0.099        | 18.0    | 0.014        | 13.7    |
| Total characterised from ERR                                | 1.268        | 34.0    | 0.306        | 55.5    | 0.047        | 47.5    |
| Total characterised from RRR                                | 0.138        | 3.7     | 0.082        | 14.8    | 0.023        | 22.8    |
| Total identified and characterised                          | 3.575        | 95.8    | 0.487        | 88.2    | 0.084        | 84.1    |
| Final residue   | 0.061        | 1.6     | 0.030        | 5.4     | 0.009        | 9.0     |
| Grand total of identified, characterised and final residues | 3.636        | 97.5    | 0.517        | 93.6    | 0.093        | 93.1    |
| <b>Cyclohexane label</b>                                    |              |         |              |         |              |         |
| M684H005  | 0.755        | 22.1    | 0.004        | 0.7     | not detected |         |
| M684H006  | 0.046        | 1.4     | 0.018        | 3.2     | not detected |         |
| M684H007  | not detected |         | 0.012        | 2.0     | not detected |         |
| M684H008  | 0.268        | 7.9     | not detected |         | not detected |         |
| M684H015  | not detected |         | 0.004        | 0.7     | not detected |         |
| M684H016  | not detected |         | 0.031        | 5.4     | not detected |         |
| M684H046  | 0.116        | 3.4     | not detected |         | not detected |         |
| M684H047  | 0.072        | 2.1     | 0.013        | 2.2     | not detected |         |
| M684H048  | 0.243        | 7.1     | 0.014        | 2.5     | not detected |         |
| M684H051  | 0.103        | 3.0     | 0.010        | 1.7     | not detected |         |
| M684H055  | not detected |         | 0.006        | 1.0     | not detected |         |
| BAS 684 H   | not detected |         | not detected |         | 0.004        | 4.3     |
| Total identified  | 1.604        | 46.9    | 0.112        | 19.4    | 0.004        | 4.3     |
| Total characterised from ERR                                | 1.343        | 39.3    | 0.357        | 61.9    | 0.044        | 52.3    |
| Total characterised from RRR                                | 0.122        | 3.6     | 0.054        | 9.4     | 0.017        | 20.2    |
| Total identified and characterised                          | 3.069        | 89.8    | 0.524        | 90.8    | 0.064        | 76.9    |
| Final residue   | 0.038        | 1.1     | 0.025        | 4.3     | 0.010        | 11.5    |
| Grand total of identified, characterised and final residues | 3.107        | 90.9    | 0.549        | 95.1    | 0.074        | 88.3    |

N.D. = Not detected

### Cleavage Experiments

Cleavage experiments were conducted with individual fractions of the water phase of the methanol extract of rape straw (phenyl label) to investigate the stability of conjugated metabolites to support the development of the residue analytical method. The metabolites observed are summarised in Table 7-42.

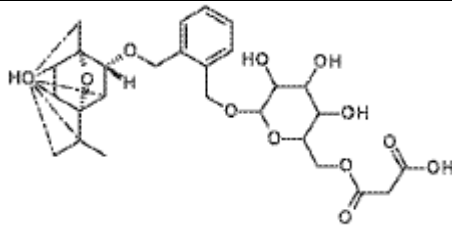
In the concentrated fraction 1 of the concentrated water phase of the rape straw methanol extract (phenyl label) a component was detected by HPLC-MS which corresponds to the molecular ion of a compound twofold hydroxylated and twofold conjugated with glucoside. The component corresponding to the molecular ion was designated as M684H051; the evidence provided is satisfactory. The eluate from LC-MS analysis of fraction 1 was collected and treated with ammonia and  $\beta$ -glucosidase, purified and analysed by LC-MS. The main compound eluting corresponds to twofold hydroxylated compounds. The retention times and MS/MS spectra do not match to those of the reference items (for example the twofold hydroxylated compounds M684H044 and Reg. No. 6059084); the component was designated as M684H039 and the evidence provided is satisfactory given M684H039 is a generic structure (hence no reference item available)\_which is consistent with the MS fragments which give evidence for hydroxylation in both the phenyl and cyclohexane rings.

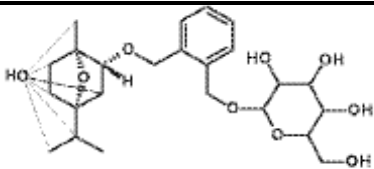
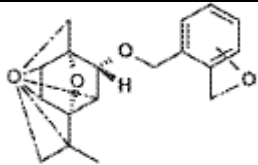
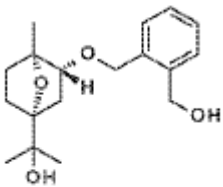
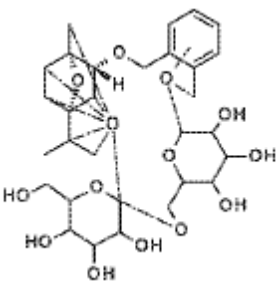
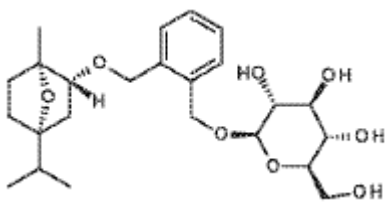
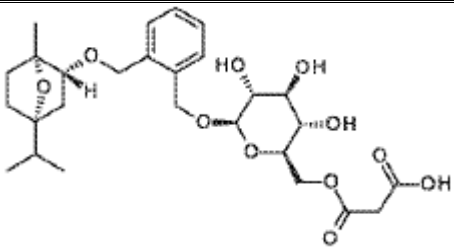
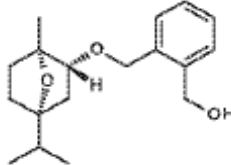
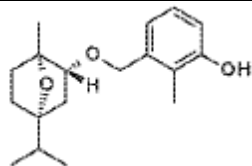
Prior to the treatment of the concentrated fraction 2 with ammonia and  $\beta$ -glucosidase the fraction was analysed by HPLC-MS/MS. The MS/MS spectrum showed several cineol fragments exhibiting an isopropenyl group; indicating the corresponding metabolite was hydroxylated at the isopropyl group of the cineol moiety. Whilst the metabolite hasn't been definitely identified the evidence provided suggests the metabolite is M684H048. Metabolite M684H048 depletes following treatment with ammonia and  $\beta$ -glucosidase and resulted in the formation of a major peak attributed to aglycone (M684H044) and two minor peaks both attributed to aglycone (M684H039). Metabolite M684H044 was identified by co-elution with reference standard using HPLC-MS method LC02. Whereas, the two minor peaks haven't been definitively identified but using the evidence provided (LC-MS spectra) the metabolite was designated as M684H039.

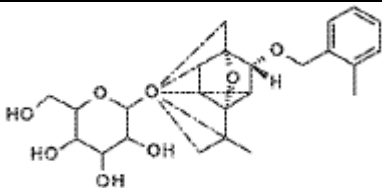
Prior to the treatment of the concentrated fraction 3 with ammonia and  $\beta$ -glucosidase the fraction was analysed by HPLC-MS method LC07. The metabolites M68H047 and M684H048 were identified by co-elution with reference standards. Incubation with ammonia and  $\beta$ -glucosidase of the concentrated fraction 3 after HPLC-MS analysis resulted in complete depletion of both metabolites M684H047 and M684H048 and in the formation of a major peak (M684H044) and a minor peak (M684H039). Both metabolites (M684H044 and M684H039) haven't been definitively identified but using the evidence provided (LC-MS spectra) the metabolites were designated as M684H039 and M684H044.

Prior to treatment of the concentrated fraction 4 with ammonia and  $\beta$ -glucosidase the fraction was analysed by HPLC-MS method LC02. The metabolite M684H005 was identified by co-elution with reference standard using HPLC-MS. Incubation with ammonia and  $\beta$ -glucosidase of the concentrated fraction 4 after HPLC-MS analysis resulted in complete depletion of M684H005 and in the formation of a major peak (M684H002) and two minor peaks (M684H046 and M684H017). The three metabolites were identified by co-elution with the reference standards using HPLC-MS.

Table 7-42 Summary of identified metabolites

| Designation | Structure  | Fraction(s) the metabolite(s) are observed in |
|-------------|--|---|
| M684H047    |  | Fraction 3                                    |

|          |  |  |
|----------|--|--|
| M684H048 |     | Fraction 2<br>Fraction 3               |
| M684H039 |     | Fraction 1<br>Fraction 2<br>Fraction 3 |
| M684H044 |     | Fraction 2<br>Fraction 3               |
| M684H051 |    | Fraction 1                             |
| M684H005 |   | Fraction 4                             |
| M684H006 |  | Fraction 4                             |
| M684H002 |   | Fraction 4                             |
| M684H017 |   | Fraction 4                             |

|          |   |            |
|----------|---|------------|
| M684H046 |  | Fraction 4 |
|----------|---|------------|

#### Chiral analysis

Chiral analysis was performed to investigate whether one enantiomer of BAS 684 H was preferably metabolised in oilseed rape (straw, hulls and seeds). BAS 684 H was not detected at sufficient levels (<0.01 mg eq/kg) in all oilseed rape matrices for both <sup>14</sup>C-labels, therefore, its main metabolite M684H005 was investigated in representative samples of straw for the phenyl label by HPLC-MS.

The subsample of the ethyl acetate phase obtained from the methanol extract of oilseed rape straw (phenyl-label) was fractionated by HPLC method LC09 to isolate the metabolite M684H005. An aliquot of the collected fraction was analysed by a chiral HPLC-MS method LC02 resulted in a pattern of two peaks corresponding to stereoisomeric ratios (Diastereomer 1 : Diastereomer 2) listed below in Table 7-43.

Table 7-43 Determination of the enantiomer ratio of M684H005 in oilseed rape straw

| Matrix              | Diastereomer 1 [% AR] | Diastereomer 2 [% AR] | SE [%] |
|---------------------|-----------------------|-----------------------|--------|
| <b>phenyl-label</b> |                       |                       |        |
| <b>Straw</b>        | 26                    | 74                    | -48    |

SE = stereoisomers excess,  $SE = [(A1\% \text{ AR} - A2\% \text{ AR}) / (A1\% \text{ AR} + A2\% \text{ AR})]$

The stereoisomeric ratio changed from 1:1 (BAS 684 H as applied) to approximately 1:3 (M684H005 in rape straw), which represents a significant change in stereoisomeric ratio upon metabolism in oilseed rape. The two diastereomers of M684H005 were not available as reference items to allow definite assignment of each peak to a diastereomer. However, given the column and solvents used were similar to that for enantiomeric analysis of parent, the order of elution may suggest that the first eluting peak, diastereomer 1 originates from the (-)-enantiomer of parent 2 from the (+)-enantiomer of parent. The inability to definitively assign a diastereomer to each peak and the significant change in stereoisomeric ratio are not considered to affect the consumer risk assessment given the toxicological evaluation of the two diastereomers concluded they are of equivalent toxicity (Vol 1 Section 2.12.3).

#### Storage stability

Rape straw, hulls and seeds were extracted up to 605 days after sampling and the extracts were analysed up to 626 days after extraction. The period from sampling to analysis was up to 645 days.

**Matrix stability:** No matrix stability experiments were undertaken. Given no label-specific metabolites were identified in the study, comparison can be made between the chromatograms for the cyclohexane label (following extraction 19-34 days after sampling) and the chromatograms for the phenyl label (following extraction 408-605 after sampling), in which a similar metabolic pattern was observed, to demonstrate stability of the matrices (for the phenyl label).

**Extract stability:** The methanol and water extracts of rape straw (phenyl label) were analysed before and after storage for up to 629 days (method LC07) and up to 629 days (method LC02). Partial conversion of M684H047 into M684H048, and M684H006 into M684H005 was observed however a similar metabolic pattern was observed. Given the matrix similarity between straw and hulls, the data is considered sufficient to cover the extract stability intervals for both straw and hulls.

For rape seeds, the extractable residues were low (0.051 – 0.063 mg eq/kg), and other than low amounts of parent BAS 684 H in the cyclohexane extract (0.004 – 0.014 mg eq/kg), no other components were identified. Additionally, a similar metabolic pattern was observed in the chromatograms of the cyclohexane label (extracted up to 21 days after sampling and analysed up to 613 days after sampling) and the phenyl label (extracted up to

443 days after sampling and analysed up to 127 days after extraction) demonstrating stability of residues in both the matrix and extracts.

Therefore it is concluded that sample integrity was maintained for the storage intervals in the study.

#### *Proposed metabolic pathway*

The proposed metabolic pathway of BAS 684 H in oilseed rape is shown in Figure 7-5. A summary of the detected metabolites is given in Table 7-42.

Almost all identified metabolites result from hydroxylation of the parent compound BAS 684 H and subsequent conjugation with glucoside or malonyl glucoside.

The primary metabolites in oilseed rape: M684H005 and M684H006, result from hydroxylation of the parent compound BAS 684 H at the methyl group of the phenyl moiety and subsequent conjugation with glucoside and malonyl glucoside, respectively (Figure 7-5: pathway A). Hydroxylation and subsequent conjugation on other positions of the phenyl moiety results in the metabolites M684H015, M684H016 and M684H007, which are present at low levels (Figure 7-5: pathway C). The parent compound was also hydroxylated and conjugated at cineol moiety resulting in metabolite M684H055. Multiple hydroxylation at both the phenyl and cineol moiety and subsequent conjugation with glucoside and malonyl glucoside result in the formation of metabolites M684H048 and M684H047, respectively. Conjugation with glucoside at both the phenyl and cineol moiety results in metabolite M684H051 (Figure 7-5: pathway B).

Metabolite M684H008 represents the generic structure of phenyl ring conjugates of glucosides and is included in the metabolic pathway for the sake of completeness.

The aglycones were identified after cleavage experiments with ammonia and  $\beta$ -glucosidase and were not identified in the solvent extracts and solubilisates of rape matrices.

#### **Conclusion**

The metabolism of BAS 684 H was investigated in oilseed rape by applying a single application of phenyl-labelled or cyclohexane-labelled BAS 684 H at a maximum rate of 250 g a.s./ha. Samples of oilseed rape straw, hulls and seeds were collected at 90 DAT (BBCH 89).

The highest and lowest TRR were determined for oilseed rape straw (up to 3.730 mg eq/kg) and oilseed rape seeds ( $\leq 0.100$  mg eq/kg), respectively. The TRR of oilseed rape hulls was 0.552 mg eq/kg and 0.577 mg eq/kg for the phenyl and the cyclohexane-label, respectively. Within the same matrix, the amount of radioactive residues was comparable for both labels.

The extractability of the oilseed rape matrices with cyclohexane, methanol and water ranged from 61.4% TRR to 89.5% TRR (0.051 – 2.323 mg eq/kg). For oilseed rape straw, the major portions of radioactive residues were extracted with methanol at 72.7% TRR (2.713 mg eq/kg) and 73.5% TRR (2.512 mg eq/kg) for the phenyl and the cyclohexane-label, respectively. For oilseed rape hulls, similar portions were extracted with methanol and water. For oilseed rape hulls, similar portions were extracted with methanol (35.7% TRR (0.197 mg eq/kg) and 35.3% TRR (0.204 mg eq/kg) for the phenyl and cyclohexane label, respectively) and water (35.9% TRR (0.198 mg eq/kg) and 45.9% TRR (0.265 mg eq/kg) for the phenyl and cyclohexane label, respectively). For oilseed rape seeds, the extractabilities of both labels were highly comparable.

The residues after solvent extraction were further solubilised by ammonia and subsequent enzyme incubations. For oilseed rape straw and hulls (both labels), the highest portions of radioactive residues were solubilised by ammonia treatment (ranging between 0.054 – 0.202 mg eq/kg or 5.4 – 9.7% TRR). For oilseed rape seeds (both labels), the highest portions of radioactive residues were solubilised by macerozyme treatment (ranging between 0.008 – 0.010 mg eq/kg (9.6 – 10.1% TRR)). For oilseed rape seeds of the phenyl label, high portions were additionally released by ammonia and amylase/  $\beta$ -amylglycosidase incubation (0.009 mg eq/kg (8.8% TRR) and 0.003 mg eq/kg (2.8% TRR) respectively). The final residues were each below or equal to 11.5% TRR (0.010 mg eq/kg) or 0.061 mg eq/kg (1.6% TRR) and are not considered to be bioavailable.

Structure elucidation was based on HPLC-MS and MS/MS analysis of the purified ethyl acetate phase and fractions of the water phase both obtained from the methanol extract of oilseed rape straw (phenyl-label). Peaks in other extracts were assigned by co-chromatography experiments with external samples from a related metabolism of BAS 684 H in wheat.

The most abundant components in oilseed rape straw were metabolites M684H005 (both labels), M684H006 (for the phenyl-label) and M684H008 (for the cyclohexane-label). The most abundant components in oilseed

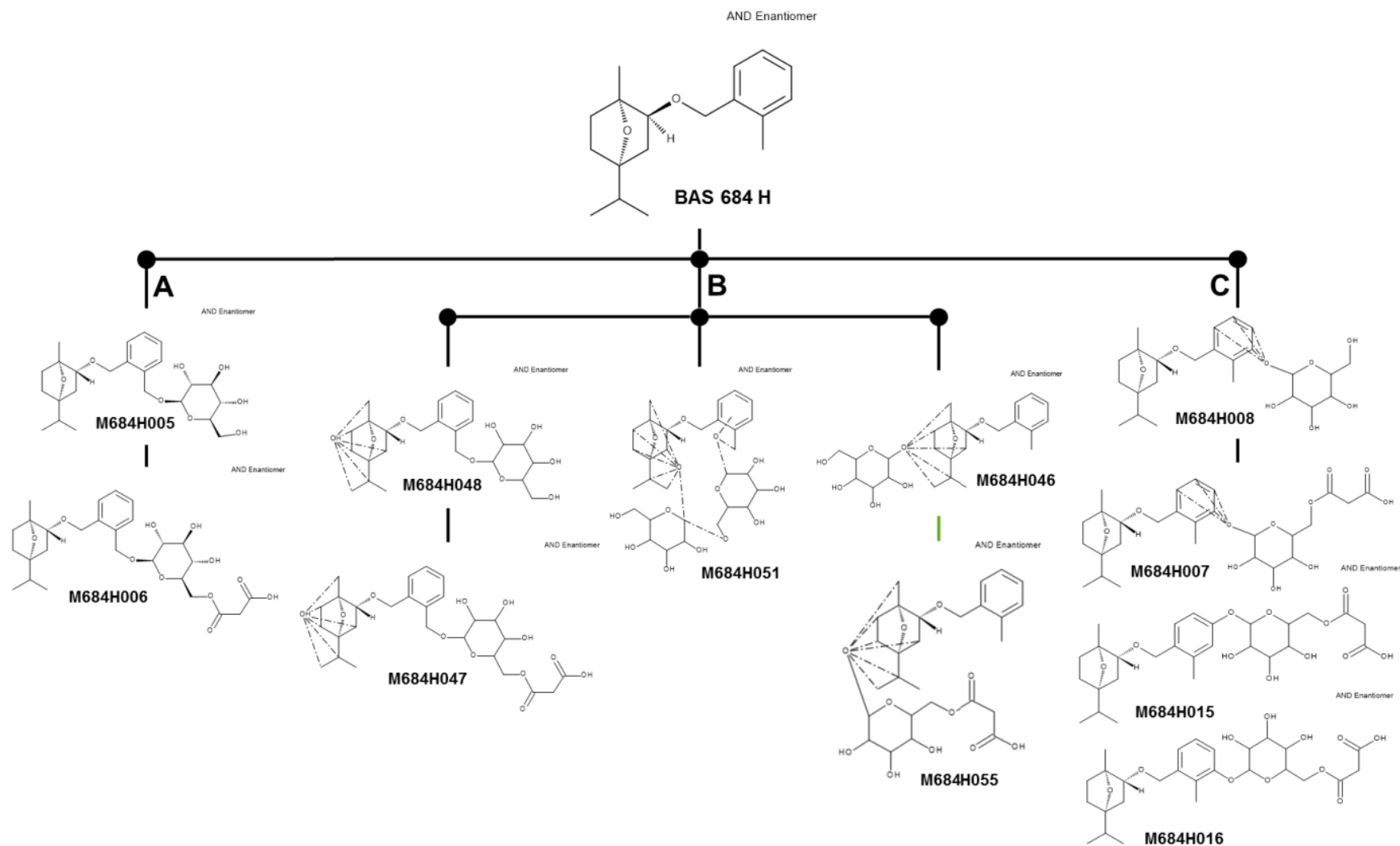
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rape hulls were metabolites M684H006 (for both labels), M684H016 (for both labels) and M684H048 (for cyclohexane label). The parent compound BAS 684 H was detected at 1.2% TRR (0.043 mg eq/kg) or below in oilseed rape straw and hulls. Other metabolites accounted for up to 22.1% TRR (0.755 mg eq/kg). In oilseed rape seeds (both labels) the parent compound was the only detected compound (4.3 – 13.7% TRR (0.004 – 0.014 mg eq/kg)).

Up to 17 peaks  $\geq 0.01$  mg/kg in the extracts of oilseed rape straw and hulls were not identified, including up to 5 unidentified peaks  $\geq 0.05$  mg/kg at a maximum of 0.096 mg/kg or 2.6% TRR. Attempts were made to identify these peaks including comparison of retention times and MS data with a range of reference items for postulated metabolites. For some extracts, poor peak resolution and co-elution of peaks hindered identification. Given oilseed rape straw and hulls are neither food nor feed commodities there is no effect on the dietary burden and overall consumer risk assessment hence the degree of characterisation and identification performed is considered acceptable.

The metabolic transformation steps of BAS 684 H in oilseed rape are hydroxylation of the parent compound at various positions and subsequent conjugation of hydroxyl groups with glycoside and malonyl glycoside. No cleavage of the molecule was observed through metabolism of BAS 684 H in oilseed rape.

Figure 7-5 Proposed pathway of BAS 684 H in oilseed rape



**B.7.2.1.3. Carrots**

|                    |   |
|--------------------|---|
| <b>Report:</b>     | CA 6.2.1/003<br>Schweda Z., Forieri I., 2018 a<br>Metabolism of $^{14}\text{C}$ -BAS 684 H in carrots<br>2017/1110861; Study ID 741153  |
| <b>Guidelines:</b> | EPA 860.1000, EPA 860.1300: Nature of the Residue in Plants Livestock, PMRA Residue Chemistry Guidelines Section 97.2 Nature of the Residue - Plants - Livestock (Canada), EEC 7028/VI/95 rev. 3 Appendix A (EU): Metabolism and distribution in plants, JMAFF 59 NohSan No 4200, Test No. 501: Metabolism in crops |
| <b>GLP:</b>        | yes   |

**Materials and methods***Materials*1. C-label BAS 684 H (CAS No. 87818-31-3)

|                              |   |
|------------------------------|---|
| <b>Description:</b>          | Phenyl- $\text{U-}^{14}\text{C}$ (spec. activity of a.s. 17.1 MBq/mg) |
| <b>Lot/Batch #:</b>          | 1147-2001   |
| <b>Radiochemical Purity:</b> | 98.9%   |
| <b>Chemical Purity:</b>      | 97.0%   |

2. C-label BAS 684 H (CAS No. 87818-31-3)

|                              |   |
|------------------------------|---|
| <b>Description:</b>          | Cyclohexane-4- $^{14}\text{C}$ (spec. activity of a.s. 8.08 MBq/mg) |
| <b>Lot/Batch #:</b>          | 1146-1001   |
| <b>Radiochemical Purity:</b> | 97.9%   |
| <b>Chemical Purity:</b>      | 95.9%   |

3. C-label BAS 684 H (CAS No. 87818-31-3)

|                         |                         |
|-------------------------|-------------------------|
| <b>Description:</b>     | Benzyl- $^{13}\text{C}$ |
| <b>Lot/Batch #:</b>     | 1159-1012               |
| <b>Chemical Purity:</b> | <b>99.6%</b>            |

4. BAS 684 H (CAS No. 87818-31-3)

|                         |                      |
|-------------------------|----------------------|
| <b>Description:</b>     | Unlabelled BAS 684 H |
| <b>Lot/Batch #:</b>     | L87-84               |
| <b>Chemical Purity:</b> | <b>99.0%</b>         |

*Methods*

A metabolism study on carrots (variety Nantaise) grown indoors in at the Agricultural Research Centre of BASF SE in Limburgerhof, Germany was carried out in 2015-2018. Plants were grown in in climatic conditions simulating natural climatic conditions of a typical carrot seeds growing area. The experiments with both labels were carried out within the same time period.

Carrot seeds were sown into 12 boxes filled with sandy loam and cultivated using normal agricultural practices. For both labels (preparation 1 and preparation 2), the crops were treated once with a single foliar application of BAS 684 H, at growth stage BBCH 12-13 (there is no intended uses proposed on carrots). The test item was applied as EC formulations at a total nominal application rate of 500.0 g a.s./ha. The actual application rates were 511.0 g a.s./ha (phenyl label) and 503.3 g ai/ha (cyclohexane label). Roots and leaves samples of both labels were generated 67 DAT at growth stage BBCH 49 for both labels. A summary of the applications in the study are given in Table 7-44.

Preparation 1: For the preparation of the application formulation of the phenyl label: phenyl- $\text{U-}^{14}\text{C}$  (dissolved in toluene), benzyl- $^{13}\text{C}$  and unlabelled BAS 684 H were mixed to obtain a ratio of approximately 1:1:1.

Preparation 2: For the preparation of the application formulation of the cyclohexane label, cyclohexane-4- $^{14}\text{C}$  (dissolved toluene) and unlabelled BAS 684 H were mixed in an approximate ratio of 1:1.

On the day of application, the mixtures were taken up in water and blank formulation assisted by ultrasonication. The purity of the application solution was confirmed using HPLC and the isotopic pattern as well as the identity was determined and verified by HPLC-MS analysis.



The structural formulae of the labelled BAS 684 H molecules are given in Figure 7-2.

Table 7-44 Study design: plant uptake part (carrots)

| Label                               | <sup>14</sup> C-Phenyl-label<br>(with <sup>13</sup> C-Benzyl-label) |    | <sup>14</sup> C-Cyclohexane-label |    |
|-------------------------------------|---|----|-----------------------------------|----|
| Intended use rate [g a.s./ha]       | 500   |    | 500                               |    |
| Actual application rate [g a.s./ha] | 511.0   |    | 503.3                             |    |
| application number                  | 1   |    | 1                                 |    |
| application growth stage            | BBCH12-13   |    | BBCH12-13                         |    |
| sampled matrices                    | Roots, leaves   |    | Roots, leaves                     |    |
| sampling [DALA] <sup>1)</sup>       | Roots   | 67 | Roots                             | 67 |
|                                     | Leaves  | 67 | Leaves                            | 67 |

1) days after last application

At growth stage BBCH 49, roots and leaves were separated. The roots were rinsed with water and chopped (designated as ‘carrot roots’). The leaves were cut into small pieces and mixed (designated as ‘carrot leaves’). All samples were weighed and stored in a freezer ( $\leq -18^{\circ}\text{C}$ ).

Carrot roots and leaves were extracted up to 79 days after sampling and the extracts were analysed up to 63 days after extraction. The period from sampling to analysis was up to 141 days. Therefore no storage stability data are required, however experiments were still performed to determine extract and matrix stability and these are summarised in the results section for completeness.

#### *Description of analytical procedures*

Homogenised solid plant samples were weighed and combusted by means of an automatic sample oxidiser. The limit of quantitation in mg eq/kg was calculated from the twofold background radioactivity level (dpm/g matrix) divided by the corresponding specific radioactivity. For the quantitation of radioactive residues in liquid samples a liquid scintillation counter (LSC) was used.

Homogenisation/solvent extraction: The samples (carrot roots and leaves) were homogenised with a mill along with a dry ice. After sublimation of the dry ice (overnight in a freezer), the samples were weighed and stored in a freezer.

The homogenised samples were extracted three times with methanol and twice with water. Each extraction was performed in an incubation shaker for 60 min.

After each extraction step, the solid material was separated from the extract by centrifugation, followed by filtration (filter paper). The resulting filtrates were made up to defined volumes. The residues after solvent extractions were transferred to a tared vessel and dried in a lyophilisation device for approximately 48 hours. Aliquots of the extracts were radio-assayed and further aliquots of all methanol extracts and of water extracts obtained from carrot leaves (both labels) were analysed by HPLC.

Solubilisation of the RRR: The RRR of carrot roots and leaves (both labels) were subjected to sequential solubilisation procedures. All incubations were carried out gently shaken and in buffered enzyme solutions and were stopped by addition of acetonitrile. After each incubation step, the solubilisate was separated from the residue by centrifugation and filtration.

The RRR after solvent extraction was extracted twice with ammonia. Thereafter, the dried residues were taken up in 1% ammonia solution and shaken for 60 min. The resulting residues after ammonia treatment were suspended in 0.1 M acetate buffer and incubated with macerozyme and cellulase at  $37^{\circ}\text{C}$  for approximately 48 hours. The residues after macerozyme/ cellulase incubation were taken up in sodium acetate buffer and incubated with  $\beta$ -glycosidase at  $37^{\circ}\text{C}$  for approximately 48 hours. For carrot roots (both labels), the residue

after  $\beta$ -glycosidase treatment was not further investigated. For carrot leaves (both labels), the residue after  $\beta$ -glycosidase treatment was dissolved in an artificial gastric juice containing pepsin and incubated at 37°C overnight. Finally, the residue after pepsin treatment was incubated in an artificial intestine fluid containing pancreatin at 37°C overnight. Aliquots of the solubilisates and residues thereof were radio-assayed. Further aliquots of the resulting ammonia solubilisates were analysed by HPLC. For carrot roots (both labels), additional aliquots of the macerozyme solubilisates were subject to HPLC analysis.

**Fermentation (carrot roots, phenyl label):** An aliquot of the methanol extract obtained from carrot roots of the phenyl label was concentrated, loaded on a preconditioned SPE column and eluted twice with water and twice with methanol. An aliquot of the water eluate was transferred into a flask and concentrated using a rotary evaporator. The resulting residue was dissolved in water, and yeast (Wieninger Hefe) was added. The fermentation mixture was incubated in a water bath at 37°C upon constant aeration, using a vacuum pump for 24 h. The resulting carbon dioxide was trapped using a sodium hydroxide solution (traps 1–3: sodium hydroxide, 0.5 M, and two drops of Mordano Orange). The water bath was swapped for an ultrasonication bath and the remaining carbon dioxide was outgassed by constant aeration for 30 min. The sodium hydroxide traps 1–3 were made up to volume and analysed by LSC. The ethanol was separated from the fermentation mixture by distillation at 78–79°C. The fermentation distillate and the remaining fermentation mixture (fermentation sludge) were made up to volume and analysed by LSC. An aliquot of the fermentation distillate was taken for co-chromatography analyses with the reference item  $^{14}\text{C}$ -ethanol. HPLC method LC08 was used to identify ethanol by co-chromatography (details below).

HPLC method LC08: A Phenomenex Rezex ROA Organic Acid column (300 x 7.8 mm, 8  $\mu\text{m}$ ) was used with a Carbo-H 4 x 3 mm (AJO-4490) pre-column. An isocratic elution was used (mobile phase: water).

The technique used to identify ethanol is considered sufficient.

**Isolation of radioactive residues:** An aliquot of the carrot leaves methanol extract (phenyl label) was partitioned three times against dichloromethane. The dichloromethane phase was evaporated to dryness, dissolved in methanol and analysed by HPLC. The water phase was loaded onto a preconditioned SPE column and eluted twice with water / acetonitrile (65 / 35, V / V) and twice with acetonitrile. Aliquots of the eluates were analysed by HPLC. The water / acetonitrile eluate was further fractionated by HPLC and seven fractions denoted as fraction 1-7 were separately collected. Aliquots of fractions 3-5 were subjected to HPLC and LC-MS analysis and further investigated by cleavage experiments.

Components of the residue were identified by HPLC-MS (LC02 and LC11 (details below)), NMR, as well as by co-chromatography and comparison of retention times by HPLC methods LC02 and LC11. In addition, for the metabolite M684H005, chiral HPLC analyses were performed in samples of the application solution, as well as extracts of carrot leaves and roots.

HPLC method LC02: A YMC Pro C18 RS column (250 x 4.6 mm, 5  $\mu\text{m}$ ) was used with a C18 (4 x 3 mm) pre-column. A gradient elution was used (mobile phase A: water:formic acid (1000:1); mobile phase B: acetonitrile:formic acid (1000:1)).

HPLC method LC11: A Phenomenex Kinetex biphenyl column (250 x 4.6 mm, 5.0  $\mu\text{m}$ ) was used with a Biphenyl 4 x 3 mm (AJO-9207) pre-column. A gradient elution was used (mobile phase A: water:formic acid (1000:1, v:v); mobile phase B: methanol:acetonitrile:formic acid (600:400:1; v:v)).

Table 7-45 How identification of metabolites was achieved

| Metabolite             | Identification  |
|------------------------|---|
| BAS 684 H              | Co-chromatography of methanol extract of carrot leaves and roots with reference standard using two sufficiently dissimilar techniques (HPLC method LC02)  |
| M684H048 (and isomers) | HPLC-MS of fractions of water/acetonitrile phase of methanol extract of carrot leaves   |
| M684H050 (and isomers) | HPLC-MS of fractions of water/acetonitrile phase of methanol extract of carrot leaves<br>Co-chromatography of methanol extract of carrot leaves with reference standard or isolated fractions of identified compounds using two sufficiently dissimilar techniques (HPLC methods LC02 and LC11) |
| M684H005               | Co-chromatography of methanol extract of carrot leaves with reference standard  |

|          |  |
|----------|--|
|          | or isolated fractions of identified compounds using two sufficiently dissimilar techniques (HPLC methods LC02 and LC11)  |
| M684H006 | Co-chromatography of methanol extract of carrot leaves with reference standard or isolated fractions of identified compounds using two sufficiently dissimilar techniques (HPLC methods LC02 and LC11) |
| M684H047 | Co-chromatography of methanol extract of carrot leaves with reference standard or isolated fractions of identified compounds using two sufficiently dissimilar techniques (HPLC methods LC02 and LC11) |
| M684H051 | Co-chromatography of methanol extract of carrot leaves with reference standard or isolated fractions of identified compounds using two sufficiently dissimilar techniques (HPLC methods LC02 and LC11) |

HPLC-MS, a technique capable of positive structural identification, and/or HPLC co-chromatography using two sufficiently dissimilar techniques has been used to identify metabolites. Therefore the techniques used to identify the metabolites are considered sufficient.

An aliquot of fraction 1 was analysed using sugar-specific HPLC method LC03 (details below) to identify D-fructose and D-glucose by co-chromatography.

HPLC method LC03: A Phenomenex Rezex RCM-Monosaccharide Ca+2 (8%) column (300 x 7.8 mm) was used with a RCM-Monosaccharide Ca+2 (8%) (50 x 7.8 mm) pre-column. An isocratic elution was used (mobile phase: water).

The technique used to identify D-fructose and D-glucose, and therefore the fraction designated as “carbohydrates”, is considered sufficient.

Cleavage experiments: Fractions 3-5 isolated from the water / acetonitrile eluate of the methanol extract obtained from carrot leaves (phenyl label) were evaporated to dryness and subjected to sequential solubilisation with ammonia and  $\beta$ -glycosidase. The purpose of these experiments was to investigate the stability of conjugated metabolites to support the development of the residue analytical method. After each incubation step, the solubilise was separated from the residue by centrifugation and filtration. The dried residues were taken up in acetonitrile and 25% ammonia solution and shaken at room temperature for 2 h. The residue after ammonia treatment was dissolved in sodium acetate buffer and incubated with  $\beta$ -glycosidase gently shaken at 37°C for 48 h. After the treatments, the samples were partitioned three times against ethyl acetate and the obtained ethyl acetate phases were analysed by HPLC by the methods above for M684H048 and M684H050.

The conditions of the applied solubilisation procedures are summarised in Table 7-46.

Table 7-46 Cleavage experiments with isolated fractions

| Method  | Conditions  |
|---|---|
| <b>Hydrolysis of ester bonds</b><br>Ammonia treatment       | Suspension in 1 mL acetonitrile and 2 mL 25% ammonia solution; incubation at RT for 2 hours on a shaker (100 rpm)   |
| <b>Hydrolysis of ester bonds</b><br>B-glycosidase treatment | Suspension in 5 mL sodium acetate buffer (70% solution A: 0.82g sodium acetate in 100 mL water and 30% solution B: 0.3g acetic acid in 50 mL water; pH 4.95), incubation with $\beta$ -glucosidase (70 U/g residue) for approx. 24 h at 37°C and 100 rpm. |

## Results and discussion

### *Total radioactive residue (TRR)*

In the present study, the TRR was calculated by summing the extractable radioactive residue (ERR) and the residual radioactive residue (RRR) after solvent extraction. There are no significant differences between the TRR measured and TRR calculated for each label and matrix.

The calculated TRR of carrot roots was 0.093 mg eq/kg and 0.152 mg eq/kg for the phenyl and cyclohexane label, respectively. The TRR of carrot leaves was 0.442 mg eq/kg and 0.571 mg eq/kg for phenyl and cyclohexane label, respectively. Within the same matrix, the amount of radioactive residues were comparable for both labels. A summary of the TRRs are presented in Table 7-47.

Table 7-47 Total radioactive residue after foliar spray application of BAS 684 H

| Matrix [BBCH]            | DALA <sup>1)</sup> | TRR measured (LSC) <sup>2)</sup> [mg eq/kg] | TRR calculated <sup>3)</sup> [mg eq/kg] |
|--------------------------|--------------------|---|---|
| <b>Phenyl label</b>      |                    |   |   |
| <b>Roots [49]</b>        | 67                 | 0.093                                       | 0.093                                   |
| <b>Leaves [49]</b>       | 67                 | 0.459                                       | 0.442                                   |
| <b>Cyclohexane label</b> |                    |   |   |
| <b>Roots [49]</b>        | 67                 | 0.155                                       | 0.152                                   |
| <b>Leaves [49]</b>       | 67                 | 0.572                                       | 0.571                                   |

1) days after last application, 2) TRR measured directly via combustion LSC, 3) TRR calculated as the sum of ERR (extractable radioactive residue) and RRR (residual radioactive residue) after extraction of the residues  
Extractability

#### Extractability of radioactive residues

The extractabilities of <sup>14</sup>C residues from carrot roots and carrot leaves are summarised in Table 7-48.

The extractability ranged from 3.1% TRR to 77.5% TRR. High extractability of <sup>14</sup>C residue was seen in leaves >76% TRR for total extract for both labels. The majority of the radioactivity was extracted with methanol (62.6-73.0 % TRR) while with subsequent water extraction resulted in additional extraction of 3.1-5.0% TRR. Solvent extraction left an RRR (residual radioactive residue) in roots of 34.3% TRR (phenyl-label 0.032 mg eq/kg) and 24.0% TRR (cyclohexane-label 0.052mg eq/kg), while the RRR in leaves amounted to 23.5% TRR (0.104 mg eq/kg, phenyl-label) and 22.5% TRR (0.128 mg eq/kg, cyclohexane-label). The RRR was therefore further investigated by enzyme treatment as discussed below.

Table 7-48 Extractability of radioactive residue from carrot roots and leaves

| Matrix            | DALA <sup>1)</sup> | TRR <sup>2)</sup> | Distribution of radioactive residues |          |                              |          |           |          |       |          |
|-------------------|--------------------|-------------------|--------------------------------------|----------|------------------------------|----------|-----------|----------|-------|----------|
|                   |                    |                   | Methanol extracts <sup>3)</sup>      |          | Water extracts <sup>3)</sup> |          | Total ERR |          | RRR   |          |
|                   |                    | mg eq/kg          | % TRR                                | mg eq/kg | % TRR                        | mg eq/kg | % TRR     | mg eq/kg | % TRR | mg eq/kg |
| Phenyl-label      |                    |                   |                                      |          |                              |          |           |          |       |          |
| Roots             | 67                 | 0.093             | 62.6                                 | 0.058    | 3.1                          | 0.003    | 65.7      | 0.061    | 34.3  | 0.032    |
| Leaves            | 67                 | 0.442             | 71.5                                 | 0.316    | 5.0                          | 0.022    | 76.5      | 0.338    | 23.5  | 0.104    |
| Cyclohexane-label |                    |                   |                                      |          |                              |          |           |          |       |          |
| Roots             | 67                 | 0.152             | 62.9                                 | 0.096    | 3.1                          | 0.005    | 66.0      | 0.100    | 34.0  | 0.052    |
| Leaves            | 67                 | 0.571             | 73.0                                 | 0.417    | 4.5                          | 0.026    | 77.5      | 0.442    | 22.5  | 0.128    |

1) days after last application, 2) TRR measured directly via combustion LSC, 3) pool of combined repetitive extracts

#### Solubilisation of radioactive residues

The residues after solvent extraction were further solubilised with ammonia and enzymes (macerozyme and glycosidase). For carrot leaves, the residues after macerozyme solubilisation were further solubilised with artificial gastric juice/ pepsin and artificial intestinal fluid/ pancreatin to assess the bioavailability of the residues. The residues are summarised in Table 7-49.

For carrot roots (both labels), the highest portions of radioactive residues were solubilised by macerozyme/cellulase treatment amounting to 19.4 % TRR for the phenyl label and 19.5% TRR for the cyclohexane label. For carrot leaves (both labels), ammonia treatment released main portions of radioactive accounting to 11.4% TRR and 9.4% TRR for phenyl- label and cyclohexane-label, respectively. As still considerable levels of radioactive residues remained in the residues of carrot leaves (both labels), pepsin and pancreatin solubilisation was applied to investigate the bioavailability of bound residues. In sum, additional 1.2% TRR (phenyl-label) and 0.8% TRR (cyclohexane-label) were released. The final residues were each below or equal to 7.0% TRR and not considered bioavailable.

Table 7-49 Characterisation of the radioactive residues after solvent extraction in carrot samples

| Designation   | Matrix                         |                                 |                                |                                 |
|---|--------------------------------|---------------------------------|--------------------------------|---------------------------------|
|   | Phenyl label                   |                                 | Cyclohexane label              |                                 |
|   | Roots<br>[mg eq/kg]<br>[% TRR] | Leaves<br>[mg eq/kg]<br>[% TRR] | Roots<br>[mg eq/kg]<br>[% TRR] | Leaves<br>[mg eq/kg]<br>[% TRR] |
| <i>Residue after solvent extraction</i>   | <i>0.032</i><br><i>34.3</i>    | <i>0.104</i><br><i>23.5</i>     | <i>0.052</i><br><i>34.0</i>    | <i>0.128</i><br><i>22.5</i>     |
| Ammonia solubilisate  | 0.003<br>3.7                   | 0.051<br>11.4                   | 0.005<br>3.4                   | 0.054<br>9.4                    |
| Macerozyme solubilisate   | 0.018<br>19.4                  | 0.007<br>1.7                    | 0.030<br>19.5                  | 0.007<br>1.3                    |
| Glycosidase solubilisate  | 0.003<br>3.3                   | 0.002<br>0.5                    | 0.005<br>3.3                   | 0.002<br>0.4                    |
| <i>Sum of solubilised residue</i>   | <i>0.025</i><br><i>26.4</i>    | <i>0.060</i><br><i>13.6</i>     | <i>0.040</i><br><i>26.1</i>    | <i>0.063</i><br><i>11.1</i>     |
| Pepsin solubilisate   | not applied                    | 0.002<br>0.4                    | not applied                    | 0.002<br>0.4                    |
| Pancreatin solubilisate   | not applied                    | 0.003<br>0.8                    | not applied                    | 0.002<br>0.4                    |
| <i>Sum of solubilised residue by<br/>simulated gastric and intestinal fluid</i> | not applied                    | <i>0.005</i><br><i>1.2</i>      | not applied                    | <i>0.005</i><br><i>0.8</i>      |
| <b>Sum of released residue</b>  | <b>0.025</b><br><b>26.4</b>    | <b>0.066</b><br><b>14.9</b>     | <b>0.040</b><br><b>26.1</b>    | <b>0.068</b><br><b>11.9</b>     |
| Final residue   | 0.004<br>4.7                   | 0.029<br>6.6                    | 0.007<br>4.5                   | 0.040<br>7.0                    |
| <b>Sum of solubilised radioactive<br/>residues + final residue</b>              | <b>0.029</b><br><b>31.1</b>    | <b>0.095</b><br><b>21.4</b>     | <b>0.047</b><br><b>30.6</b>    | <b>0.108</b><br><b>18.9</b>     |

Values in italics were not considered for calculation of the “sum solubilised radioactive residues and the “sum solubilised radioactive residues + final residue”

#### Characterisation, Identification and Quantification of Radioactive Residues in Carrot Matrices

The parent compound BAS 684 H and the metabolites in carrot leaves and roots identified by HPLC-MS (HPLC method LC07 is a quantitative method and LC02 as a confirmatory method. An overview of the components of the extractable residue is given below in Table 7-50 to Table 7-58. Structures of the metabolites are outlined in Table 7-1.0

##### Roots

##### Phenyl-label

The chromatogram of methanol extract of carrot roots (LC02) shows two peaks. The polar peak was designated as carbohydrates, classified as identified and accounted for 0.055 mg eq/kg or 59.0% TRR. The parent compound BAS 684 H was also identified and accounted for 0.003 mg eq/kg or 3.5% TRR. The total radioactivity present in the concentrated methanol extract of carrot roots (phenyl label) was 0.058 mg eq/kg (62.6% TRR) and 100% of that has been conclusively identified.

BAS 684 H and carbohydrates were confirmed used HPLC-MS method LC11. The parent compound was assigned by comparison of the retention times with those of the HPLC-MS analyses of the root and leaf methanol extracts of the cyclohexane label. The major polar peaks ( $t_r \leq 6$  min) were detected in the chromatograms of the methanol extracts by HPLC-MS method LC02 and LC11. Thus, the polar fraction of the methanol extracts was separated by SPE fractionation. For carrot roots of the phenyl label, the polar fraction was used for fermentation and distillation processes. Ethanol, which derives from the fermentation of carbohydrates, was identified by co-chromatography analysis with a reference item thereof in the fermentation distillate using HPLC-MS method LC08. Additionally, the isolated polar fractions were taken for co-chromatography analyses with the reference items D-fructose and D-glucose using the sugar-specific HPLC-MS method LC03. Both sugars were identified in the isolated polar fractions. Overall, the polar peaks in the chromatograms of methanol extracts of carrot roots (phenyl label) were designated as carbohydrates and classified as identified.

In the chromatogram of ammonia solubilisate of carrot roots (LC02), the polar peak was classified as polar components and accounted for 0.003 mg eq/kg or 3.7% TRR. The total radioactivity present in the concentrated ammonia solubilisate of carrot roots (phenyl label) was 0.003 mg eq/kg (3.7% TRR) and 100% of that has been conclusively characterised. Polar components were confirmed using HPLC-MS method LC11. Analysis of the ammonia solubilisate with HPLC-MS methods LC02 and LC11 resulted in up to two polar peaks. BASF concluded that ammonia solubilisation does not specifically release sugars but rather unspecific biomolecules, the polar peaks were designated as polar components and classified as characterised.

In the chromatogram of the macerozyme solubilisate of carrot roots (LC02), the early-eluting peak was designated as a carbohydrate, classified as identified and accounted for 0.018 mg eq/kg or 19.4% TRR. The total radioactivity present in the concentrated macerozyme solubilisate of carrots roots (phenyl label) was 0.018 mg eq/kg (19.4% TRR) and 100% of that has been conclusively identified. Carbohydrates were confirmed using HPLC-MS method LC11.

For the carrot roots (phenyl label), one polar peak was detected in the chromatogram of the macerozyme solubilisate with HPLC-MS methods LC02 and LC11. Since the macerozyme treatment specifically releases sugars from cellulose of cells walls, the macerozyme solubilisate was taken for sugar-specific analysis using HPLC-MS method LC03. In the chromatograms from sugar-specific HPLC method LC03 all radioactivity was on retention and the major amount eluted in the same range as the sugars. D-fructose and D-glucose were assigned to the corresponding peaks by comparison with the co-chromatography analyses of the SPE water eluates. Thus, BASF concluded the polar peaks in the chromatograms of the macerozyme solubilisate was designated as carbohydrates and classified as identified.

In sum, 0.086 mg eq/kg or 92.1% TRR were identified and characterised in the ERR and RRR of carrot roots for the phenyl label. The water extract of carrot roots (phenyl label) was not analysed by HPLC-MS due to the low total amount of radioactive present (0.003 mg eq/kg or 3.1% TRR).

**Table 7-50 Summary of identified and characterised residues in the ERR of carrot roots (phenyl label)**

| Designation                                 | Extracts<br>Methanol extracts |       | Sum of methanol and water extracts |             |
|---|-------------------------------|-------|------------------------------------|-------------|
|   | mg eq/kg                      | % TRR | mg eq/kg                           | % TRR       |
| Total radioactive residues                  |                               |       | 0.093                              | 100         |
| Identified                                  |                               |       |                                    |             |
| BAS 684 H                                   | 0.003                         | 3.5   | 0.003                              | 3.5         |
| Carbohydrates                               | 0.055                         | 59.0  | 0.055                              | 59.0        |
| <b>Total identified</b>                     |                               |       | <b>0.058</b>                       | <b>62.6</b> |
| Characterised                               |                               |       |                                    |             |
| Number of additionally characterised peaks  | 0                             |       | -                                  | -           |
| Maximum of additionally characterised peaks | N.D.                          | N.D.  | -                                  | -           |
| Sum of additionally characterised peaks     | N.D.                          | N.D.  | N.D.                               | N.D.        |



|  |              |              |
|--|--------------|--------------|
| Total characterised by HPLC                  | N.D.         | N.D.         |
| Water extract                                | 0.003        | 3.1          |
| <b>Total characterised</b>                   | <b>0.061</b> | <b>65.7</b>  |
| Residual radioactive residue (RRR)           | 0.032        | 34.3         |
| <b>Total identified+ characterised + RRR</b> | <b>0.093</b> | <b>100.0</b> |

N.D = Not detected

Table 7-51 Summary of identified and characterised residues in the RRR of carrot roots (phenyl Label)

| Designation   | Solubilisates      |       |                       |       | Sum of solubilisates |             |
|---|--------------------|-------|-----------------------|-------|----------------------|-------------|
|   | Ammonia solubilise |       | Macerozyme solubilise |       | mg eq/kg             | % TRR       |
|   | mg eq/kg           | % TRR | mg eq/kg              | % TRR |                      |             |
| Residual radioactive residue                              |                    |       |                       |       | 0.032                | 34.3        |
| Identified  |                    |       |                       |       |                      |             |
| Carbohydrates   | N.D.               | N.D.  | 0.018                 | 19.4  | 0.018                | 19.4        |
| Total identified by HPLC in ERR                           |                    |       |                       |       | <b>0.018</b>         | <b>19.4</b> |
| Characterised   |                    |       |                       |       |                      |             |
| Polar components  | 0.003              | 3.7   | N.D.                  | N.D.  | 0.003                | 3.7         |
| Number of additionally characterised peaks                | 0                  |       | 0                     |       | -                    | -           |
| Maximum of additionally characterised peaks               | N.D.               | N.D.  | N.D.                  | N.D.  | -                    | -           |
| Sum of additionally characterised peaks                   | N.D.               | N.D.  | N.D.                  | N.D.  | -                    | -           |
| Total characterised by HPLC                               |                    |       |                       |       | 0.003                | 3.5         |
| Glucosidase solubilise                                    |                    |       |                       |       | 0.003                | 3.5         |
| <b>Total characterised</b>                                |                    |       |                       |       | <b>0.007</b>         | <b>7.0</b>  |
| <b>Total identified and characterised</b>                 |                    |       |                       |       | <b>0.025</b>         | <b>26.4</b> |
| Final residue   |                    |       |                       |       | 0.004                | 4.7         |
| <b>Total identified and characterised + final residue</b> |                    |       |                       |       | <b>0.029</b>         | <b>31.1</b> |

N.D = Not detected

#### Cyclohexane-label

The chromatogram of the methanol extracts of carrot roots (LC02) shows two peaks. The polar peak was designated as carbohydrates, classified as identified and accounted for 0.084 mg eq/kg or 55.1% TRR. The parent compound BAS 684 H was also identified and accounted for 0.012 mg eq/kg or 7.9% TRR. The total radioactivity present in the concentrated methanol extract of carrot roots (cyclohexane label) was 0.096 mg eq/kg (62.9% TRR) and 100% of that has been conclusively identified.

Major polar peaks ( $t_r \leq 6$  min) were detected in the chromatograms of the methanol extracts by analysis with HPLC-MS methods LC02 and LC11. Thus, the polar fractions of the methanol extracts were separated by SPE fractionation. The isolated polar fractions were taken for co-chromatography analysis with the reference items D-fructose and D-glucose using sugar-specific HPLC method LC03. Both sugars were identified in the isolated polar fractions. Therefore, the polar peaks in the chromatograms of methanol extracts of carrot roots (cyclohexane label) were designated as carbohydrates and classified as identified. The peak assignment of the parent compound BAS 684 H was based on the co-chromatography analysis of the methanol extracts of carrot roots with a reference item thereof using HPLC method LC02. Furthermore, method LC11 confirmed the presence of carbohydrates and the parent compound BAS 684 H in methanol extracts of carrot roots (cyclohexane label).

In the chromatogram of ammonia solubilise of carrot roots (LC02), the polar peak was designated as a polar component, classified as characterised and accounted for 0.005 mg eq/kg or 3.4% TRR. The total radioactivity

present in the concentrated ammonia solubilisate of carrot roots (cyclohexane label) was 0.005 mg eq/kg (3.4% TRR) and 100% of that has been conclusively characterised. Polar components were confirmed using HPLC-MS method LC11. Analysis of the ammonia solubilisate with HPLC-MS methods LC02 and LC11 resulted in up to two polar peaks. BASF concluded that ammonia solubilisation does not specifically release sugars but rather unspecific biomolecules, the polar peaks were designated as polar components and classified as characterised.

In the chromatogram of macerozyme solubilisate (LC02), the polar peak was identified as a carbohydrate and accounted for 0.029 mg eq/kg or 19.0% TRR. The total radioactivity present in the concentrated macerozyme solubilisate of carrots roots (cyclohexane label) was 0.030 mg eq/kg (19.5% TRR) and 100% of that has been conclusively characterised. Carbohydrates were confirmed using HPLC-MS methods LC11.

For the carrot roots (cyclohexane label), one polar peak was detected in the chromatogram of the macerozyme solubilisate with HPLC-MS methods LC02 and LC11. Since the macerozyme treatment specifically releases sugars from cellulose of cells walls, the macerozyme solubilisate was taken for sugar-specific analysis using HPLC-MS method LC03. In the chromatograms from sugar-specific HPLC method LC03 all radioactivity was on retention and the major amount eluted in the same range as the sugars. D-fructose and D-glucose were assigned to the corresponding peaks by comparison with the co-chromatography analyses of the SPE water eluates. Thus, BASF concluded the polar peaks in the chromatograms of the macerozyme solubilisate was designated as carbohydrates and classified as identified.

In sum, 0.140 mg eq/kg or 92.1% TRR were identified and characterised in the ERR and RRR of carrot roots for the cyclohexane label. The water extract of carrots roots (cyclohexane label) was not analysed by HPLC-MS due to low total amount of radioactivity present (0.005 mg eq/kg or 3.1% TRR).

Table 7-52 Summary of identified and characterised residues in the ERR of carrot roots (cyclohexane label)

| Designation                                 | Extracts          |       | Sum of methanol and water extracts |              |
|---|-------------------|-------|------------------------------------|--------------|
|   | Methanol extracts |       |                                    |              |
|   | mg eq/kg          | % TRR | mg eq/kg                           | % TRR        |
| Total radioactive residues                  |                   |       | 0.152                              | 100          |
| Identified                                  |                   |       |                                    |              |
| BAS 684 H                                   | 0.012             | 7.9   | 0.012                              | 7.9          |
| Carbohydrates                               | 0.084             | 55.1  | 0.084                              | 55.1         |
| <b>Total identified</b>                     |                   |       | <b>0.096</b>                       | <b>62.9</b>  |
| Characterised                               |                   |       |                                    |              |
| Number of additionally characterised peaks  | 0                 |       | -                                  | -            |
| Maximum of additionally characterised peaks | N.D.              | N.D.  | -                                  | -            |
| Sum of additionally characterised peaks     | N.D.              | N.D.  | N.D.                               | N.D.         |
| Total characterised by HPLC                 |                   |       | N.D.                               | N.D.         |
| Water extract                               |                   |       | 0.005                              | 3.1          |
| <b>Total characterised</b>                  |                   |       | <b>0.005</b>                       | <b>3.1</b>   |
| <b>Total identified and characterised</b>   |                   |       | <b>0.100</b>                       | <b>66.0</b>  |
| Residual radioactive residue (RRR)          |                   |       | 0.052                              | 34.0         |
| <b>Total identified+ characterised+ RRR</b> |                   |       | <b>0.152</b>                       | <b>100.0</b> |

N.D = Not detected

Table 7-53 Summary of identified and characterised residues in the RRR of carrot Roots (cyclohexane label)

| Designation                  | Solubilisates        |       |                         |       | Sum of solubilisates |       |
|------------------------------|----------------------|-------|-------------------------|-------|----------------------|-------|
|                              | Ammonia solubilisate |       | Macerozyme solubilisate |       | mg eq/kg             | % TRR |
|                              | mg eq/kg             | % TRR | mg eq/kg                | % TRR |                      |       |
| Residual radioactive residue |                      |       |                         |       | 0.052                | 34.0  |
| Identified                   |                      |       |                         |       |                      |       |



|   |       |      |       |      |              |             |
|---|-------|------|-------|------|--------------|-------------|
| Carbohydrates   | N.D.  | N.D. | 0.029 | 19.0 | 0.029        | 19.0        |
| Total identified by HPLC in ERR                           |       |      |       |      | <b>0.029</b> | <b>19.0</b> |
| Characterised   |       |      |       |      |              |             |
| Polar components  | 0.005 | 3.4  | N.D.  | N.D. | 0.005        | 3.4         |
| Number of additionally characterised peaks                | 0     |      | 1     |      | -            | -           |
| Maximum of additionally characterised peaks               | N.D.  | N.D. | 0.001 | 0.4  | -            | -           |
| -Sum of additionally characterised peaks                  | N.D.  | N.D. | 0.001 | 0.4  | 0.001        | 0.4         |
| Total characterised by HPLP                               |       |      |       |      | 0.006        | 3.8         |
| Glucosidase solubilise                                    |       |      |       |      | 0.005        | 3.3         |
| <b>Total characterised</b>                                |       |      |       |      | <b>0.011</b> | <b>7.1</b>  |
| <b>Total identified and characterised</b>                 |       |      |       |      | <b>0.04</b>  | <b>26.1</b> |
| Final residue   |       |      |       |      | 0.007        | 4.5         |
| <b>Total identified and characterised + final residue</b> |       |      |       |      | <b>0.047</b> | <b>30.6</b> |

N.D = Not detected

### Leaves

#### Phenyl-label

Analysis of the methanol extract of carrot leaves with quantitative HPLC-MS method LC02 resulted in a pattern of 31 peaks, of which nine were identified. The parent compound BAS 684 H was identified as the most abundant compound and accounted for 0.107 mg eq/kg (24.1% TRR). The second most abundant compound identified was one isomer of M684H050 and accounted for 0.032 mg eq/kg or 7.3% TRR. The polar peak was designated as a polar component, classified as characterised and accounted for 0.012 mg eq/kg or 2.6% TRR. Additionally, the metabolites M684H005, M684H006, M684H047, M684H050 and an area containing two isomers of M684H048 and two isomers of M684H050, were identified in the methanol extract of carrot leaves. The total radioactivity present in the concentrated methanol extract of carrot leaves (phenyl label) was 0.316 mg eq/kg (or 71.5% TRR) and 44.5% of that has been conclusively identified. BAS 684 H, metabolites: M684H051, M684H050, M684H047, M684H005, M684H006, the area containing two isomers of M684H048 and two isomers of M684H050 and polar components were confirmed using HPLC-MS method LC11. The remaining components were detected up to 0.018 mg eq/kg (4.1% TRR) and summed up to 0.108 mg eq/kg (24.5% TRR) and were classified as characterised. 21 additionally characterised peaks were detected, 3 of which were  $\geq 0.01$  mg eq/kg. The nineteen unidentified radioactive metabolites within the range of 0.001 – 0.018 mg eq/kg (0.2 – 4.1% TRR) are classified as characterised and identification is not required.

The peak assignment for the methanol extract of carrot leaves (phenyl-label) was mainly based on co-chromatography experiments using HPLC-MS methods LC02 and LC11. The reference items for the co-chromatography experiments were either MS samples generated from the methanol extract of carrot leaves of the phenyl label via fractionation or an external reference standard. Co-chromatography of the methanol extract of carrot leaves (phenyl label) with the reference standard M684H050 was positive using HPLC-MS method LC02 and LC11. HPLC-MS analysis of isolated fractions of the methanol extract of carrot leaves (phenyl label) resulted in the identification of the two additional isomers of metabolite M684H050 and two isomers of metabolite M684H048. Although it was not feasible to assign each of the M684H050 and M684H048 isomers to a discrete peak, the isomers could be assigned to an area consisting of three peaks using HPLC-MS method LC02. Minor polar peaks ( $t_r \leq 6$  min) were detected in the chromatograms of the methanol extracts by analysis with HPLC-MS methods LC02 and LC11. The polar fractions of the methanol extracts were fractionated via HPLC-MS method LC11 and the radioactivity found in these polar peaks likely consists of carbohydrates and it was classified as characterised.

In the chromatogram of the water extract of carrot leaves (LC02), the polar peak group was designated as polar components, classified as characterised and accounted in total for 0.015 mg eq/kg or 3.4% TRR. The total radioactivity present in the concentrated water extract of carrot leaves (phenyl label) was 0.022 mg eq/kg (5.0% TRR) and 7.7% of that has been conclusively characterised as polar components. The polar components were confirmed using HPLC-MS method LC11. The remaining components were detected up to 0.002 mg eq/kg (0.4% TRR) and summed up to 0.007 mg eq/kg (1.5% TRR) and were classified as characterised. The seven

unidentified radioactive metabolites within the range of <0.001 – 0.002 mg eq/kg (0.1 – 0.4% TRR) are classified as characterised and identification is not required. Minor polar peaks ( $t_r \leq 6$  min) were detected in the chromatograms of the water extracts by analysis with HPLC-MS methods LC02 and LC11. Thus, the polar fractions of the methanol extracts were separated fraction.

In the chromatogram of ammonia solubilisate of carrot leaves (LC02), the polar peak group was designated as polar components, classified as characterised and accounted for 0.042 mg eq/kg or 9.6% TRR. The total radioactivity present in ammonia solubilisate of carrot leaves (phenyl label) was 0.051 mg eq/kg (11.4% TRR) and 84.3% of that has been conclusively characterised as polar components. The polar components were confirmed using HPLC-MS method LC11. The remaining components were detected up to 0.006 mg eq/kg (1.4% TRR) and summed up to 0.008 mg eq/kg (1.9% TRR) and were classified as characterised. The two unidentified radioactive metabolites within the range of 0.002 – 0.006 mg eq/kg (0.4 – 1.4% TRR) are classified as characterised and identification is not required.

In sum, 0.404 mg eq/kg or 91.4% TRR were identified and characterised in the ERR and RRR of carrot leaves for the phenyl label.

Table 7-54 Summary of identified and characterised residues in the ERR of carrot leaves (phenyl Label)

| Designation   | Extracts                     |       |               |       | Sum of methanol and water extracts |       |
|---|------------------------------|-------|---------------|-------|------------------------------------|-------|
|   | Methanol extract             |       | Water extract |       |                                    |       |
|   | mg eq/kg                     | % TRR | mg eq/kg      | % TRR | mg eq/kg                           | % TRR |
| Total radioactive residues                              |                              |       |               |       | 0.459                              | 100   |
| Identified  |                              |       |               |       |                                    |       |
| M684H005  | 0.008                        | 1.8   | N.D.          | N.D.  | 0.008                              | 1.8   |
| M684H006  | 0.011                        | 2.6   | N.D.          | N.D.  | 0.011                              | 2.6   |
| M684H047  | 0.005                        | 1.2   | N.D.          | N.D.  | 0.005                              | 1.2   |
| M684H050 (one isomer)                                   | 0.032                        | 7.3   | N.D.          | N.D.  | 0.032                              | 7.3   |
| Sum of M684H048 (two isomers) and M684050 (two isomers) | 0.031                        | 7.1   | N.D.          | N.D.  | 0.031                              | 7.1   |
| M684H051  | 0.002                        | 0.4   | N.D.          | N.D.  | 0.002                              | 0.4   |
| BAS 684 H   | 0.107                        | 24.1  | N.D.          | N.D.  | 0.107                              | 24.1  |
| Total identified  |                              |       |               |       | 0.196                              | 44.4  |
| Characterised   |                              |       |               |       |                                    |       |
| Polar components  | 0.012                        | 2.6   | 0.015         | 3.4   | 0.027                              | 6.1   |
| Number of additionally characterised peaks              | 21 (3 peaks ≥ 0.01 mg eq/kg) |       | 7             |       | -                                  | -     |
| Maximum of additionally characterised peaks             | 0.018                        | 4.1   | 0.002         | 0.4   | -                                  | -     |
| Sum of additionally characterised peaks                 | 0.108                        | 24.5  | 0.007         | 1.5   | 0.115                              | 26.1  |
| Total characterised by HPLC                             |                              |       |               |       | 0.142                              | 32.1  |
| Total identified and characterised                      |                              |       |               |       | 0.338                              | 76.5  |
| Residual radioactive residue (RRR)                      |                              |       |               |       | 0.104                              | 23.5  |
| Total identified and characterised + RRR                |                              |       |               |       | 0.442                              | 100.0 |

N.D = Not detected

Table 7-55 Summary of identified and characterised residues in the RRR of carrot leaves (phenyl label)

| Designation  | Ammonia solubilisate |       | Sum of solubilisates |       |
|--|----------------------|-------|----------------------|-------|
|  | mg eq/kg             | % TRR | mg eq/kg             | % TRR |
| Residual radioactive residue                       |                      |       | 0.104                | 23.5  |
| Identified   |                      |       |                      |       |
| Total identified                                   |                      |       | N.D.                 | N.D.  |
| Characterised                                      |                      |       |                      |       |
| Polar compounds                                    | 0.042                | 9.6   | 0.042                | 9.6   |
| Number of additionally characterised peaks         | 2                    |       | -                    | -     |
| Maximum of additionally characterised peaks        | 0.006                | 1.31  | -                    | -     |
| Sum of additionally characterised peaks            | 0.008                | 1.9   | 0.008                | 1.9   |
| Total characterised by HPLC                        |                      |       | 0.051                | 11.4  |
| Macerozyme solubilisate                            |                      |       | 0.007                | 1.7   |
| Glucosidase solubilisate                           |                      |       | 0.002                | 0.5   |
| Pepsin solubilisate                                |                      |       | 0.002                | 0.4   |
| Pancreatin solubilisate                            |                      |       | 0.003                | 0.8   |
| Total characterised in RRR                         |                      |       | 0.066                | 14.9  |
| Total identified and characterised                 |                      |       | 0.066                | 14.9  |
| Final residue                                      |                      |       | 0.029                | 6.6   |
| Total identified and characterised + final residue |                      |       | 0.095                | 21.4  |

N.D = Not detected

*Cyclohexane label*

Analysis of the methanol extract of carrot leaves with quantitative HPLC-MS method LC02 resulted in a pattern of 36 peaks, of which seven were identified. The parent compound BAS 684 H was identified as the most abundant compound and accounted for 0.159 mg eq/kg or 27.9% TRR. Additionally, the metabolites M684H005, M684H006, M684H047, M684H050 and an area containing two isomers of M684H048 and two isomers of M684H050 were identified in the methanol extract of carrot leaves. M684H050 accounted for 0.015 mg eq/kg or 2.7% TRR. The polar peak group was designated as polar components, classified as characterised and accounted for up to 0.018 mg eq/kg or 3.1% TRR. The peak assignment of metabolites: M684H047, M684H051, M684H050, M684H006, M684H005, and the area containing two isomers of M684H048 and two isomers of M684H050, for the methanol extract of carrot leaves (cyclohexane label), using HPLC-MS methods LC02 and LC11, was based on comparison of the retention times and metabolites patterns with those of the HPLC-MS analyses of the methanol extract of carrot leaves (phenyl label).

The total radioactivity present in concentrated methanol extract of carrot leaves (cyclohexane label) was 0.417 mg eq/kg (73.0% TRR) and 55.4% of that has been conclusively identified. The parent compound, polar components and metabolites: M684H050, M684H047, M684H005, M684H006, M684H048 isomers and M684H050 isomers were confirmed using HPLC-MS method LC11. The remaining components were detected up to 0.03 mg eq/kg (5.3% TRR) and summed up to 0.185 mg eq/kg (32.5% TRR) and were classified as characterised. The twenty-seven unidentified radioactive metabolites within the range of 0.030 – 0.001 mg eq/kg (5.3 – 0.1% TRR) (8 of which were > 0.01 mg/kg) are classified as characterised and identification is not required.

In the chromatogram of the water extract of carrot leaves (LC02), the polar peak group was designated as polar components, classified as characterised and accounted in total for 0.018 mg eq/kg or 3.1% TRR. The total radioactivity present in concentrated water extract of carrot leaves (cyclohexane label) was 0.026 mg eq/kg (4.5% TRR) and 69.2% of that has been conclusively characterised as polar components. The polar components were confirmed using HPLC-MS method LC11. The remaining components were detected up to 0.002 mg eq/kg (0.3% TRR) and summed up to 0.008 mg eq/kg (1.4% TRR) and were classified as characterised. The eight unidentified radioactive metabolites within the range of <0.001– 0.002 mg eq/kg (0.1 – 0.3% TRR) are classified as characterised and identification is not required.

In the chromatogram of the ammonia solubilisate of carrot leaves (LC02) the polar peak was designated as polar components, classified as characterised and accounted for 0.045 mg eq/kg or 7.8% TRR. The total radioactivity present in ammonia solubilisate of carrot leaves (cyclohexane label) was 0.054 mg eq/kg (9.4% TRR) and 83.3% of that has been conclusively characterised as polar components. The polar components were confirmed using HPLC-MS method LC11. The remaining components were detected up to 0.007 mg eq/kg (1.2% TRR) and summed up to 0.009 mg eq/kg (1.6% TRR) and were classified as characterised. The two unidentified radioactive metabolites within the range of 0.002 – 0.007 mg eq/kg (0.4- 1.2% TRR) are classified as characterised and identification is not required.

In sum, 0.510 mg eq/kg or 89.4% TRR was identified and characterised in the ERR and RRR of carrot leaves for cyclohexane label.

Table 7-56 Summary of identified and characterised residues in the ERR of carrot leaves (cyclohexane Label)

| Designation   | Extracts                     |       |               |       | Sum of methanol and water extracts |       |
|---|------------------------------|-------|---------------|-------|------------------------------------|-------|
|   | Methanol extract             |       | Water extract |       |                                    |       |
|   | mg eq/kg                     | % TRR | mg eq/kg      | % TRR | mg eq/kg                           | % TRR |
| Total radioactive residues                              |                              |       |               |       | 0.571                              | 100   |
| Identified  |                              |       |               |       |                                    |       |
| M684H005  | 0.002                        | 0.4   | N.D.          | N.D.  | 0.002                              | 0.4   |
| M684H006  | 0.014                        | 2.4   | N.D.          | N.D.  | 0.014                              | 2.4   |
| M684H047  | 0.011                        | 2.0   | N.D.          | N.D.  | 0.011                              | 2.0   |
| M684H050 (one isomer)                                   | 0.015                        | 2.7   | N.D.          | N.D.  | 0.015                              | 2.7   |
| Sum of M684H048 (two isomers) and M684050 (two isomers) | 0.012                        | 2.1   | N.D.          | N.D.  | 0.012                              | 2.1   |
| BAS 684 H   | 0.159                        | 27.9  | N.D.          | N.D.  | 0.159                              | 27.9  |
| Total identified  |                              |       |               |       | 0.213                              | 37.4  |
| Characterised   |                              |       |               |       |                                    |       |
| Polar components  | 0.018                        | 3.1   | 0.018         | 3.1   | 0.036                              | 6.3   |
| Number of additionally characterised peaks              | 27 (8 peaks ≥ 0.01 mg eq/kg) |       | 8             |       | -                                  | -     |
| Maximum of additionally characterised peaks             | 0.030                        | 5.3   | 0.002         | 0.3   | -                                  | -     |
| Sum of additionally characterised peaks                 | 0.185                        | 32.5  | 0.008         | 1.4   | 0.193                              | 33.9  |
| Total characterised by HPLC                             |                              |       |               |       | 0.229                              | 40.1  |
| Total identified and characterised                      |                              |       |               |       | 0.442                              | 77.5  |
| Residual radioactive residue (RRR)                      |                              |       |               |       | 0.128                              | 22.5  |
| Total identified and characterised + RRR                |                              |       |               |       | 0.571                              | 100.0 |

N.D = Not detected

Table 7-57 Summary of identified and characterised residues in the RRR of carrot leaves (cyclohexane label)

| Designation   | Ammonia solubilisate |       | Sum of solubilisates |             |
|---|----------------------|-------|----------------------|-------------|
|   | mg eq/kg             | % TRR | mg eq/kg             | % TRR       |
| Residual radioactive residue                              |                      |       | 0.128                | 22.5        |
| Identified  |                      |       |                      |             |
| <b>Total identified</b>                                   |                      |       | <b>N.D.</b>          | <b>N.D.</b> |
| Characterised   |                      |       |                      |             |
| Polar compounds   | 0.045                | 7.8   | 0.045                | 7.8         |
| Number of additionally characterised peaks                | 2                    |       | -                    | -           |
| Maximum of additionally characterised peaks               | 0.007                | 1.2   | -                    | -           |
| Sum of additionally characterised peaks                   | 0.009                | 1.6   | 0.009                | 1.6         |
| Total characterised by HPLC                               |                      |       | 0.054                | 9.4         |
| Macerozyme solubilisate                                   |                      |       | 0.007                | 1.3         |
| Glucosidase solubilisate                                  |                      |       | 0.002                | 0.4         |
| Pepsin solubilisate                                       |                      |       | 0.002                | 0.4         |
| Pancreatin solubilisate                                   |                      |       | 0.002                | 0.4         |
| Total characterised in RRR                                |                      |       | 0.068                | 11.9        |
| <b>Total identified and characterised</b>                 |                      |       | <b>0.068</b>         | <b>11.9</b> |
| Final residue   |                      |       | 0.040                | 7.0         |
| <b>Total identified and characterised + final residue</b> |                      |       | <b>0.108</b>         | <b>18.9</b> |

N.D = Not detected

The methanol and water extracts and the ammonia solubilisates obtained from carrot leaves of both labels were analysed by HPLC. The most abundant components in carrot leaves was the parent compound accounting to 0.107 mg eq/kg or 23.31% TRR for the phenyl label and 0.159 mg eq/kg or 27.80% TRR for the cyclohexane label. Metabolite M684H050 was the second most abundant component accounting for 0.032 mg eq/kg or 6.97% TRR for the phenyl-label and 0.015 mg eq/kg or 2.62% TRR for the cyclohexane label. Additionally, metabolites M684H005, M684H006, M684H047, M684H048, M684H051 (only for the phenyl label), and the area containing two isomers of M684H048 and two isomers of M684H050, were identified and accounted for up to 0.031 mg eq/kg or 6.75% TRR.

In total, 0.404 mg eq/kg or 88.02% TRR (phenyl-label) and 0.510 mg eq/kg or 86.16% TRR (cyclohexane-label) were identified and characterised, whereby each characterised peak, fraction, water extract and solubilisate was below or equal to 0.229 mg eq/kg or 40.1% TRR.

The results of the phenyl label are consistent with those of the cyclohexane label. All components, which were detected in the phenyl label, were also recovered in the cyclohexane label at similar levels.

Furthermore, cleavage experiments were conducted with three individual fractions (fraction 2-4) of the water/ acetonitrile eluate obtained from the methanol extract of carrot leaves (phenyl label). The experiments showed that metabolites conjugated with (malonyl) glycoside can be degraded to aglycones in presence of ammonia and  $\beta$ -glycosidase. The aglycones were only identified after cleavage and were not identified in the extracts and solubilisates, therefore the glycoside metabolites are stable in carrot matrices.

Table 7-58 Summary of identified/characterised components in carrots

| Designation  | Carrot roots |         | Carrot leaves |         |
|--|--------------|---------|---------------|---------|
|  | [mg eq/kg]   | [% TRR] | [mg eq/kg]    | [% TRR] |
| <b>Phenyl label</b>  |              |         |               |         |
| M684H005   | not detected |         | 0.008         | 1.8     |
| M684H006   | not detected |         | 0.011         | 2.6     |
| M684H047   | not detected |         | 0.005         | 1.2     |
| Sum of M684H048 (2 isomers) and M684H050 (2 isomers)       | not detected |         | 0.031         | 7.1     |
| M684H050   | not detected |         | 0.032         | 7.3     |
| M684H051   | not detected |         | 0.002         | 0.4     |
| BAS 684 H  | 0.003        | 3.5     | 0.107         | 24.1    |
| Carbohydrates <sup>1</sup>                                 | 0.073        | 78.4    | not detected  |         |
| Total identified   | 0.076        | 82.0    | 0.196         | 44.4    |
| Total characterised from ERR                               | 0.003        | 3.1     | 0.142         | 32.1    |
| Total characterised from RRR                               | 0.007        | 7.0     | 0.066         | 14.9    |
| Total identified and characterised                         | 0.086        | 92.1    | 0.404         | 91.4    |
| Final residue  | 0.004        | 4.7     | 0.029         | 6.6     |
| Grand total of identified, characterised and final residue | 0.090        | 96.8    | 0.433         | 98.0    |
| <b>Cyclohexane label</b>                                   |              |         |               |         |
| M684H005   | not detected |         | 0.002         | 0.4     |
| M684H006   | not detected |         | 0.014         | 2.4     |
| M684H047   | not detected |         | 0.011         | 2.0     |
| Sum of M684H048 (2 isomers) and M684H050 (2 isomers)       | not detected |         | 0.012         | 2.1     |
| M684H050   | not detected |         | 0.015         | 2.7     |
| M684H051   | not detected |         | not detected  |         |
| BAS 684 H  | 0.012        | 7.9     | 0.159         | 27.9    |
| Carbohydrates  | 0.113        | 74.1    | n.d.          | n.d.    |
| Total identified   | 0.125        | 82.0    | 0.213         | 37.4    |
| Total characterised from ERR                               | 0.005        | 3.1     | 0.229         | 40.1    |
| Total characterised from RRR                               | 0.011        | 7.1     | 0.068         | 11.9    |
| Total identified and characterised                         | 0.140        | 92.1    | 0.510         | 89.4    |
| Final residue  | 0.007        | 4.5     | 0.040         | 7.0     |
| Grand total of identified, characterised and final residue | 0.147        | 96.7    | 0.550         | 96.4    |

1 Including amounts of carbohydrates, which were detected in the macerozyme / cellulase solubilisates

### Cleavage Experiments

Cleavage experiments were conducted with three individual fractions (fraction 3-5) of the water/ acetonitrile eluate of the methanol extract of carrot leaves (phenyl label) to investigate the stability of conjugated metabolites to support the development of the residue analytical method. The metabolites observed are summarised in Table 7-59.

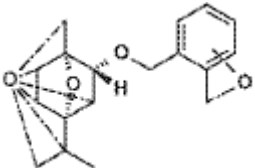
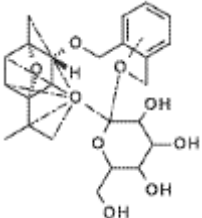
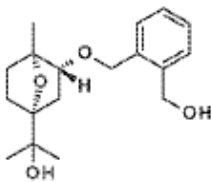
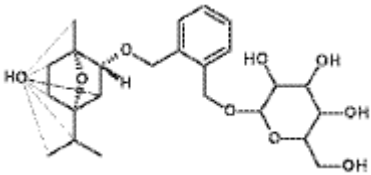
In the concentrated fraction 3 of the concentrated water and acetonitrile phase of the methanol extract of carrot leaves (phenyl label) a component was detected by HPLC-MS (method LC02) corresponding to M684H050. Incubation with ammonia and  $\beta$ -glucosidase of fraction 3 (LC02) resulted in the complete depletion of M684H050 and the formation of M684H039. The retention times and MS/MS spectra do not match to those the

reference items (for example the twofold hydroxylated compounds M684H044 and Reg. No. 6059084); however, the component was designated as M684H039 and the evidence provided is satisfactory given M684H039 is a generic structure (hence no reference item available) which is consistent with the MS fragments which give evidence for hydroxylation in both the phenyl and cyclohexane rings.

Prior to the treatment of the concentrated fraction 4 with ammonia and  $\beta$ -glucosidase the fraction was analysed by HPLC-MS/MS. The MS/MS spectrum showed two major peaks M684H048 and M684H050 and isomers thereof. Incubation with ammonia and  $\beta$ -glucosidase of fraction 4 (LC02) resulted in the complete depletion of the two peaks while three peaks emerged. The peaks are attributed to aglycone M684H039 and aglycone M684H044. For M684H039, the retention times and MS/MS spectra do not match to those the reference items (for example the twofold hydroxylated compounds M684H044 and Reg. No. 6059084); however, the component was designated as M684H039 and the evidence provided is satisfactory given M684H039 is a generic structure (hence no reference item available) which is consistent with the MS fragments which give evidence for hydroxylation in both the phenyl and cyclohexane rings.

In the concentrated fraction 5 of the concentrated water and acetonitrile phase of the methanol extract of carrot leaves (phenyl label) components were detected by HPLC-MS (LC02) corresponding to M684H048, M684H050 and isomers thereof. Treatment of fraction 5 with ammonia and  $\beta$ -glucosidase resulted in the complete depletion of the three peaks containing metabolite M684H048, M684H050 and isomers thereof, while three major peaks at emerged. The three peaks were assigned to aglycone M684H039 and M684H044. For M684H039, the retention times and MS/MS spectra do not match to those of the reference items (for example the twofold hydroxylated compounds M684H044 and Reg. No. 6059084); however, the component was designated as M684H039 and the evidence provided is satisfactory given M684H039 is a generic structure (hence no reference item available) which is consistent with the MS fragments which give evidence for hydroxylation in both the phenyl and cyclohexane rings.

Table 7-59 Summary of identified metabolites

| Designation   | Structure   | Fraction(s) the metabolite(s) are observed in |
|---|---|---|
| M684H039  |  | Fraction 3<br>Fraction 4<br>Fraction 5        |
| M684H050 (glucose conjugates of M684H039)             |  | Fraction 3<br>Fraction 4                      |
| M684H044  |  | Fraction 4<br>Fraction 5                      |
| M684H048 (glucose conjugates of M684H044 and isomers) |  | Fraction 4<br>Fraction 5                      |



*Chiral Analysis*

Enantiomer-specific analysis was performed to investigate whether one enantiomer of BAS 684 H was preferably metabolised in carrot leaves (using both  $^{14}\text{C}$ -labels). The analysis was not performed in carrot roots since BAS 684 H was only detected at sufficient levels in carrot leaves. The methanol extracts of carrot leaves were fractionated using HPLC-MS method LC09. The analysis of these fractions with enantiomer-specific HPLC-MS method LC010 resulted in a pattern of two peaks corresponding to enantiomeric ratios ((-)/ (+) enantiomer) listed below in Table 7-60. The enantiomers of BAS 684 H were identified by co-elution with reference standards from a metabolism study of BAS 684 H in rats using HPLC-MS method LC10.

Table 7-60 Determination of the enantiomer ratio of BAS 684 H

| Matrix            | (-) stereoisomer A1 [%AR] | (+) stereoisomer A2 [%AR] | SE [%] | SE Change |
|-------------------|---------------------------|---------------------------|--------|-----------|
| Test item         | 51.0                      | 49.0                      | 2      | 0         |
| Phenyl-label      |                           |                           |        |           |
| Leaves            | 40.6                      | 59.4                      | 18     | -16       |
| Cyclohexane-label |                           |                           |        |           |
| Leaves            | 43.0                      | 57.0                      | 14     | -14       |

SE = stereoisomers excess,  $SE = [(A1\% \text{ AR} - A2\% \text{ AR}) / (A1\% \text{ AR} + A2\% \text{ AR})]$

The analyses with enantiomer-specific HPLC method of the isolated fractions demonstrate that changes in the stereoisomeric excess (SE) are >10%; this represents a significant change in stereoisomeric ratio upon metabolism in carrot. The toxicological evaluation of the two enantiomers concluded they are of equivalent toxicity (Vol 1 Section 2.12.3).

*Storage stability*

Carrot roots and leaves were extracted up to 79 days after sampling and the extracts were analysed up to 63 days after extraction. The period from sampling to analysis was up to 141 days. Therefore no storage stability data are required, however experiments were still performed to determine extract and matrix stability and these are summarised below for completeness.

*Extract stability:* The methanol extracts of carrot roots leaves (cyclohexane label) were analysed before and after storage for 61-62 days and the chromatograms showed very similar metabolic patterns.

*Matrix stability:* The homogenised carrot roots and leaves (cyclohexane label) were re-extracted 163 days after sampling. The chromatograms of the methanol extracts showed very similar metabolic patterns before and after storage.

Therefore it is concluded that sample integrity was maintained for the storage intervals in the study.

*Proposed metabolic pathway*

The proposed metabolic pathway of BAS 684 H in carrot is shown in Figure 7-6 below. A summary of the detected metabolites is given in Table 7-58.

Almost all identified metabolites result from hydroxylation of the parent compound BAS 684 H and subsequent conjugation with glucoside or malonyl glucoside.

The metabolites M684H005 and M684H006 result from hydroxylation of the parent compound BAS 684 H at the methyl group of the phenyl moiety and subsequent conjugation with glucoside and malonyl glucoside, respectively.



Multiple hydroxylation at both the phenyl and cyclohexane moiety and subsequent conjugation with glucoside result in the formation of metabolites M684H048 and M684H050. Multiple hydroxylation at both the phenyl and cyclohexane moiety and subsequent conjugation with malonyl glucoside result in the formation of metabolite M684H047. Conjugation with glucoside at both the phenyl and cyclohexane moiety results in metabolite M684H051.

The aglycones identified after cleavage experiments with ammonia and  $\beta$ -glucosidase (M684H039 and M684H044) were not identified in the solvent extracts and solubilisates of carrot matrices. This indicates that the glucosidic metabolites (M684H048 and M684H050) are stable in the carrot matrices.

### *Conclusion*

The metabolism of BAS 684 H was investigated in carrot by applying a single foliar spray application of phenyl-labelled or cyclohexane-labelled BAS 684 H at a maximum rate of 500 g a.s./ha. Samples of carrot leaves and roots were collected at 67 DAT (BBCH 49).

The TRR of carrot roots was 0.093 mg eq/kg and 0.152 mg eq/kg for the phenyl and cyclohexane label, respectively. The TRR of carrot leaves 0.442 mg eq/kg and 0.571 mg eq/kg for the phenyl and cyclohexane label, respectively. Within the same matrix, the amount of radioactive residues was comparable for both labels.

Carrot roots and leaves were extracted with methanol and water, where major portions of radioactive residues were extracted with methanol (up to 73.0% TRR (0.417 mg eq/kg)). Smaller amounts were subsequently extracted with water (below or equal to 5.0% TRR (or 0.026 mg eq/kg)).

The residues after solvent extraction were further solubilised by ammonia and enzyme incubations. For carrot roots, the highest portions of radioactive residues were solubilised by macerozyme/ cellulase treatment (up to 19.5% TRR (0.030 mg eq/kg)). For carrot leaves (both labels), ammonia treatment released main portions of radioactive residues (up to 11.4% TRR or 0.054 mg eq/kg). The residues of carrot leaves (both labels) were further sequentially incubated with pepsin and pancreatin to investigate the bioavailability of bound residues, which released additional 1.2% TRR (0.005 mg eq/kg) (phenyl-label) and 0.8% TRR (0.005 mg eq/kg) (cyclohexane label). The final residues were each below or equal to 7.0% TRR (0.040 mg eq/kg) and are not considered to be bioavailable.

Structure elucidation was mainly based on HPLC-MS and MS/MS analysis of fractions isolated from water/ acetonitrile eluate of the methanol extract obtained from carrot leaves (phenyl-label), which resulted in the identification of the metabolites M684H048, M684H050 (and isomers thereof). Metabolites M684H005, M684H006, M68H047 and M68H051 were assigned by co-chromatography with external samples from related metabolism studies of BAS 684 H. The parent compound was assigned by co-chromatography with a reference item thereof. The peak assignment for polar fractions of the methanol extract of carrot roots was based on fermentation and distillation experiments and on co-chromatography analyses with sugar reference items.

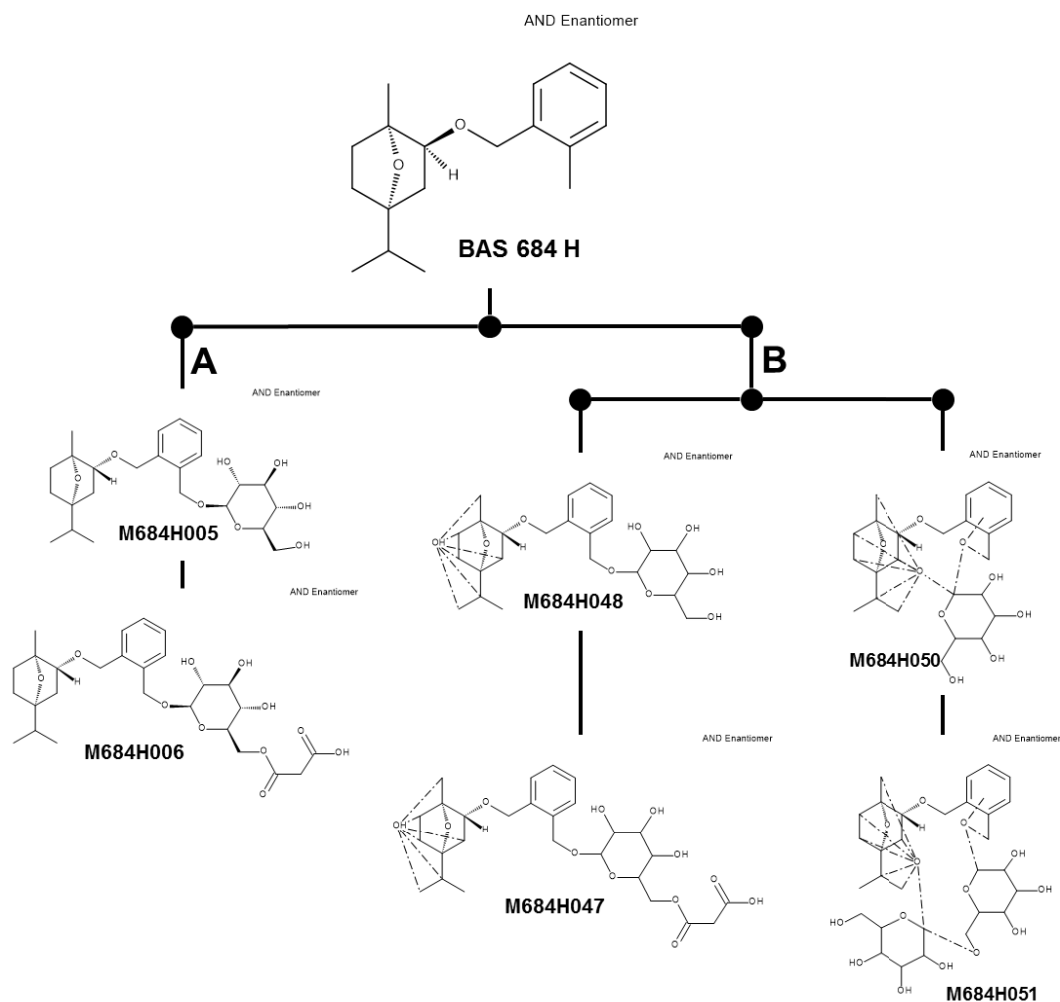
The most abundant components in carrot roots were carbohydrates (up to 78.4% TRR or 0.113 mg eq/kg), followed by BAS 684 H (3.5% TRR (0.003 mg eq/kg) for phenyl label and 7.9% TRR (0.012 mg eq/kg) for cyclohexane label). The most abundant components in carrot leaves were the parent compound (up to 27.9% TRR (0.1589 mg eq/kg)) and metabolite M684H050 (up to 7.3% TRR). Other metabolites accounted in carrot leaves for up to 7.3% TRR (0.032 mg eq/kg). Up to 8 unidentified peaks  $\geq$  0.01 mg/kg were additionally characterised, however these were at a maximum of 0.030 mg eq/kg or 5.3 % TRR and sufficient characterisation and identification was performed.

The metabolic transformation steps of BAS 684 H in carrots is hydroxylation of the parent compound at various positions and subsequent conjugation of hydroxyl groups with glycoside and malonyl glycoside. No cleavage of the molecule was observed through metabolism of BAS 684 H in carrot.

The identification of a major proportion of the residues in carrot root as carbohydrates (up to 78.4% TRR) is considered acceptable. Given application was made at BBCH 12-13, a significant proportion of the active substance will have reached the soil (crop interception value of 25%, EFSA Journal 2014;12(5):3662). Harvest of root samples was made 67 days after application allowing sufficient time for some degradation of BAS 684 H in soil to occur given the DT<sub>50</sub> for BAS 684 H is 53.9 days (Volume 3 CA B.8.1). The aerobic soil metabolism study shows no major metabolites are formed, rather numerous unidentified small degradation products are formed (each <5% of the applied radioactivity). This is also consistent with the results of the rotational crop

metabolism study in which no major metabolites are observed in following crops (Section CA B.7.6.1). It is concluded that the soil degradation products are taken up by the carrot root and metabolised further. The identification of glucose and fructose in the root extracts and ethanol after fermentation provide evidence for the radioactive soil degradation products entering sugar biosynthesis. Given almost 60% of the RRR in roots was solubilised by macerozyme/cellulase, and that glucose and its derivatives are used in the synthesis of cell walls, this provides further evidence for the formation of carbohydrates.

Figure 7-6 Proposed pathway of BAS 684 H in carrot



#### B.7.2.1.4. Plants – Soybean

The metabolism of BAS 684 H in soybean has been reported in two parts: a soil-grown study (part 1) and a hydroponic study (part 2). The studies on metabolism of soybean were not performed to GLP and have major deviations to current OECD guidelines (see conclusions). It was therefore decided not to rely on these data in the current submission and the study is seen as supporting information.

#### Part 1: soil-grown study

Report: CA 6.2.1/4  
CA 6.2.1/5  
Woodward M., 1984a  
Metabolism of sd95481 in soybeans 1. Quantitation and fractionation of residues; 2. Characterisation and identification of the principal metabolites from foliage CI-640-001 and CI-640-002

Guidelines: No

GLP: No

## Materials:

### 1. C-label BAS 684 H

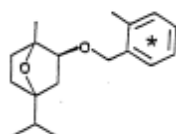
**Description:** Phenyl-U-<sup>14</sup>C (spec. activity of a.s. 57.7 MBq/mg)

**Lot/Batch #:** 8212

**Radiochemical Purity:** 98.5%

**Chemical Purity:** not stated

**Structure:**



## Method

A metabolism study on soybean grown outdoors was carried out in the summer of 1983 in Modesto, California, USA. Soybean seeds were sown into nine containers (one control and eight treated) filled with British sandy loam soil. The containers were steel drums 18 3/4 inches in diameter by 36 inches high. The crops were treated by application to the soil and the soil was thoroughly mixed before layering it on top of the untreated soil already in the soil container. The test item was applied at a total nominal application rate of 1.12 kg a.s./ha. <sup>14</sup>C-BAS 684H was applied diluted with acetone.

## Sampling

It is unclear at what growth stage the samples were taken, other than immature samples were taken 30, 60 and 90 days after application and harvest samples were taken at 120 days after application. Soybean plants were cut off about 1 inch above the soil line and were rinsed with water, to remove any soil particles adhering to leaves, stems or pods, in the laboratory. The immature plant samples were pulverised in liquid nitrogen and subsamples were combusted and extracted. During the course of the experiment, tissue dry weights were determined by heating subsamples to 70°C for 24 hours or until a constant weight was obtained. The remainder of the sample was frozen at -20°C. At the later time points, immature and/or mature pods were removed and separated into seeds and empty pods. These were pulverised in liquid nitrogen, subsamples combusted and the remainder of the samples frozen. The storage interval between sampling and analysis is not reported.

## Description of analytical procedures

Homogenised solid plant samples were weighed and combusted by means of an automatic sample oxidiser. Quantitation of metabolites was carried out using thin-layer chromatography followed by autoradiography and liquid scintillation counting.

**Homogenisation/plant extraction:** Soybean plants were frozen in liquid nitrogen and ground into a powder in a mortar and pestle. A 5g subsample of tissue was weighed and homogenised in 75mL of 90% acetonitrile for 2 minutes at high speed. The tissue was allowed to settle; the liquid was decanted and filtered through Whatmann No. 54 paper on a Buchner funnel. The homogenisation was carried out two more times with 75mL of 90% acetonitrile. After the final extraction, the residue was rinsed on filter paper. The solid residue was analysed by oxygen combustion followed by liquid scintillation counting (LSC).

**Preliminary Fractionation of the Plant Extract on C-18 Bond Elut Columns:** The aqueous organic plant extract was concentrated on a steam table with air blowing over the sample to aid evaporation. The extract was concentrated to approximately 25 mL and 25 mL of water were added. The acetonitrile concentration in this solution was approximately 30-40%. This extract (50 mL) was passed through a 1g C-18 Bond Elut column pre-equilibrated with water. The column and reservoir were rinsed with 10 mL 10% acetonitrile. Then, the column was dried under high vacuum for 3 minutes. The elution sequence was 15 mL 5% ethyl acetate in hexane, 5 mL ethyl acetate, and 5 mL methanol. Recovery of radioactivity from the column was consistently about 95% (range 92-100%). Nearly the entire radioactivity eluted under these conditions was in the flow-

through fraction. The radioactivity in the flow-through represented primarily conjugated and or other polar products.

Incubation of the Flow-Through of the C-18 Column with Beta-Glucuronidase and Cellulase: To examine the nature of the water-soluble conjugates in the flow-through fraction of the C-18 column, this fraction was concentrated and the remaining aqueous solution was adjusted to pH 5.0 with sodium acetate-acetic acid buffer (final concentration 50 mM). The volume was adjusted to 40 mL with water. Numerous enzymes were tested but only beta-glucosidase, crude beta-glucuronidase and crude cellulase showed cleavage activity. A mixture of beta-glucuronidase (Sigma G-0376, 102,000 units/mL, 50  $\mu$ L) and cellulase (Sigma C-7377, 1.35 units/mg, 10 mg) were added. These crude enzymes also have activity on para-nitrophenol-beta-D-glucopyranoside, the standard substrate for beta-glucosidase. The solution was allowed to incubate at 37°C overnight. The solution was adjusted to pH 3 and centrifuged to remove the protein precipitate. The clarified extract was ready for fractionation on a second C-8 Bond Elut column.

Extraction of Dry Plant Foliage, Seeds and Empty Pods: A samples of each of the harvest dry plant foliage, seeds and empty pods was dry-blended into a fine powder. 1g aliquots of each of the above were weighed into cellulose extraction thimbles (25 x 80 mm). Each was extracted with 100 mL of boiling solvent (hexane, acetone, methanol or aqueous acetonitrile (9:1)). The organic extract was concentrated and quantitated by LSC. The unextractable residue was combusted and quantitated in the same manner.

Radioassay: Radioactivity was quantified by using a Packard Model 2660 Liquid Scintillation Spectrometer. Counting efficiency determination was carried out by using the external standard ratio (ESR) technique. The actual quench curve was determined at various time intervals to ensure its validity. Radioactivity present in most liquids was analysed by taking two aliquots of the solution and dissolving each in 15 mL of Aquasol-2 scintillation cocktail.

Radioactive areas on TLC plates after development were removed by scraping and analysing for radioactivity in an Aquasol-2: water (11:4) gel system. The percentage of a given component on the TLC plate was determined by taking the dpms on the plate for that component and dividing by the total dpms on the plate).

Plant tissue samples (pulverised in liquid nitrogen) and unextractable plant residue were analysed by weighing subsamples into a Combusto-Cone sample holder (Packard Instrument Co., Downers Grove, IL) followed by combustion in a Packard Model 306 TriCarb sample oxidiser. The counting cocktail included Carbo-Sorb I and Permafluor V in a 10:12 ratio.

All samples except TLC plate scrapings were counted for 50 minutes or until 10,000 gross counts were obtained (a 2-sigma value of 2%). TLC plate scrapings were counted for 20 minutes or until 40,000 gross counts were obtained. Counting data were obtained without correction for background (i.e. gross dpms). Backgrounds were determined for the various solvents and control plant tissues used in the study and there were subtracted from the gross dpms in the calculated data presented in this report.

#### Thin-Layer Chromatography (TLC)

Plates used for TLC were silica gel F-254, 20 cm x 20 cm, 0.25 mm thickness. Five solvent systems were selected for TLC:

- 1) toluene: ethyl acetate, 17:3 (v/v)
- 2) hexane: methylene chloride: diethyl ether, 6:3:1 (v/v/v)
- 3) heptane: isopropanol: acetic acid (150: 20: 1)
- 4) hexane: isopropanol: acetic acid (160: 40: 2)

Developing tanks contained ca. 200 mL of the appropriate solvent system mixture. All plates were spotted 3 cm from the bottom edge of the plate. Plates were scribed 3 cm from the top making a total migration distance of 14 cm. Plant extracts were generally chromatographed in systems 1 and 3 or systems 4 and 5.

Autoradiography was performed on Kodak SB-5 single-coated, blue-sensitive X-ray film.

Reference Standards: Synthetic standards of numerous known and/or suspected degradation products of BAS 684 H have been prepared. The structures of these compounds are shown in Table 7-61. The  $R_f$  values of these compounds in several solvent systems are shown in Table 7-62.  $R_f$ 's were found to vary with amount of sample spotted and also varied with the age of the solvent system. The  $R_f$ 's reported are intended to provide a guide to

the reader for the relative position of these compounds in these solvent systems. Reference standards were visualised under UV light.

**Radiolabelled Reference Compounds:** Some of the degradation products of BAS 684 H have been isolated from other studies and identified by spectroscopic and co-chromatographic techniques. These include BAS 684 H, M684H014 and M684H004 (205558), all isolated from the soil metabolism study (RIR-22-005-83 (part II)).

**Gas Chromatography/ Mass Spectroscopy (GC/MS):** All mass spectra were obtained on either the Finnigan 1020 or 4500. The GC column used was a fused-silica, wall-coated SE-34 column (20-30 m x 0.25 mm). Temperature programs varied and are indicated on the chromatogram. The carrier gas was helium at a flow rate of 1 mL/min. The split at the inlet was variable and ranged from 20 to 1 to 60 to 1. The 1020 mass spectrometer operating parameters were: (a) ionisation mode – electron impact, (b) ionisation voltage – 70 eV, (c) emission current – 0.85 mA, (d) sensitivity –  $10^{-7}$  A/V, (e) electron multiplier voltage – 2000 V. Spectra were obtained at a rate of 1 per second. Sample volumes injected ranged from 1 to 5  $\mu$ L. Dihydroxylated metabolites were analysed underivatized and as trimethylsilyl derivatives.

Direct comparisons between synthetic standards and isolated metabolites were made using GC-MS-SIM (selected ion monitoring) with a mass selective detector. Four major ions were examined from each metabolite. The gas chromatographic column was fused-silica, wall coated SE-54 column (50 m x 0.25 mm). The flow rate was 1 mL helium/min and the oven temperature was maintained at 220°C and the injector was 250°C. The split was approximately 10:1. The ionisation voltage was 70 eV and the electron multiplier voltage was 2200 eV.

Table 7-61 Structures of Reference Compounds

| Metabolite           | Structure | Chemical Name   |
|----------------------|-----------|---|
| BAS 684 H (SD 95481) |           | 7-oxabicyclo[2.2.1]heptane, 1-methyl-4-(1-methylethyl)-2-[(2-methylphenyl)methoxy]-exo-             |
| M684H014 (SD 204328) |           | Benzoic acid, 2-methyl-, 1-methyl-4-(1-methylethyl)-7-oxabicyclo[2.2.1]hept-2-yl-ester,exo-         |
| M684H004 (SD 205588) |           | 7-oxabicyclo[2.2.1]heptane-1-methanol, alpha, alpha, 4-trimethyl-3-[(2-methyl-phenyl)methoxy]-,exo- |
| SD 207430            |           | 7-oxabicyclo[2.2.1]heptane-1-ethanol, beta, 4-dimethyl-3-((2-methylphenyl)methoxy)-exo-             |
| SD 207322            |           | 7-oxabicyclo[2.2.1]heptane-1-methanol, 4-(1-methylethyl)-2-((2-methylphenyl)methoxy)-exo-           |

|                      |  |   |
|----------------------|--|---|
| SD 207850            |  | 7-oxabicyclo[2.2.1]heptan-2-ol, 4-methyl-1-(1-methylethyl)-5-((2-methylphenyl)-methoxy)-, exo, exo-   |
| M684H002 (SD 207856) |  | Benzenemethanol, 2-((1-methyl-4-(1-methyl-ethyl)-7-oxabicyclo[2.2.1]-hept-2-yloxy)-methyl)-.exo-      |
| M684H019 (SD 211368) |  | Phenol, 3-methyl-4-((1-methyl-4-(1-methyl-ethyl)-7-oxabicyclo[2.2.1]-hept-2-yloxy)-methyl)-           |
| SD 211646            |  | 7-oxabicyclo[2.2.1]heptan-2-ol.4-methyl-1-(1-methylethyl)-5-((2-methylphenyl)-methoxy)-,2-endo,5-exo- |
| M684H018 (SD 211647) |  | Phenol, 4-methyl-3-((1-methyl-4-(1-methyl-ethyl)-7-oxabicyclo[2.2.1]-hept-2-yloxy)-methyl)-, exo-     |

Table 7-62 R<sub>f</sub> Values of BAS 684 H and Related Compounds on Silica Gel F-254 Plates <sup>a)</sup>

| Metabolite           | TLC Solvent System |      |      |      |      |
|----------------------|--------------------|------|------|------|------|
|                      | 1                  | 2    | 3    | 4    | 5    |
| BAS 684 H (SD 95481) | 0.60               | 0.57 | 0.68 | 0.82 | 0.86 |
| M684H014 (SD 204328) | 0.71               | 0.65 | 0.64 | 0.86 | 0.89 |
| M684H004 (SD 205588) | 0.14               | 0.10 | 0.4  | 0.49 | 0.76 |
| M684H024 (SD 207430) | 0.10               | 0.06 | 0.30 | 0.44 | 0.69 |
| SD 207522            | 0.14               | 0.09 | 0.34 | 0.46 | 0.72 |
| SD 207850            | 0.09               | 0.06 | 0.31 | 0.44 | 0.71 |
| M684H002 (SD 207856) | 0.14               | 0.08 | 0.29 | 0.46 | 0.72 |
| M684H019 (SD 211368) | 0.23               | 0.13 | 0.30 | 0.48 | 0.75 |
| SD 211646            | 0.20               | 0.13 | 0.34 | 0.46 | 0.76 |
| M684H018 (SD 211647) | 0.24               | 0.12 | 0.34 | 0.50 | 0.30 |

a) The values reported above represented ca. 10 µg spotted and solvent systems were freshly prepared and tanks were not pre-equilibrated



## Results and Discussion

**Whole Plant Autoradiography:** Whole plant autoradiograms were made for plants harvested 30 days after planting. For comparison is an autoradiogram of a plant treated with 10 MicroCuries of BAS 684 H as a soil drench but grown in a 4 x 4 inch pot. The autoradiograms show that BAS 684 H is taken up by the roots and translocated apoplastically to the leaves. The younger leaves contained much less radioactivity than the older leaves. The roots showed the highest level of radioactivity.

**Residue Levels in Soybean Plants During the Growing Season:** Levels of  $^{14}\text{C}$ -BAS 684 H-derived residues were measured throughout the growing season Table 7-63. Levels in plants increased during the first three month and decreased at harvest. The increase of residues levels in seeds and from Day-91 to harvest was likely due to the normal dehydration of the seeds and pods during maturation. A quantitative analysis of total radioactivity in the various parts of the plant at harvest is presented in Table 7-64. Greater than 80% of the total radioactivity in plants at harvest was in the stems and leaves (still attached to the plant) with only about 10% in seeds and 7% in pods. Based on weights of the various plant parts, it is clearly evident that the  $^{14}\text{C}$ -residues remain primarily in the vegetative plant parts with no apparent accumulation of residues in seeds or pods.

Table 7-63  $^{14}\text{C}$ -BAS 684 H-derived Residue Levels in Soyabean Plants<sup>a)</sup>

| Tissue Type                       | Day         |             |             |             |
|-----------------------------------|-------------|-------------|-------------|-------------|
|                                   | 30          | 60          | 90/91       | 120         |
| Green Plant Tissue (% dry matter) | 0.4<br>(19) | 1.0<br>(26) | 3.2<br>(31) | 1.9<br>(30) |
| Dry Plant Foliage (% dry matter)  | -           | -           | 6.0<br>(91) | -           |
| Seeds (% dry matter)              | -           | -           | 0.3<br>(60) | 0.6<br>(91) |
| Pods (% dry matter)               | -           | -           | 0.5<br>(50) | 0.8<br>(39) |

a) The ppm values (fresh weight basis) were obtained by combustion of subsamples (100 mg each).

Table 7-64 Comparison of the Distribution of Radioactivity at Harvest and Weights of Various Plant Parts

| Percent of Total |                          |                      |
|------------------|--------------------------|----------------------|
| Tissue Type      | Radioactivity            | Weight <sup>a)</sup> |
| Plant Tissue     | 81.3 ± 3.6 <sup>b)</sup> | 62.7 ± 3.9           |
| Seeds            | 11.9 ± 2.7               | 25.5 ± 3.1           |
| Pods             | 6.8 ± 1.1                | 11.8 ± 1.3           |

a) Percent of total fresh weight in each fraction

b) Mean and standard deviation (n=8)

### Extraction and Fractionation of $^{14}\text{C}$ -BAS 684 H Derived Residue in Soybean Plants

Extracts representing 5g fresh weight were prepared. These extracts were concentrated to ca. 30-40% acetonitrile prior to application to a 1g C-18 Bond Elute column. The column was eluted with 15 mL 5% EtOAc in hexane and then with 5 mL of ethyl acetate and 5 mL methanol. It was demonstrated that acetonitrile concentration of less than 20% would leave much of the radioactivity on the column. The distribution of radioactivity in the various fractions is shown in Table 7-65. Approximately 65% of the radioactivity was extracted using this procedure. Nearly the entire radioactivity was found in the flow-through of the C-18 column. This fact indicated that the extractable residue was primarily water-soluble conjugates and other polar metabolites. After removal of all of the acetonitrile, the samples were buffered and incubated with crude beta-

glucuronidase and cellulose enzymes overnight at 37°C. These samples were then adjusted to pH 3 and centrifuged to remove any insoluble material. In order to characterise the metabolites recovered by the enzyme hydrolysis, the solution was then applied to a 1g C-18 Bond Elut column, followed by 10 mL water, 15 mL 5% ethyl acetate in hexane, and then (sometimes) with 5 mL 20% ethyl acetate in hexane, followed by 5 mL of ethyl acetate and 5 mL methanol. These results are presented in Table 7-66. Approximately 50% of the radioactivity in the sample is eluted with either 5% ethyl acetate or 20% ethyl acetate. These fractions contain the metabolites released from conjugates by enzymes hydrolysis. The 5% ethyl acetate fraction contains predominantly the monohydroxy metabolites. The 20% ethyl acetate fraction contains predominantly the dihydroxy metabolites. (The characterisation data on which these statements are based is presented in Part II of this report). At harvest 26% of the radioactivity is in the fraction which contains the monohydroxylated metabolites. Little radioactivity was recovered in the flow-through fraction after enzyme hydrolysis indicating the effectiveness in cleaving the polar conjugates.

Table 7-65 Distribution of Radioactivity in mg eq/kg in Extracts of Soybean Foliage Treated with BAS 684 H in Soil prior to enzyme treatment

|  | Day  |      |      |      |
|--|------|------|------|------|
|  | 30   | 60   | 91   | 120  |
| <b>Extractable Residue</b>                         | 0.33 | 0.80 | 2.43 | 1.45 |
| <b>Conjugated and Polar Products <sup>a)</sup></b> | 0.30 | 0.80 | 2.32 | 1.33 |
| <b>Free Metabolites <sup>b)</sup></b>              | 0.02 | 0.00 | 0.03 | 0.01 |
| <b>Un-extractable Residue</b>                      | 0.08 | 0.14 | 0.45 | 0.24 |

a) C-18 column flow-through plus C-18 column bond material

b) C-18 column 5% ethyl acetate in hexane fraction

c) Based on dividing by the sum of the extractable and the unextractable and multiplying by 100

Table 7-66 Distribution of Radioactivity Eluted From 1-C C-18 Bond Elut Columns After Enzyme Treatment <sup>a)</sup>

|  | Day |    |    |     |
|--|-----|----|----|-----|
|  | 30  | 60 | 91 | 120 |
| <b>5% EtOAc/ Hexane <sup>b)</sup></b>  | 54  | 28 | 34 | 26  |
| <b>20% EtOAc/ Hexane <sup>c)</sup></b> | 37  | 69 | 22 | 28  |
| <b>Ethyl acetate <sup>d)</sup></b>     |     |    | 41 | 42  |
| <b>MeOH <sup>d)</sup></b>              |     |    |    |     |
| <b>Flow-Through <sup>d)</sup></b>      | 5   | 4  | 4  | 5   |

a) Percent of recovered radioactivity (i.e. C-18 flow-through in Table 7-65).

b) This fraction contains the monohydroxy metabolites of BAS 684 H

c) This fraction contains the dihydroxy metabolites of BAS 684 H

d) These fractions contain polar products

#### Extractability of <sup>14</sup>C-Residues From Dry Plant Foliage, Seeds and Empty Pods

Extraction of soybean seeds by the same procedures used for plant foliage (green or dry) resulted in the recovery of less than 10% of the radioactivity. A series of solvents were tried including hexane, acetone, methanol, acetonitrile/water, and water which yielded the highest amount extractable residue (42 and 47% in two tests). The other solvents extracted ca. 10% of the radioactivity with the sequence of hexane and methanol extracting ca. 20% of the radioactivity. The extractability of <sup>14</sup>C-residues in dry foliage and pods were also examined. In both cases, acetonitrile/water (9:1) was superior to the other solvents. The amount obtained from foliage was ca. 75% and from pods ca. 30%.

#### Fractionation of the Extractable Radioactivity From Soybean Seeds

Since less than 10% of the total <sup>14</sup>C-residue in the soybean seed was recovered by aqueous acetonitrile extraction, a hot water extraction was made in attempt to characterise a larger portion of the residue in the soybean seed. Hot water extraction recovered 47% of the radioactivity in the seeds. For comparative purposes,



an aliquot of radioactive residue from the foliage was also tested. In order to demonstrate that the extractable components from the soybean seeds did not inhibit or interfere with the enzymatic cleavage of the conjugated products, the hot water extract of control soybean seed was mixed with the radioactive residue from the foliage which had been cleaned up on the initial C-18 column to remove chlorophylls.

From the C-18 column chromatographic patterns of the extracts from the treated plants (foliage and the seeds with the addition of the control seed extract) prior to enzyme hydrolysis, it can be seen that the majority of the radioactivity from the foliage extract is retained on the column and eluted with ethyl acetate. It has already been demonstrated that at least half of the radioactivity is not retained by the column (i.e., the flow-through fraction) and in the precipitate. This demonstrates the extremely polar nature of the seed residue and its qualitative difference from the foliage residue. The precipitate contains primarily high molecular weight storage proteins and this indicates that a major portion of the  $^{14}\text{C}$ -residue in the soybean seed is protein bound.

Table 7-67 Summary of the Characterisation of Soybean Seed Residue

| No. | Treatment   | Mono and/or Dihydroxy Metabolites <sup>a)</sup> |                 |
|-----|---|---|-----------------|
|     |   | Seed extract                                    | Foliage Extract |
| (1) | Esterase at pH 8.0, 37°C, 16 hr                     | No  | No              |
| (2) | Protease at pH 7.5, 37°C, 16 hr                     | No  | No              |
| (3) | Acid at pH 1, 35°C, 1 hr                            | No  | No              |
| (4) | Base at pH 13, 85°C, 1 hr                           | No  | No              |
| (5) | Glucuronidase +<br>Cellulase at pH 5.0, 37°C, 16 hr | No  | Yes             |
| (6) | Control, 37°C, 16 hr                                | No  | No              |

a) Metabolites hydrolysed from conjugates and/or polar products by each treatment.

The two seed extracts (i.e. with and without foliage extract) were divided into six portions and treated as shown in Table 7-67. These samples after incubation were analysed by C-18 fractionation and the results are shown in Table 7-67. The results of this experiment are summarised in the following statements:

1. Treatment 5 successfully released monohydroxy and dihydroxy metabolites from the foliage extract but failed in the seed extract. This indicates the qualitative difference in the chemical nature between the foliage and the seed residue.
2. Esterase and acid and base hydrolysis were ineffective in both types of extracts.
3. Protease changed the distribution pattern of the radioactivity in the treated seed extract but not in the foliage extract. Protease catalyses the hydrolysis of the soybean seed protein into soluble peptides. The shift in the  $^{14}\text{C}$ -residue profile after protease treatment indicates that the  $^{14}\text{C}$ -residue is bound to peptides which are not retained on the C-18 column (i.e. are found in the flow-through fraction). The high level of radioactivity seen in the precipitate in the seed extract appears to be tightly bound to the protein precipitate and together with the protease data suggesting that the residue in seeds is protein bound. No further work was undertaken regarding the nature of the aqueous extractable or the unextractable residue in soybean seeds.

#### Distribution and Quantitation of Metabolites in Immature and Mature Soybean Plants

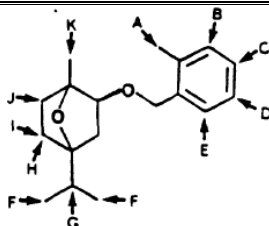
In order to obtain sufficient quantities of metabolites to carry out quantitation and characterisation of individual metabolites, 100 g samples of soybean stems, petioles, and foliage were processed using procedures similar to those used for small samples reported in Part 1 of this report. The aqueous acetonitrile extractable materials (ca. 90% of total radioactivity) were concentrated to ca. 3% acetonitrile and partially purified by C-18 column chromatography. The flow-through fraction from this column contained essentially all of the radioactivity in the sample (>98%). After removal of the organic solvents and enzyme treatment, the aqueous sample was adsorbed on a second C-18 column (5 g). The elution sequence was 5% ethyl acetate in hexane, 20% ethyl acetate in hexane, ethyl acetate, and methanol. The 5% ethyl acetate in hexane fraction was shown to contain primarily monohydroxy metabolites, the 20% ethyl acetate fraction contained primarily dihydroxy metabolites, and the ethyl acetate, methanol, and flow-through contains beta-glucoside conjugates (not cleaved by the enzymes) and other polar metabolites.

Extracts were prepared from immature (day-60) and harvest plants (Day 120). Data from Day-60 and Day-120 are shown in Table 7-68. Metabolites recovered after enzyme hydrolysis ranged from 26-36% as compared to

greater than 50% for the small samples reported above. The proportion of radioactivity in the methanol and flow-through also suggested that the enzyme hydrolysis was incomplete. No attempt was made to further hydrolyse additional conjugates from these samples.

Thin-layer chromatography was carried out on the 5% ethyl acetate and 20% ethyl acetate fractions and the plates were subjected to autoradiograph. Quantitation of the various mono- and dihydroxy metabolites observed are summarised in Table 7-68. The major monohydroxy metabolites include M684H002 (SD 207356 (A)), and three phenolic products: M684H017 (M684H017 (SD 211648)) (B-proposed structure), M684H019 (SD 211368) (C), and M684H018 (SD 211647) (D).

Table 7-68 Distribution of Radioactivity Eluted From 5g C-18 Bond Elut Columns from 100g Plant Samples after Enzyme Treatment

|  | <div></div> <p>positions :</p> |                    |      |      |
|--|---|--------------------|------|------|
| Fraction   | Metabolite  | Spot <sup>a)</sup> | Day  |      |
|  |   |                    | 60   | 120  |
| 5% EtOAc/ Hexane<br>(monohydroxy<br>metabolites) | % extractable radioactivity   |                    | 16.8 | 20.8 |
|  | M684H004 (SD 205588)  | G                  | 0.1  | 0.3  |
|  | M684H017 (SD 211648)  | B <sup>b)</sup>    | 1.2  | 2.5  |
|  | M684H018 (SD 211647)  | D                  | 4.6  | 5.9  |
|  | M684H019 (SD 211368)  | C                  | 0.4  | 2.1  |
|  | SD 211892   | A <sup>c)</sup>    | 0.4  | 0.6  |
|  | M684H002 (SD 207856)  | A                  | 3.2  | 3.1  |
|  | SD 207850   | I <sup>d)</sup>    | 1.9  | 1.6  |
|  | Other <sup>e)</sup>   |                    | 5.0  | 4.7  |
| 20% EtOAc/ Hexane<br>(dihydroxy<br>metabolites)  | % extractable radioactivity   |                    | 9.6  | 15.5 |
|  | SD 211733   | D/G <sup>b)</sup>  | 3.4  | 6.8  |
|  | M684H044 (SD 207855)  | A/G <sup>b)</sup>  | 1.7  | 4.5  |
|  | SD 211731   | A/I <sup>b)</sup>  | 1.4  | 1.0  |
|  | Other <sup>e)</sup>   |                    | 3.1  | 3.2  |
| EtOAc <sup>f)</sup>                              |   |                    | 38.5 | 30.0 |
| MeOH <sup>f)</sup>                               |   |                    | 16.1 | 17.1 |
| Flow-Through <sup>f)</sup>                       |   |                    | 19.0 | 16.8 |

- Letters correspond to position of hydroxylation
- Proposed structure
- Appears to be a monohydroxy metabolite with a double bond on the cineole side
- Mixture of at least two components
- Remaining radioactivity
- These fractions contain polar products

Preparative TLC of the 5% Ethyl Acetate in Hexane Fraction (Monohydroxy Metabolites)

The radiolabelled components in the 5% ethyl acetate in hexane fraction were separated by preparative TLC using solvent system 1. After removing these components from the silica, the samples were subjected to

preparative TLC in solvent system 3. Seven components (A, A', B, C, D, G and I) were separated and five (A, C, D, G and I) were compared with standards by TLC, GC and selected ion monitoring (SIM). (Component A' appears to be a monohydroxy metabolite (hydroxylation at A) with a double bond on the cineole side of the molecule; however, no standard was available. Component B is a phenolic metabolite; however, the hydroxylation position on the ring has yet to be confirmed.) The data supporting the identification are summarised in Table 7-69 and the TLC and SIM chromatograms which provide the direct evidence.

Table 7-69 Data supporting the identification of the monohydroxy metabolites of BAS 684 H obtained from soybean foliage after enzyme hydrolysis

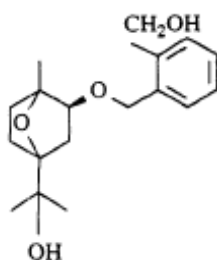
| Metabolite              | Spot | Chromatography TLC System |   | GC Retention Time |          | Ions Monitored     |
|-------------------------|------|---------------------------|---|-------------------|----------|--------------------|
|                         |      | 1                         | 3 | Standard          | Isolated |                    |
| M684H002<br>(SD 207856) | A    | X                         | X | 7.76              | 7.75     | 91, 93, 123, 171   |
| M684H019<br>(SD 211368) | C    | X                         | X | 9.07              | 9.12     | 121, 91, 123, 107  |
| M684H018<br>(SD 211647) | D    | X                         | X | 8.91              | 6.91     | 121, 120, 107, 290 |
| M684H004<br>(SD 205588) | G    |                           |   | 5.70              | 5.73     | 105, 107, 121, 122 |
| SD 207850               | I    | X                         | X | 6.55              | 5.56     | 105, 71, 79, 91    |

Preparative TLC of the 20% Ethyl Acetate in Hexane Fraction (Dihydroxy Metabolites)

The radiolabelled components in the 20% ethyl acetate in hexane fraction were separated by preparative TLC using solvent system 4. Four bands were removed and subjected to preparative TLC in solvent system 5. The two major metabolites in this group were analysed by radio-GLC and by GC-MS on a Hewlett-Packard Model 5992B GC-MS system.

The component designated A/G was examined by GC-MS. The underivatised material had fragment ions at m/z 169, 168, 167, 122, 121, 107, 93, 91 and 59. The ion at m/z 59 is indicative of a hydroxyl group located at the tertiary carbon of the isopropyl group. The lack of a major ion at m/z 105 or 121 is indicative of hydroxylation on the aromatic methyl group. This component was also analysed as the TMS (trimethylsiloxy) derivative. The diagnostic ions appear to be m/z 360 ([M-90]+), 257, 241, 193, 192, 131, and 119. The ion at m/z 131 is characteristic of compounds with a trimethylsilyloxy group at the tertiary carbon of this isopropyl group. This observation is consistent with analysis of various standards as TMS derivatives. The ion at m/z 257 appears to represent the cineole portion of the molecule carrying 38 mass units (OTMS-H) more than the parent. The ions at m/z 193 and 192 are characteristic of compounds with a TMS group on the aromatic hydroxyl methyl (mass shift from 105 to 193). These ions, which were suspected of having a TMS group, were confirmed as having a TMS group by using D9-TMS and observing the appropriate mass shifts. Based on the mass spectral data this metabolite is tentatively identified as M684H044 (SD 207855) (hydroxylations at A and G, structure shown in Figure 7-7). No reference material was available to confirm the identification.

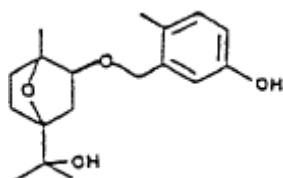
Figure 7-7 Structure of M684H044 (SD 207855)



The component designated D/G was analysed as the underivatised material. Diagnostic ions were seen at m/z 167, 140, 139, 122, 121 and 59. The ion at m/z 121 is indicative of aromatic ring hydroxylation. The ion at m/z

59 is indicative of hydroxylation at the tertiary carbon in the isopropyl group. The mass spectrum has diagnostic ions at  $m/z$  450 ( $M^+$ ), 360, 257, 193 and 131. The ions at  $m/z$  131 and 257 would appear to represent the same fragments as observed in SD 207833. The intense ion at  $m/z$  193 is indicative of a trimethylsilyloxy group on the aromatic ring. The position of the hydroxyl group on the aromatic ring cannot be determined with certainty. The predominant phenolic metabolite (mono-substituted) was M684H018 (SD 211647) with hydroxyl in position 3. Based primarily on this evidence, the structure of this metabolite is proposed as SD 211733 (hydroxylations at D and G, structure shown in Figure 7-8).

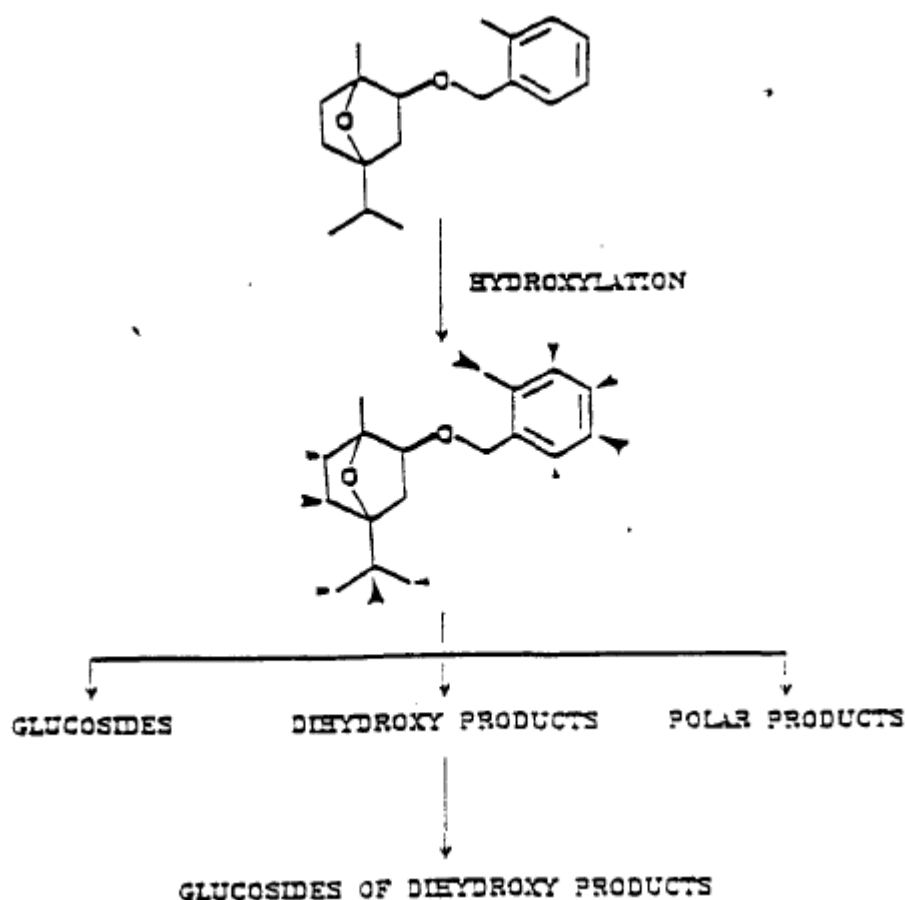
Figure 7-8 Structure of SD 211733



#### Proposed Metabolic Fate of BAS 684 H in Soybean Plants

BAS 84 H is rapidly metabolised in soybean plants to a wide variety of monohydroxyl products. These products were not found as free metabolites but rather as beta-1,4-glucosides and other types of conjugates. The monohydroxylated products and/or their glycoside conjugates may undergo a subsequent hydroxylation leading to a variety of dihydroxylated products. No carboxylic acids were detected either as free metabolites or glycoside conjugates. No evidence was obtained indicating that the benzylic ether is oxidised (to the ester) or cleaved. The general pathway of metabolism of BAS 684 H in soybean plants is shown below in Figure 7-9 with the size of the arrows indicating the relative importance of the various sites for hydroxylation.

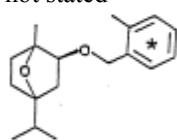
Figure 7-9 Metabolism of BAS 684 H in Soybean Plants



**Part 2**

|             |   |
|-------------|---|
| Report:     | CA 6.2.1/6<br>CA 6.2.1/7<br>CA 6.2.1/8<br>Woodward M., 1984 a, 1984b, 1984c<br>Metabolism of sd95481 in soybeans 1. Identification of the principal metabolites of sd95481 from the hydroponic growth medium of soybean plants 2. Characterisation and identification of the principal metabolites of sd95481 in soybean plants; 3. Characterisation and identification of the principal metabolites in a pilot study<br>CI-640-003; CI-640-004; CI-640-015 |
| Guidelines: | No  |
| GLP:        | No  |

**Materials**

|                              |  |
|------------------------------|--|
| <b>Description:</b>          | Phenyl-U- <sup>14</sup> C (spec. activity of a.s. 57.7 MBq/mg)                     |
| <b>Lot/Batch #:</b>          | 8206   |
| <b>Radiochemical Purity:</b> | 97%  |
| <b>Chemical Purity:</b>      | not stated   |
| Structure:                   |  |

**Methods**

Soybean plants (cultivar Williams) were grown in sand and after 14 - 20 days the plants (at BBCH 11) were transferred to laboratory hydroponic apparatus in Hoagland's solution. After a 2-day equilibration period, the Hoagland's solution was removed and replaced with fresh Hoagland's solution containing ca. 10 ppm <sup>14</sup>C-SD 95481 (ca. 25 microCuries/L).

It is unclear at what growth stage the samples were taken other than plants being removed after 7 days. The unused Hoagland's solution was filtered and extracted with chloroform. The plants were frozen at -10 °C until extraction. The storage interval between sampling and analysis is not reported. The shoots were powdered in liquid nitrogen and extracted with acetone and water. The organic extract was concentrated and partitioned with hexane. The aqueous phase was diluted in acetonitrile and passed through a 1-g C-18 Bond Elut column with acetonitrile and concentrated. The aqueous fraction was made to pH 5 with acetate buffer and incubated with a mixture of beta-glucuronidase and cellulase. The extract was adjusted to pH 3 with concentrated hydrochloric acid and centrifuged to remove the precipitate. The extract was diluted in acetonitrile and passed through a 1-g C-18 column, eluted with acetonitrile/water. The eluate was applied to a 1-g C-18 column and eluted with 5-50% ethyl acetate/hexane, ethyl acetate and methanol. Identification was performed by GC-MS, two-dimensional TLC and comparison with reference standards. Radioassay, TLC, R<sub>f</sub> values, reference standards, GC-MS were performed as stated in Part 1 (CA 6.2.1/4) above.

**Results***Soybean plants*

The aqueous and organic extractable radioactivity of the soybean plants represented 98.6% of the total radioactivity in the plants. The distribution of radioactivity between metabolites following enzyme treatment is shown in

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Table 7-70. Monohydroxy metabolites (free and enzyme hydrolysed) represented 27.9 % of the extractable radioactivity, parent accounted for 7.0% of the extractable radioactivity, and dihydroxy metabolites accounted for 9.3% of the radioactivity. The remaining 56 % of the radioactivity was polar products which were not hydrolysed by enzymes, no further analysis of this fraction was performed.

Table 7-70: Extractable radioactivity found as monohydroxy metabolites in soybean plants

| Position of hydroxyl group | SD No.                   | % extractable radioactivity | How identification/ characterisation was achieved |
|----------------------------|--------------------------|-----------------------------|---|
| A <sup>a)</sup>            | 211892                   | 0.8                         | TLC, GC, MS                                       |
| A                          | 207856                   | 13.3                        | TLC, GC, MS                                       |
| B <sup>b)</sup>            | 211648                   | 0.9                         | GC, MS  |
| C                          | M684H019 (211368)        | 0.7                         | TLC, GC, MS                                       |
| D                          | M684H018 (211647)        | 0.3                         | TLC, GC, MS                                       |
| F+J                        | M684H024 (207430)+211732 | 1.1                         | GC, MS  |
| G                          | M684H004 (205588)        | 0.6                         | TLC, GC, MS                                       |
| I                          | 207850                   | 7.2                         | GC, MS  |
| Total                      |                          | 27.9                        |   |

a) appears to be a dehydro analog of SD 207856

b) proposed structure

Further fractions following enzyme treatment were analysed by 2D TLC and proposed as dihydroxy metabolites by MS: M684H044 (SD 207855), 211733, 211731 and 211729, although there was insufficient material to confirm the locations of the hydroxyl groups, no reference standards were available, and the amounts were not quantified.

#### *Hydroponic growth medium*

The amount of radioactivity found in the Hoagland's solution after extraction with chloroform is shown in Table 7-71. The organic extraction removed >97% radioactivity from the aqueous solution.

Table 7-71: Radioactivity extracted from remaining treatment solution

| Fraction                               | Percent of total radioactivity |
|--|--------------------------------|
| Total applied                          | 100                            |
| Remaining treatment solution           | 15.8                           |
| Organic (chloroform) extractable, pH 2 | 15.4                           |
| Aqueous residue                        | 0.4                            |

The distribution of components by 2D TLC is shown in

---

Table 7-72. The major component observed was parent SD 95481 (BAS 684 H). The second most major product was SD 751, however this was also present as a radiochemical impurity in the applied material and a photolysis product, which accounted for for 2.2 % of the applied radioactivity. All other components represented < 1% of the total applied radioactivity. Other radiochemical impurities/photolysis products were M684H059 (SD 637) and M684H014 (SD 204328). Except for M684H001 (SD 202193), the metabolites identified are all hydroxylated products of BAS 684 H.



Table 7-72: Distribution of components in the remaining treatment solution by 2D TLC

| Spot no             | Identification       | Percent of total radioactivity on the TLC plate | Percent of applied radioactivity | How identification/characterisation was achieved        |
|---------------------|----------------------|---|----------------------------------|---|
| 1                   | SD 95481 (BAS 684 H) | 65.7  | 10.12                            | Not stated  |
| 2                   | -                    | 0.9   | 0.14                             | -   |
| 3                   | M684H059 (SD 637)    | 0.8   | 0.13                             | TLC retention time match with reference standard, GC-MS |
| 4                   | M684H004 (SD 205588) | 1.3   | 0.19                             | TLC retention time match with reference standard, GC-MS |
| 5                   | M684H002 (SD 207856) | 2.5   | 0.39                             | TLC retention time match with reference standard, GC-MS |
| 6                   | SD 207850            | 4.7   | 0.72                             | NMR, GC-MS  |
| 7                   | SD 751               | 14.0  | 2.16                             | TLC retention time match with reference standard, GC-MS |
| 8                   | M684H001 (SD 202193) | 2.3   | 0.35                             | TLC retention time match with reference standard, GC-MS |
| 9                   | -                    | 1.2   | 0.19                             | -   |
| 10                  | -                    | 1.0   | 0.15                             | -   |
| Other <sup>a)</sup> | -                    | 5.7   | 0.87                             | -   |

a) Radioactivity associated with the origin of the TLC plate and other minor components

Further purification in solvent systems 1 and 3 and examination by GC-MS identified two minor components, the two diastereomers of M684H024 (SD 207430) by comparison of MS with reference standards, and tentatively SD 211732 by MS and NMR (tentative as the location of the hydroxy group on the cineole ring is not confirmed).

The study concludes that the components observed in the Hoagland's solution appear to result from metabolism of parent by plant roots or enzymes released from plant roots with an unknown contribution from microbes associated with the roots.

### Conclusion

The metabolism of BAS 684 H in hydroponically-grown soybean plants investigated by these studies show a majority of the residues were present as polar conjugates which, following enzymatic hydrolysis, were identified/proposed as monohydroxy or dihydroxy derivatives. These metabolites were characterised by TLC and GC/MS. Reference standards of most of the monohydroxylated metabolites were used to confirm the identifications.

**HSE Comment on all soybean studies:** The studies were published in the 1980s and were not performed to the OECD Guideline 501 nor to GLP. Only a single ring was labelled (Phenyl- $U-^{14}C$ ) and was applied to soybean seeds at a total nominal application rate of approximately 1.12 kg a.s./ha (soil study) or 10 ppm a.s./L Hoagland's solution (hydroponic study). Samples were stored frozen prior to analysis but the lengths of storage are not clear and no additional data to address the stability were provided in the studies.

For the soil-grown soybean study, the amount of radioactivity extracted (mg eq/kg or %TRR) was not presented in the reports. Compounds: M684H002, M684H019 (SD 211368), M684H018 (SD 211647), M684H004 (SD 205588) and SD 207850 were sufficiently identified in soybean foliage after enzyme hydrolysis by co-chromatography with acceptably derived reference standards by TLC and GC-SIM. There was no mention of identification of the parent compound in extracts. The TLC autoradiograms of the 5% ethyl acetate in hexane fraction were characterised as monohydroxy derivative and phenolic metabolite (band A' and band b respectively) by TLC and GC-MS; however no reference standards were available to confirm the identification. The TLC autoradiograms of the 20% ethyl acetate in hexane fraction were characterised as TMS derivatives and dihydroxy metabolites by TLC and GC-MS; however no reference standards were available to confirm the identification.

The results of the hydroponic study are less relevant given the growth conditions, however the metabolism was similar to the soil-grown soybean study with the majority of residues being polar conjugates which, upon enzymatic hydrolysis, were identified as mono- and di-hydroxy derivatives of parent BAS 684 H.

Owing to the issues raised the soybean studies cannot be quantitatively relied upon but can be considered as supporting information given they provide some qualitative information on metabolites which have been identified in a primary crop.

A comparison of the metabolites identified in the soybean studies with the metabolites identified in the new studies is made in [Table 7-73](#). The metabolic pathway in the old soybean studies is similar to the new oilseed rape study and there is a good correlation between the metabolite structures in the old and new studies, considering the new studies identified conjugated metabolites prior to further extraction whereas the 1980s studies mostly identified metabolites after deconjugation. As the new studies used LC-MS/MS for identification, the exact positions of hydroxylation were uncertain given regioisomers have the same m/z ratio and typical key fragments.

Table 7-73 Comparison of metabolites identified in old and new studies

| Metabolites identified in old soybean (pulses and oilseeds) study | Correlation in new studies (unconjugated) | Correlating metabolites identified in new oilseed rape (pulses and oilseeds) study   |
|---|---|--|
| SD 211368   | M684H019                                  | Direct: M684H015<br>One of the possible isomers of M684H007 and M684H008   |
| SD 211647   | M684H018                                  | One of the possible isomers of M684H007 and M684H008   |
| SD 211648   | M684H017                                  | Direct: M684H016<br>One of the possible isomers of M684H007 and M684H008   |
| SD 207856   | M684H002                                  | Direct: M684H005 and M684H006  |
| SD 207850   | -----                                     | One of the possible isomers of M684H046 and M684H055   |
| SD 205588   | M684H004                                  | One of the possible isomers of M684H046 and M684H055   |
| SD 211733   | M684H039                                  | One of the possible isomers of M684H051  |
| SD 211731   | M684H039                                  | One of the possible isomers of M684H051  |
| SD 207855   | M684H044                                  | One of the possible isomers of M684H051  |
| SD 202193   | M684H001                                  | None (only observed in rat, livestock)<br><br>Metabolite is formed from oxidation of M684H002, but in new studies M684H002 is conjugated with sugars |

#### ***B.7.2.1.5. Plants – Peanut***

The metabolism of BAS 684 H in peanuts has been reported in three parts. The first part of this report summarised the residue information and examines the fractionation of extractable residues. Whereas, the second and third parts deals with the characterisation and identification of the individual metabolites from both a soil-grown and a hydroponic study. The study on metabolism of peanuts was conducted in 1984, was not performed under GLP and has major deviations to current OECD guidelines (see conclusions section). It was therefore decided not to rely on this data in the current submission and the study is seen as supporting information.

#### **Part 1**

Report: CA 6.2.1/9  
Woodward M., 1984e-f  
Metabolism of sd95481 in peanuts 1. Quantitation and fractionation of residues; CI

640-008

Guidelines: No

GLP: No

**Materials:**1. C-label BAS 684 H

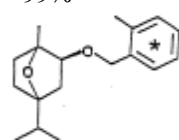
**Description:** Phenyl-U-<sup>14</sup>C (spec. activity of a.s. 57.7 MBq/mg)

**Lot/Batch #:** 8212

**Radiochemical Purity:** 98.5%

**Chemical Purity:** >99%

**Structure:**

*Method*

A metabolism study in peanut plants grown outdoors was carried out in the summer of 1984 in Modesto, California, USA. Peanut seeds were sown into barrels filled with sandy loam soil and the physical characteristics of the test soil are shown in Table 7-74. The containers used in this study were 80 gallon steel drums (18 3/4 inches in diameter by 36 inches high). The crops were treated by application to the soil and the soil was thoroughly mixed before layering it on top of the untreated soil already in the soil container. The test item was applied at total nominal application rate of approximately 1.12 kg/ha (there is no intended uses proposed on peanut). <sup>14</sup>C-BAS 684H was applied diluted with acetone.

Table 7-74 Physical Characteristics of the Test Soil<sup>a)</sup>

|                       |             |                            |            |
|-----------------------|-------------|----------------------------|------------|
| <b>Location</b>       | Modesto, CA | <b>C.E.C (Meq/ 100g)</b>   | 7.4        |
| <b>Sand</b>           | 62%         | <b>pH</b>                  | 6.4        |
| <b>Silt</b>           | 27%         | <b>Field Capacity (%)</b>  | 11.2       |
| <b>Clay</b>           | 11%         | <b>Bulk Density (g/cc)</b> | 1.25       |
| <b>Organic Matter</b> | 0.7%        | <b>Texture</b>             | Sandy Loam |

a) Soil was analysed by A & L Midwest Agricultural Laboratories, inc., 13611 Elm Street, Omaha, No 6d144

*Sampling*

The exact growth stage at which sampling occurred is not stated other than immature samples were taken 30, 60, 90, 120 and 161 days after treatment and mature samples were taken 169 days after treatment. Peanut plants were cut off about 1 inch above the soil line, brought into the laboratory and rinsed with water to remove any soil particles adhering to leaves, stems, or pods. The immature plant samples were pulverised in liquid nitrogen and subsamples were combusted and extracted. During the course of the experiment, tissue dry weights were determined by heating subsamples to 70°C for 24 hours or until a constant weight was obtained. The remainder of the sample was frozen at -20°C. At the later time points, immature and/or mature pods were removed and separated into seeds and empty pods. These were pulverised in liquid nitrogen, subsamples combusted and the remainder of the samples frozen. The storage interval between sampling and analysis is not reported.

*Description of analytical procedures*

Homogenised solid plant samples were weighed and combusted by means of an automatic sample oxidiser. Quantitation of metabolites was carried out using thin-layer chromatography followed by autoradiography and liquid scintillation counting.

Homogenisation/plant extraction: Peanut plants were frozen in liquid nitrogen and ground into a powder in a mortar and pestle. A 5g subsample of tissue was weighed and placed in a waring blender and homogenised in 75 mL of 90% acetonitrile for 2 minutes a high speed. The tissue was allowed to settle, the liquid was decanted and filtered through Whatmann No. 34 paper on a Buchner funnel. The homogenisation was carried out two more times with 75 mL 90% acetonitrile. After the final extraction, the residue was rinsed on the filter paper. The solid residue was analysed by oxygen combustion followed by liquid scintillation counting.

Preliminary Fractionation of the Plant Extract on C-13 Bond Elut Columns: The aqueous organic plant extract was concentrated on a steam table with air blowing over the sample to aid evaporation. The extract was concentrated to approximately 25 mL and 25 mL of water were added. The acetonitrile concentration in this solution was approximately 30-40%. This extract (50 mL) was passed through a 1g C-18 Bond Elut column pre-equilibrated with water. The column and reservoir were rinsed with 10 mL 10% acetonitrile. Then, the column was dried under high vacuum for 3 minutes. The elution sequence was 15 mL 5% ethyl acetate in hexane, 5 mL ethyl acetate and 5 mL methanol. Recovery of radioactivity from the column was consistently about 95% (range 92-100%). Nearly all of the radioactivity eluted under these conditions was in the flow-through fraction. The radioactivity in the flow-through represented primarily conjugated and/or other polar products.

Incubation of the Flow-Through Fraction of the C-18 Column with Beta-Glucuronidase and Cellulase: To examine the nature of the water-soluble conjugates in the flow-through fraction of the C-18 column, this fraction was concentrated and the remaining aqueous solution was adjusted to pH 5.0 with sodium acetate-acetic acid buffer (final concentration 50 mM). The volume was adjusted to 40 mL with water. Numerous enzymes were tested but only beta-glucosidase, crude beta-glucuronidase and crude cellulase showed cleavage activity. A mixture of 50 µL beta-glucuronidase and 10 mg cellulase were added. These crude enzymes also had activity on para-nitrophenol-beta-glucopyranoside, the standard substrate for beta-glucosidase. The solution was allowed to incubate at 37°C overnight. The solution was adjusted to pH 5 and centrifuged to remove the protein precipitate. The clarified extract was ready for fractionation on a second C-18 Bond Elut column.

Extraction of Dry Plant Foliage, Seeds and Empty Pods: A sample of each of the harvest dry plant foliage, seeds, an empty pod was dry-blended into a fine powder. 1g aliquots of each of the above were weighted into cellulose extraction thimbles (25 x 60 mm). Each was extracted with 100 mL of boiling solvent (hexane, acetone, methanol, or aqueous acetonitrile (9:1)). The organic extract was concentrated and quantitated by LSC. The unextractable residue was combusted and quantitated in the same manner.

Radioassay: Radioactivity was quantified by using a Packard model 2660 Liquid Scintillation Spectrometer. Counting efficiency determination was carried out by using the external standard ratio (LSR) technique. The actual quench curve was determined at various time intervals to ensure its validity. Radioactivity present in most liquids was analysed by taking two aliquots of the solution and dissolving each in 15 mL of aquasol-1 scintillation cocktail.

Radioactive areas on TLC plates after development were removed by scraping and analysed for radioactivity in an Aquasol-2: water (11:4) gel system. The percentage of a given component on the TLC plate was determined by taking the dpm's on the plate for that component and dividing by the total dpm's on the plate (i.e. the sum of all individual spots plus any other radioactivity on the plate).

All samples except TLC plate scrapings were counted for 50 minutes or until 10,000 gross counts were obtained (a 2 sigma value of 2%). TLC plate scraping were counted for 20 minutes or until 40,000 gross counts were obtained. Counting data were obtained without correction for background (i.e. gross dpm's). Backgrounds were determined for the various solvents and control plant tissues used in the study and these were subtracted from the gross dpm's in the calculated data presented in this report.

## Results and Discussion

### Whole Plant Autoradiography

Whole plant autoradiograms were made for plants harvested 30 days after planting. The autoradiograms show that BAS 684 H is taken up by the roots and translocated apoplastically to the leaves. The younger leaves contained much less radioactivity than the older leaves. The roots showed the highest level of radioactivity.

Residue Levels in Peanut Plants During the Growing Season:

Levels of  $^{14}\text{C}$ -BAS 684 H derived residues were measured throughout the growing season (Table 7-75). Levels in plants increased during the first three months and decreased at harvest. The increase of residue levels in seeds and pods from day-120 to harvest was due to the normal dehydration of the seeds and pods during maturation. A quantitative analysis of total radioactivity in the various parts of the plant at harvest is presented in Table 7-76. Approximately 93% of the total radioactivity in plants at harvest was in the stems and leaves with only about 4% in seeds and 3% in pods. Based on weights of the various plants parts, it is clearly evident that the  $^{14}\text{C}$ -residues remain primarily in the vegetative plant parts with no apparent accumulation of residues in seeds or pods.

Table 7-75  $^{14}\text{C}$ -BAS 684 H Derived Residue Levels in Peanut Plants<sup>a)</sup>

| Tissue Type                       | Day          |              |              |              |              |              |
|-----------------------------------|--------------|--------------|--------------|--------------|--------------|--------------|
|                                   | 30           | 60           | 90           | 120          | 161          | 169          |
| Green Plant Tissue (% dry matter) | 0.29<br>(18) | 0.44<br>(19) | 0.56<br>(24) | 0.34<br>(20) | 0.34<br>(29) | -<br>-       |
| Dry Plant Foliage (% dry matter)  | -            | -            | -            | -            | -            | 1.54<br>(94) |
| Seeds (% dry matter)              | -            | -            | -            | 0.03<br>(16) | 0.04<br>(40) | 0.12<br>(96) |
| Pods (% dry matter)               | -            | -            | -            | -            | 0.04<br>(22) | 0.25<br>(94) |

a) The ppm values (fresh weight basis) were obtained by combustion of subsamples (100 mg each).

Table 7-76 Comparison of the Distribution of Radioactivity at Harvest and Weights of Various Plant Parts

| Tissue Type  | Percent of Total Radioactivity | Percent of Total Harvest Weight |
|--------------|--------------------------------|---------------------------------|
| Plant Tissue | 93.0                           | 57.3                            |
| Seeds        | 4.0                            | 31.1                            |
| Pods         | 3.0                            | 11.0                            |

Extraction and Fractionation of  $^{14}\text{C}$ -BAS 684 H Derived Residues in Peanut Plants

Extracts representing 5 g fresh weight were prepared. These extracts were concentrated to ca. 30-40% acetonitrile prior to application to a 1g C-18 Bond Elut column. The column was eluted with 15 mL 5% EtOAc in hexane and then with 5 mL of ethyl acetate and 5 mL of methanol. It was demonstrated that acetonitrile concentrations of less than 20% would leave much of the radioactivity on the column. The distribution of radioactivity in the various fractions is shown in

Table 7-77. Approximately 85% of the radioactivity was extracted using this procedure. Nearly all of the radioactivity was found in the flow-through of the C-18 column. This fact indicated that the extractable residue was primarily water-soluble conjugates and other polar metabolites. After removal of all of the acetonitrile, the samples were buffered and incubated with the crude beta-glucuronidase and crude cellulase enzymes overnight at 37°C. These samples were then adjusted to pH 8 and centrifuged to remove any insoluble material. In order to characterise the metabolites recovered by the enzyme hydrolysis, the solution was then applied to a 1g C-18 Bond Elut column, followed by 10 mL water, 15 mL 5% ethyl acetate in hexane, and then (sometimes) with 5 mL 20% ethyl acetate in hexane, followed by 5 mL of ethyl acetate and 5 mL methanol. These results are presented in Table 7-78. Approximately 50-60% of the radioactivity in the sample is eluted with 5% ethyl acetate or 20% ethyl acetate. These fractions contain the metabolites released from conjugates by enzyme hydrolysis. The 3% ethyl acetate fraction contains predominantly the monohydroxy metabolites. The 20% ethyl acetate fraction contains predominantly the dihydroxy metabolites. . At harvest ca. 30% of the radioactivity is in the fraction which contains the monohydroxylated metabolites. Little radioactivity was recovered in the flow-through fraction after enzyme hydrolysis indicating the effectiveness of the enzymes in cleaving the polar conjugates.

Table 7-77 Distribution of Radioactivity in mg eq/kg in Extracts of Peanut Foliage Treated with BAS 684 H in Soil at 1.12 kg a.s./ha prior to Enzyme Treatment

|  | Day  |      |      |      |      |      |
|--|------|------|------|------|------|------|
|  | 30   | 60   | 91   | 120  | 161  | 169  |
| <b>Extractable Residue</b>                         | 0.33 | 0.40 | 0.37 | 0.34 | 0.31 | 1.18 |
| <b>Conjugated and Polar Products <sup>a)</sup></b> | 0.31 | 0.41 | 0.36 | 0.33 | 0.32 | 1.13 |
| <b>Free Metabolites <sup>b)</sup></b>              | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.01 |
| <b>Unextractable</b>                               | 0.06 | 0.12 | 0.06 | 0.05 | 0.07 | 0.21 |
| <b>% Extracted <sup>c)</sup></b>                   | 84.5 | 76.9 | 06.0 | 87.1 | 81.6 | 84.9 |

a) C-18 column flow-through and column bound material

b) C-18 column 5% ethyl acetate in hexane fraction

c) Based on dividing the extractable residue by the sum of the extractable and the unextractable residue and multiplying by 100

Table 7-78 Distribution of Radioactivity Eluted from 1-C C-18 Bond Elut Columns After Enzyme Treatment of 5g Peanut Plant Samples

|                                      | Day              |    |    |     |     |     |
|--------------------------------------|------------------|----|----|-----|-----|-----|
|                                      | 30               | 60 | 90 | 120 | 161 | 169 |
| <b>5% EtOAc/Hexane<sup>a)</sup></b>  | 48 <sup>b)</sup> | 31 | 27 | 30  | 27  | 32  |
| <b>20% EtOAc/Hexane<sup>c)</sup></b> | 42               | 61 | 60 | 28  | 33  | 25  |
| <b>Ethyl Acetate<sup>d)</sup></b>    |                  |    |    | 37  | 30  | 22  |
| <b>MeOH<sup>d)</sup></b>             | 4                | 5  | 6  |     | 6   | 13  |
| <b>Flow-Through<sup>d)</sup></b>     | 6                | 2  | 7  | 5   | 5   | 6   |

a) This fraction contains the monohydroxy metabolites of BAS 684 H

b) Percent of recovered radioactivity (i.e., C-13 flow-through in Table 4)

c) This fraction contains the dihydroxy metabolites of BAS 684 H

d) These fractions contain polar products

#### Extractability of <sup>14</sup>C-Residues From Dry Plant Foliage, Seeds, and Empty Pods

Extraction of peanut seeds by the same procedures used for plant foliage (green or dry) resulted in the recovery of <10% of the radioactivity. A series of solvents was tried including hexane, acetone, methanol, acetonitrile/water, and water. It was found that hexane and acetone yielded the highest amount extractable (32 and 31% respectively); however, these extracts represented ca. 60% of the weight of the seeds. The extractability of <sup>14</sup>C-residues in dry foliage was also examined. In both cases, acetonitrile/water (9:1) was superior to the other solvents. The amount obtained from foliage was ca. 63% and from pods ca. 52%. Dry plant foliage when extracted as in the “Methods” section gave 55% extraction of radioactivity (



Table 7-77).

## **Part 2**

|             |  |
|-------------|--|
| Report:     | CA 6.2.1/10<br>Woodward M., 1984f<br>Metabolism of sd95481 in peanuts 2. Characterisation and identification of the principal metabolites from foliage<br>CI-640-009 |
| Guidelines: | No   |
| GLP:        | No   |

Extraction of Large Samples of Peanut Foliage: A 300 g portion of dry leaves and stems and a 700 g sample of fresh leaves and stems were extracted individually with acetonitrile/water (9:1), concentrated, run through two 5g C-18 Bond Elut column and further concentrated prior to enzyme incubation. The solution was incubated with 2 mL of beta-glucuronidase and 25 mg of cellulase overnight at 37°C. (Both of these enzymes appeared to have greater activity on the radioactive metabolites than did beta-glucosidase. Numerous other enzymes were inactive. These two enzymes also appeared to have higher activity on para-nitrophenol-beta-D-glucopyranoside than did beta-glucosidase). The pH was adjusted to 3; any insoluble material was removed by centrifugation and the sample was passed through another 5g C-13 Bond Elut column. Elution was with 75 mL of 5% EtOAc in hexane followed usually with 25 mL of 20% EtOAc in hexane and then 25 mL of EtOAc in hexane and then 25 mL of EtOAc and 25 mL of methanol. Recovery of radioactivity from the columns was invariably 95-99%. It has been found that monohydroxylated metabolites were in the first cut (5% EtOAc), dihydroxy metabolites were in the second cut (20% EtOAc) and the more polar conjugated products were in the EtOAc, methanol and flow-through of the column.

Radioassay: Radioactivity was quantified by using a Packard model 2660 Liquid Scintillation Spectrometer. Counting efficiency determination was carried out by using the external standard ratio (LSR) technique. The actual quench curve was determined at various time intervals to ensure its validity. Radioactivity present in most liquids was analysed by taking two aliquots of the solution and dissolving each in 15 mL of Aquasol-2 scintillation cocktail.

Radioactive areas on TLC plates after development were removed by scraping and analysed for radioactivity in an Aquasol-2: water (11:4) gel system. The percentage of a given component on the TLC plate was determined by taking the dpm's on the plate for that component and dividing by the total dpm's on the plate (i.e. the sum of all individual spots plus any other radioactivity on the plate).

Plant tissue samples (pulverised in liquid nitrogen) and unextractable plant residue were analysed by weighing subsamples into a Combusto-Cone sample holder and followed by combustion in a Packard Mofel 306 TriCarb sample oxidiser. The counting cocktail included Carbo-Sorb I and Permaflour V in a 10:12 ratio.

All samples except TLC plate scrapings were counted for 50 minutes or until 10,000 gross counts were obtained (a 2 sigma value of 2%). TLC plate scraping were counted for 20 minutes or until 40,000 gross counts were obtained. Counting data were obtained without correction for background (i.e. gross dpm's). Backgrounds were determined for the various solvents and control plant tissues used in the study and these were subtracted from the gross dpm's in the calculated data presented in this report.

### Thin-Layer Chromatography (TLC)

Plates used for TLC were silica gel F-254, 20 cm x 20 cm, 0.25 mm thickness. Four solvent systems were selected for TLC:

- 1) toluene: ethyl acetate, 17:3 (v/v)
- 2) hexane: methylene chloride: diethyl ether, 6:3:1 (v/v/v)
- 3) heptane: isopropanol: acetic acid (150: 20: 1.5)
- 4) hexane: isopropanol: acetic acid (160: 40: 2)

Developing tanks contained ca. 200 mL of the appropriate solvent system mixture. All plates were spotted 3 cm from the bottom edge of the plate. Plates were scribed 3 cm from the top making a total migration distance of 14 cm. Plant extracts were generally chromatographed in systems 1 and 3 or systems 4 and 5. Autoradiography was performed on kodak SB-5 single-coated, blue-sensitive X-ray film.



**Reference Standards:** Synthetic standards of numerous known and/or suspected degradation products of BAS 684 H have been prepared. The structures of these compounds are shown in Table 7-79. The  $R_f$  values of these compounds in several solvent systems are shown in Table 7-80.  $R_f$ 's were found to vary with amount of sample spotted and also varied with the age of the solvent system. The  $R_f$ 's reported are intended to provide a guide to the reader for the relative position of these compounds in these solvent systems. Reference standards were visualised under UV light.

**Radiolabelled Reference Compounds:** Some of the degradation products of BAS 684 H have been isolated from other studies and identified by spectroscopic and co-chromatographic techniques. These include BAS 684 H, M684H014 and M684H004 (205588), all isolated from the soil metabolism study (RIR-22-005-83 (part II)).

**Gas Chromatography/ Mass Spectroscopy (GC/MS):** All mass spectra were obtained on either the Finnigan 1020 or 4500. The GC column used was a fused-silica, wall-coated SE-54 column (20-30 m x 0.25 mm). Temperature programs varied and are indicated on the chromatogram. The carrier gas was helium at a flow rate of 1 mL/min. The split at the inlet was variable and ranged from 20 to 1 to 60 to 1. The 1020 mass spectrometer operating parameters were: (a) ionisation mode – electron impact, (b) ionisation voltage – 70 eV, (c) emission current – 0.85 mA, (d) sensitivity –  $10^{-7}$  A/V, (e) electron multiplier voltage – 2000 V. Spectra were obtained at a rate of 1 per second. Sample volumes injected ranged from 1 to 5  $\mu$ L. Dihydroxylated metabolites were analysed underivatized and as trimethylsilyl derivatives.

Direct comparisons between synthetic standards and isolated metabolites were made using GC-MS-SIM (selected ion monitoring) with a mass selective detector. Four major ions were examined from each metabolite. The gas chromatographic column was fused-silica, wall coated SE-54 column (50 m x 0.25 mm). The flow rate was 1 mL helium/min and the oven temperature was maintained at 220°C and the injector was 250°C. The split was approximately 10:1. The ionisation voltage was 70 eV and the electron multiplier voltage was 2200 eV.

Table 7-79 Structures of Reference Compounds

| Metabolite           | Structure | Chemical Name   |
|----------------------|-----------|---|
| BAS 684 H (SD 95481) |           | 7-oxabicyclo[2.2.1]heptane, 1-methyl-4-(1-methylethyl)-2-[(2-methylphenyl)methoxy]-exo-         |
| M684H014 (SD 204328) |           | Benzoic acid, 2-methyl-,1-methyl-4-(1-methylethyl)-7-oxabicyclo[2.2.1]hept-2-yl-ester,exo-      |
| M684H004 (SD 205588) |           | 7-oxabicyclo[2.2.1]heptane-1-methanol,alpha,alpha,4-trimethyl-3-[2-methyl-phenyl)methoxy]-,exo- |
| M684H024 (SD 207430) |           | 7-oxabicyclo[2.2.1]heptane-1-ethanol,beta,4-dimethyl-3-((2-methylphenyl)methoxy)-exo-           |

|                      |  |   |
|----------------------|--|---|
| SD 207322            |  | 7-oxabicyclo[2.2.1]heptane-1-methanol,4-((1-methylethyl)-2-((2-methylphenyl)methoxy)-,exo-            |
| SD 207850            |  | 7-oxabicyclo[2.2.1]heptan-2-ol, 4-methyl-1-(1-methylethyl)-5-((2-methylphenyl)-methoxy)-, exo, exo-   |
| M684H002 (SD 207856) |  | Benzenemethanol, 2-((1-methyl-4-(1-methyl-ethyl)-7-oxabicyclo[2.2.1]-hept-2-yloxy)-methyl)-.exo-      |
| M684H019 (SD 211368) |  | Phenol, 3-methyl-4-((1-methyl-4-(1-methyl-ethyl)-7-oxabicyclo[2.2.1]hept-2-yloxy)-methyl)-            |
| SD 211646            |  | 7-oxabicyclo[2.2.1]heptan-2-ol.4-methyl-1-(1-methylethyl)-5-((2-methylphenyl)-methoxy)-,2-endo,5-exo- |
| M684H018 (SD 211647) |  | Phenol, 4-methyl-3-((1-methyl-4-(1-methyl-ethyl)-7-oxabicyclo[2.2.1]hept-2-yloxy)-methyl)-, exo-      |

Table 7-80 R<sub>f</sub> Values of BAS 684 H and Related Compounds on Silica Gel F-254 Plates <sup>a)</sup>

| Metabolite           | TLC Solvent System |      |      |      |      |
|----------------------|--------------------|------|------|------|------|
|                      | 1                  | 2    | 3    | 4    | 5    |
| BAS 684 H (SD 95481) | 0.60               | 0.57 | 0.68 | 0.82 | 0.86 |
| M684H014 (SD 204328) | 0.71               | 0.65 | 0.64 | 0.86 | 0.89 |
| M684H004 (SD 205588) | 0.14               | 0.10 | 0.38 | 0.49 | 0.76 |
| M684H024 (SD 207430) | 0.10               | 0.06 | 0.30 | 0.44 | 0.69 |
| SD 207522            | 0.14               | 0.09 | 0.34 | 0.46 | 0.72 |
| SD 207850            | 0.09               | 0.06 | 0.31 | 0.44 | 0.71 |
| M684H002 (SD 207856) | 0.14               | 0.08 | 0.29 | 0.46 | 0.72 |
| M684H019 (SD 211368) | 0.23               | 0.13 | 0.30 | 0.48 | 0.75 |

|                         |      |      |      |      |      |
|-------------------------|------|------|------|------|------|
| SD 211646               | 0.20 | 0.13 | 0.34 | 0.46 | 0.75 |
| M684H018<br>(SD 211647) | 0.24 | 0.12 | 0.34 | 0.50 | 0.80 |

a) The values reported above represented ca. 10 µg spotted and solvent systems were freshly prepared and tanks were not pre-equilibrated

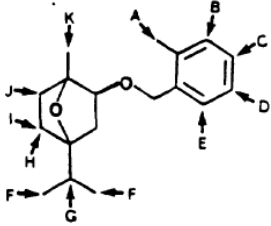
#### Distribution and Quantitation of Metabolites in Mature Green and Dry Plant Foliage

In order to obtain sufficient quantities of metabolites to carry out quantitation and characterisation of individual metabolites, large samples of peanut stems, petioles, and foliage were processed using procedures similar to those used for small samples reported in part 1 of this report. The aqueous organic extractable materials (ca. 80-90% of total radioactivity) were concentrated to ca. 35% acetonitrile and partially purified by C-18 column chromatography. The flow-through of this column contained essentially all of the radioactivity in the sample (>98%). After removal of the organic solvents and enzyme treatment, the aqueous samples was adsorbed on a second C-18 column (5g). Elution was with 5% ethyl acetate in hexane, 20% ethyl acetate in hexane, ethyl acetate and methanol. The 5% ethyl acetate in hexane fraction was shown to contain primarily monohydroxy metabolites, the 20% ethyl acetate fraction contained primarily dihydroxy metabolites, and the ethyl acetate, methanol, and flow-through contain beta-glucoside conjugates (not cleaved by the enzymes) and other polar metabolites.

Extracts were prepared from mature plants harvested fresh and dry. These data are shown in Table 7-81. Metabolites freed by enzyme treatment ranged from 51-58% which compares favourably to the results obtained for the small samples reported in Part 1 of this report.

Thin-layer chromatography was carried out on the 5% ethyl acetate and 20% ethyl acetate fractions. The plates were subjected to autoradiography. Quantitation of the various mono- and dihydroxy metabolites is shown in Table 7-81. The major monohydroxy metabolites include M684H002 (SD 207856), M684H017 (SD 211648), M684H004 (SD 205588) and SD 207850.

Table 7-81 Distribution of Radioactivity Eluted From 5g C-18 Bond Elut Columns from 300g and 700g Plant Samples after Enzyme Treatment

|   |  <p>positions :</p> |                    |  |  |
|---|--|--------------------|--|--|
| Fraction                                      | Metabolite   | Spot <sup>a)</sup> | Extractable Radioactivity (%)            |  |
|   |  |                    | 161 days after application <sup>b)</sup> | 169 days after application <sup>b)</sup> |
| 5% EtOAc/ Hexane<br>(monohydroxy metabolites) | % extractable radioactivity  |                    | 22.0                                     | 25.5                                     |
|   | M684H004<br>(SD 205588)  | G                  | 2.9                                      | 0.7                                      |
|   | M684H017<br>(SD 211648)  | B <sup>c)</sup>    | 2.3                                      | 2.4                                      |
|   | M684H018<br>(SD 211647)  | D                  | 1.3                                      | 1.3                                      |
|   | M684H019<br>(SD 211368)  | C                  | 0.5                                      | 0.4                                      |
|   | SD 211892  | A <sup>d)</sup>    | 0.7                                      | 0.6                                      |
|   | M684H002<br>(SD 207856)  | A                  | 5.0                                      | 5.5                                      |
|   | SD 207850  | I                  | 1.8                                      | 2.7                                      |
|   | M684H024<br>(SD 207430)  | F                  | 1.0                                      | 1.0                                      |

|   |                             |                     |      |      |
|---|-----------------------------|---------------------|------|------|
|   |                             | Other <sup>c)</sup> | 6.5  | 10.9 |
| 20% EtOAc/ Hexane<br>(dihydroxy<br>metabolites) | % extractable radioactivity |                     | 29.1 | 32.7 |
|   | SD 213323                   | B/G <sup>c,f)</sup> | 7.2  | 7.3  |
|   | M684H044<br>(SD 207855)     | A/G <sup>c,g)</sup> | 12.5 | 12.3 |
|   | SD 211731                   | A/I <sup>c)</sup>   | 2.5  | 4.1  |
|   |                             | Other <sup>f)</sup> | 6.3  | 9.0  |
| EtOAc <sup>g)</sup>                             |                             |                     | 17.0 | 23.4 |
| MeOH <sup>g)</sup>                              |                             |                     | 11.6 | 12.1 |
| Flow-Through <sup>g)</sup>                      |                             |                     | 20.3 | 6.3  |

- a) Letters correspond to position of hydroxylation  
b) Total extractable radioactivity equalled 0.2 mg eq/kg and 0.94 mg eq/kg at Day 161 and Day 169 respectively  
c) Proposed structure  
d) Appears to be a monohydroxy metabolite with a double bond on the cineole side  
e) Mixture of at least two components  
f) Remaining radioactivity  
g) These fractions contain polar products

#### Preparative TLC of the 5% Ethyl Acetate in Hexane Fraction (Monohydroxy Metabolites)

The radiolabelled components in the 5% ethyl acetate in hexane fraction were separated by preparative TLC using solvent system 1. The autoradiogram of the thin-layer plate is shown in figure 2. After removing these components from the silica, the samples were subjected to preparative TLC in solvent system 3. Eight components (A, A', B, C, D, F, G and I) were separated and six were compared with standards by TLC, GC and/or selected ion monitoring (SIM). (Component A' appears to be a monohydroxy metabolite containing a double bond on the cineole side of the molecule; however, no standard was available. Component B is a phenolic metabolite; however, the hydroxylation position on the ring has yet to be confirmed). The data supporting the identification is summarised in Table 7-82.

Table 7-82 Data Supporting the Identification of Six Monohydroxy Metabolites of BAS 684 H obtained from peanut Foliage After Enzyme Hydrolysis

| Compound                | Spot            | Chromatography System |   | GC Retention Time  |          | Ions Monitored     |
|-------------------------|-----------------|-----------------------|---|--------------------|----------|--------------------|
|                         |                 | 1                     | 3 | Standard           | Isolated |                    |
| M684H002<br>(SD 207856) | A               | X                     | X | 7.76               | 7.75     | 91, 93, 123, 171   |
| M684H019<br>(SD 211368) | C               | X                     | X | 9.07               | 9.11     | 121, 91, 123, 107  |
| M684H018<br>(SD 211647) | D               | X                     | X | 8.91               | 8.90     | 121, 123, 107, 290 |
| M684H024<br>(SD 207430) | F               |                       |   | 8.50 <sup>a)</sup> | 8.49     | 105, 91, 109, 167  |
| M684H004<br>(SD 205588) | G               | X                     | X | 5.70               | 5.72     | 105, 107, 121, 122 |
| SD 207850               | I <sup>b)</sup> | X                     | X | 6.55               | 6.53     | 105, 169, 185, 232 |

- a) Later Eluting Diastereomer  
b) Mixture shown to contain M684H002 (SD 207856) as major radiolabelled impurity

#### Preparative TLC of the 20% Ethyl Acetate in Hexane Fraction (Dihydroxy Metabolites)

The radiolabelled components in the 20% ethyl acetate in hexane fraction were separated by preparative TLC using solvent system 4. Three bands were removed and subjected to preparative TLC in solvent system 5. These components were further purified in solvent system 7 and then in solvent system 6. The major metabolites in this group were analysed by GC-MS on a Hewlett-Packard Model 3992B GC-MS system. Each of the four major components was shown to be a dihydroxy metabolite of BAS 684 H by examination of the electron-impact mass spectrum of the trimethylsilyl derivative. Each component was shown to possess a single

hydroxyl group on the benzyl side of the molecule and another on the cineole side. The proposed locations of the hydroxyl groups were based on mass spectral evidence as well as GC and TLC information. Reference standards of dihydroxy compounds were not available for comparison purposes; however, the monohydroxy analogues proved very useful in the analysis of the data.

The GC and TLC data are summarised in

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Table 7-83. The TLC pattern in solvent system 6 shows a similar pattern to the corresponding monohydroxy metabolites in a similar solvent system. The GC retention times for the three underivatised components A/G, B/G, and D/G were also in a logical sequence based on the retention times of the corresponding monohydroxy metabolites A, B and D show in Table 7-82. Components A/G and A/I also elute in the expected sequence based on the corresponding monohydroxy metabolites G and I shown in Table 7-82.

Table 7-83 TLC and GC Data on the Dihydroxy Metabolites

| Band              | SD Number            | TLC R <sub>f</sub> System 6 | GC Retention Time (min) |                     |
|-------------------|----------------------|-----------------------------|-------------------------|---------------------|
|                   |                      |                             | Underivatised           | TMS                 |
| B/G <sup>a)</sup> | 213323               | 0.44                        | 15.3 <sup>b)</sup>      | 13.26 <sup>c)</sup> |
| D/G               | 211733               | 0.37                        | 16.1                    | 13.13               |
| A/G               | M684H044<br>(207855) | 0.18                        | 12.7                    | 12.97               |
| A/I               | 211731               | 0.13                        | 15.8                    | 13.33               |

a) Indicates the position of hydroxylation (see Table 3)

b) DB-5 column 30 m x 0.32 mm at 180°C for a flow rate of 2 mL/min

c) Permabond methyl silicon 1.90 x 2 mm with a starting temperature of 50°C for 2 minutes and a temperature program of 15°C/min to 320°C. The carrier gas was He at a flow rate of ca. 30 mL/min

The substitution patterns of metabolites B/G and D/G are proposed based on the diagnostic ions in the mass spectra (Table 7-84) and the TLC and GC data of the underivatised compounds (

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Table 7-83). The diagnostic ions included  $m/z$  131 (hydroxyl substitution at C) and  $m/z$  195 with the absence of major ions at  $m/z$  192 and 119. These ions confirm the dihydroxy nature of the metabolites and indicated substitution on the aromatic ring. The TLC  $R_f$  data in solvent system 6 indicated that the higher  $R_f$  component was substituted at B and the lower  $R_f$  component was substituted at C or D. Position D (SD 211733) is proposed based on the mobility of this component in TLC system 7 (identical to B/G), which is identical to the corresponding monohydroxy compounds pictured in system 3.

The major dihydroxy metabolites appears to be substituted at A/G based on the mass spectral properties of the TMS derivative (Table 7-84). The metabolite shows the diagnostic ions at  $m/z$  131 (hydroxyl substitution at G) and  $m/z$  193, 192 and 119 (hydroxyl substitution at A). The TLC and GC data in



Table 7-83 support the proposed structure as M684H044 (SD 207855).

Table 7-84 Relative Intensities of Diagnostic Ions in the Mass Spectra of the Trimethylsilyl Derivatives of the Dihydroxy Plant Metabolites and the Monohydroxy Reference Compounds.

| Metabolite           | Substitution <sup>a)</sup> | Ion            |                     |                  |     |     |     |     |     |     |    |
|----------------------|----------------------------|----------------|---------------------|------------------|-----|-----|-----|-----|-----|-----|----|
|                      |                            | M <sup>+</sup> | [M-90] <sup>+</sup> | 257              | 241 | 193 | 192 | 167 | 131 | 119 | 71 |
| M684H002 (SD 207856) | A                          | 0.2            | 3                   | ND <sup>b)</sup> | 0.2 | 25  | 48  | 0.7 | 1   | 26  | 22 |
| M684H019 (SD 211368) | C                          | 2              | ND                  | ND               | ND  | 100 | ND  | 0.2 | 0.3 | 1   | 9  |
| M684H018 (SD 211647) | D                          | 10             | ND                  | ND               | 0.2 | 41  | 9   | 0.2 | 0.2 | 3   | 18 |
| M684H024 (SD 207430) | F                          | 3              | 0.4                 | 7                | 2   | 2   | 0.2 | 32  | 5   | 5   | 2  |
| M684H004 (SD 205588) | G                          | ND             | 1                   | 0.7              | 0.2 | 0.6 | ND  | 2   | 18  | 1   | 2  |
| SD 207850            | I                          | 2              | 0.1                 | 5                | 0.3 | ND  | ND  | 3   | 0.5 | 0.8 | 13 |
| M684H044 (SD 207855) | A/G                        | ND             | 2                   | 2                | 5   | 91  | 31  | 6   | 29  | 27  | 3  |
| SD 213323            | B/G                        | 2              | 8                   | 2                | 0.2 | 100 | ND  | 10  | 25  | 5   | 2  |
| SD 211733            | D/G                        | 3              | 6                   | 3                | 0.1 | 100 | 7   | 10  | 24  | 3   | 3  |
| SD 211731            | A/I                        | 1              | 2                   | 18               | 12  | 100 | 22  | 7   | 0.7 | 27  | 26 |

a) Positions of substitution are explained in Table 3

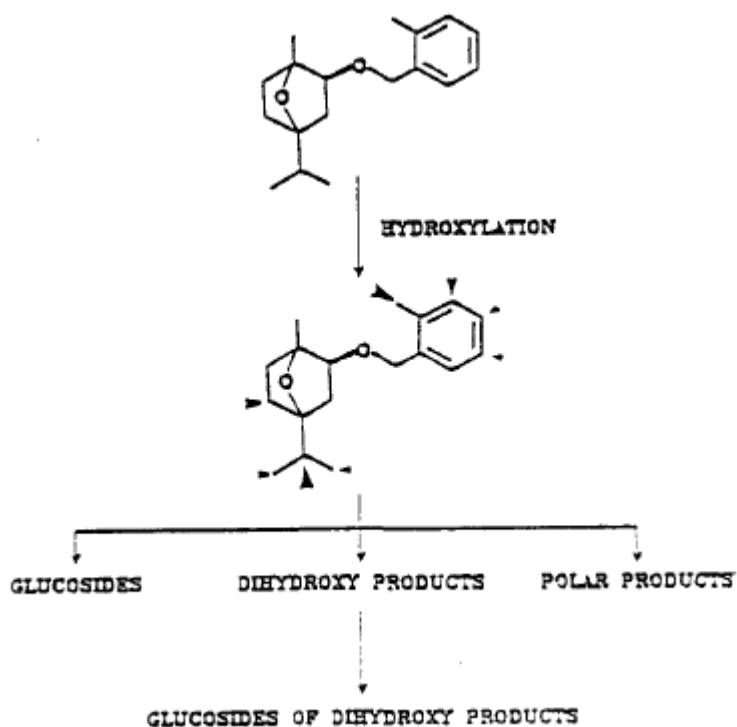
b) Not detected

The dihydroxy metabolite designated A/I has diagnostic ions at m/z 193, 192 and 119 indicating substitution at A and an additional diagnostic ion at m/z 71. Only compounds with an unsubstituted isopropyl group have an abundant ion at m/z 71. Also, characteristic are the relative intensities of the ions at m/z 257, 241, 169 and 167. The relative intensities of these ions are also consistent with hydroxylation at I rather than at F giving SD 211731.

#### Proposed Metabolic Fate of BAS 684 G in Peanut Plants

BAS 684 H is rapidly metabolised in peanut plants to a wide variety of monohydroxylated products. These products were not found as free metabolites but rather as beta-1,4-glucosides and other types of conjugates. The monohydroxylated products and/or their glycoside conjugates may undergo a subsequent hydroxylations leading to a variety of dihydroxylation products. No evidence was obtained indicating that the benzylic either is oxidised (to the ester) or cleaved. The general pathway of metabolism of BAS 684 H in peanuts is shown below (Figure 7-10) with the size of the arrows indicating the relative importance of the various sites for hydroxylation.

Figure 7-10 Metabolism of BAS 684 H in Peanut Plants



### Part 3

Report: CA 6.2.1/11  
 Woodward M., 1984d  
 Metabolism of sd95481 in peanuts: 3. characterisation  
 and identification of the principal metabolites in a  
 pilot study  
 CI-640-016

Guidelines: No

GLP: No

*Materials:* As per Part 1 (CA 6.2.1/9) above.

*Methods:* The methods section is as per Part 2 above (CA 6.2.1/10) with the following amendments. Peanut seeds (cultivar NC-7) were grown in sand and after 14 – 20 days the plants (at BBCH 13) were transferred to laboratory hydroponic apparatus in Hoagland's solution. After a 2-3 day equilibration period, the Hoagland's solution was removed and replaced with fresh Hoagland's solution containing ca. 10 ppm  $^{14}\text{C}$ -SD 95481 (ca. 25 microCuries/L). It is unclear at what growth stage the samples were taken other than plants being removed after 5 days.

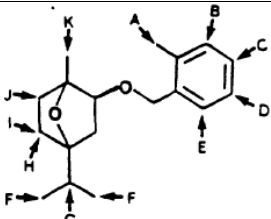
The sampled peanut shoots were frozen at  $-10\text{ }^{\circ}\text{C}$  until extraction. The storage interval between sampling and analysis is not reported. Extraction was performed as per Part 2 above (CA 6.2.1/10) except the column was eluted with 75 mL 5 % ethyl acetate in hexane, 25 mL 20 % ethyl acetate in hexane, 25 mL ethyl acetate and 25 mL. the incubation of the flow-through was performed with 200  $\mu\text{L}$  beta-glucuronidase and 25 mg cellulase. After incubation, the extract was adjusted to pH 3 before fractionation with C-18 columns. The dihydroxylated metabolites were analysed underivatized and as trimethylsilyl derivatives. Identification was performed as stated in Part 1 (CA 6.2.1/9) above.

### Results and Discussion

### Analysis of the Fractions Obtained From the C-18 Column after Enzyme Treatment

Fractions obtained from the enzyme hydrolysis of conjugates of peanut metabolite were subject to two-dimensional TLC. The organic and aqueous extractable radioactivity of the laboratory-grown peanut plants represented 96.8% of the total radioactivity in the plants. A distribution of the radioactivity as the individual metabolites is shown in Table 7-85. The monohydroxy metabolites (free and enzyme hydrolysed) represented 34.8% of the extractable radioactivity in the laboratory-grown peanuts. The parent accounted for 9.2% of the radioactivity. The dihydroxy metabolites accounted for 10% of the extractable radioactivity. The remaining 39.3% of the radioactivity was polar products which were not cleaved by the enzymes. This polar fraction was further retreated with the same enzymes and essentially the entire radioactivity was found as mono- and dihydroxy metabolites. The applicant concluded that the first hydrolysis was incomplete.

Table 7-85 Percent of Extractable Radioactivity was found as monohydroxy metabolites of BAS 684 H after one enzyme hydrolysis

|                             |  <p>Substitution positions :</p> |                                |   |
|-----------------------------|---|--------------------------------|---|
| Metabolite                  | Position of Hydroxyl Group  | % of Extractable Radioactivity | How identification/ characterisation was achieved   |
| SD 211892                   | A <sup>a)</sup>   | 1.0                            | How identification was achieved is not mentioned.   |
| M684H002 (SD 207856)        | A   | 15.9                           | GLC retention time and EI MS consistent with reference standard   |
| M684H017 (SD 211648)        | B <sup>b)</sup>   | 4.0                            | GLC retention time similar to the reference compound M684H018 (SD 211647) and the general tendency of plants to hydroxylate aromatic rings in positions 3, 4 and 5; furthermore, no reference standard was available. |
| M684H019 (SD 211368)        | C   | 0.9                            | GLC retention time and EI MS consistent with reference standard   |
| M684H018 (SD 211647)        | D   | 2.9                            | GLC retention time and EI MS consistent with reference standard   |
| M684H024 (SD 207430)+211732 | F+J   | 3.0                            | GLC retention time and EI MS consistent with reference standard   |
| M684H004 (SD 205588)        | G   | 2.1                            | GLC retention time and EI MS consistent with reference standard   |
| SD 207850                   | I   | 5.0                            | GLC retention time and EI MS consistent with reference standard   |
|                             | Total   | 34.8                           |   |

### Separation of the Components in Monohydroxy Metabolites of BAS 684 H after enzyme hydrolysis (5% Ethyl Acetate Fraction)

The radiolabelled components present in this fraction were separated by preparative TLC in solvent system 1. The bands were removed and the components eluted with ethyl acetate. Each of these components was further purified by preparative TLC in solvent system 3.

### Identification of Bands G and A as M684H004 (SD 205588) and M684H002 (SD 207856)

The minor component (band G) on the TLC behaved like M684H004 (SD 205588) and the major component (band A) like M684H002. These notions were tested by TLC co-chromatography with labelled standards. The components of interest co-migrated with labelled standards. The components were further characterised by GC-MS; the mass spectra are compared and the minor component spectra and the standard M684H004 (SD 205588) spectra are identical. Based on TLC and GC-MS properties band G is confirmed as M684H004 (SD 205588).

Band A was compared with a M684H002 standard by GC-MS. The retention times and mass spectra are identical; therefore, based on TLC and GC-MS properties, the radiolabelled component of Band A is confirmed as M684H002.

#### Identification of Bands D and C as M684H018 (SD 211647) and M684H019 (SD 211368)

The two purified radiolabelled components (bands D and C) were compared to reference standards of M684H019 (SD 211368) and M684H018 (SD 211647) by TLC. Band D co-chromatographed with M684H018 (SD 211647) and band C co-chromatographed with M684H019 (SD 211368). These components were examined by GC-MS; the mass spectra are compared and the retention times and mass spectra are identical between band D and the standard M684H019 (SD 211368) and between band C and M684H019 (SD 211368). Band C is therefore identified as M684H019 (SD 211368) based on the co-chromatography on TLC and the identical mass spectra of the isolated material and the M684H019 (SD 211368) standard. Band D is therefore identified as M684H019 (SD 211368) based on the chromatography on TLC and the identical mass spectra of the isolated material and the M684H019 (SD 211368) standard.

#### Proposed Structure of M684H017 (SD 211648)

The radiolabelled component noted as band C on the TLC was compared to the corresponding component from the soybean plant metabolism study (B.7.2.1.4 part 1 and part 2). Component B was also examined by GC-MS, where the mass spectrum of this component is nearly identical to the mass spectrum of the reference standard for M684H018 (SD 211647). The only obvious differences are the relative intensities of the  $[M-15]^+$  and  $[M-18]^+$  ions in the two spectra. The applicant proposed this metabolite as M684H017 (SD 211648) due to the similarity to the reference compound M684H018 (SD 211647) and the general tendency of plants to hydroxylate aromatic rings in positions 3, 4 and 5; furthermore, no reference standard was available.

#### Identification of Bands I, J and F

The major component was examined by capillary GC; the major component co-chromatography with synthetic standard of SD 207850 on two columns. A minor component appeared to elute at about the same retention time as one of the diastereomers of M684H024 (SD 207430). This sample was also analysed by TLC. The total ion current chromatogram of this sample was compared to the chromatogram of the SD 207850 reference standard. mass spectra of SD 207850 reference standard; the mass spectra are identical. Based on this evidence, the major component is confirmed as SD 207850 and the minor component identified as M684H024 (SD 207430).

The peaks labelled I and F1 were identified as SD 207850 and M684H024 (SD 207430), respectively by co-chromatography identification using GC-MS where there was a positive retention time match of metabolites with reference standards. Peak F2 was identified as the diastereomer M684H024 (SD 207430) based on its retention time and mass spectrum match to the reference standard of M684H024 (SD 207430). For the peak labelled J the mass spectrum indicated that the aromatic portion of the molecule is unchanged and that a hydroxyl group is on the cineole portion of the molecule. The NMR spectrum of this component indicated that the hydroxyl group was on the C-6 of the cineole ring. The structure of this minor metabolite was proposed as SD 211732 and no standard was available to confirm this structure of this minor metabolite.

**HSE Comment on CA 6.2.1/9-11:** The studies were published in 1984 and were not performed to the OECD Guideline 501 nor to GLP. Only a single ring was labelled (Phenyl- $U-^{14}C$ ) and was applied to peanut seeds at total nominal application rate of approximately 1.12 kg/ha (there are no intended uses proposed on peanut). Samples were stored frozen prior to analysis but the length of storage is not clear and no additional data to address the stability was provided in the study.

The amount of radioactivity extracted was not presented in mg eq/kg but as a percentage of total radioactivity extracted (>97%) from the plant with organic solvents. The vast majority of radioactivity was present as polar conjugates. Following treatment with enzymes (beta-glucuronidase and cellulase) 34.8% of the total radioactivity was no longer conjugated. The results showed that no parent was detected, and the major metabolite extracted at 15.9% was M684H002.

The identification on the basis of co-chromatography is sufficient as at least two dissimilar analytical systems were used (TLC using 5 different solvent systems and co-chromatography with acceptably derived reference standards and GC-MS was also used). This resulted in 9 metabolites being sufficiently identified: SD 211892, M684H002, M684H017 (SD 211648), M684H019 (SD 211368), M684H018 (SD 211647), M684H024 (SD 207430), SD211732, M684H004 (SD 205588) and SD 207850. The remaining radioactivity was characterised

as dihydroxy metabolites of BAS 684 H. The results were similar for the soil-grown and hydroponic study; the main metabolites identified being mono- and di-hydroxy derivatives of parent BAS 684 H.

Owing to the issues raised the peanut studies cannot be quantitatively relied upon but can be considered as supporting information given they provide some qualitative information on metabolites which have been identified in a primary crop.

A comparison of the metabolites identified in the peanut study with the metabolites identified in the new studies is made in Table 7-86. The metabolic pathway in the 1980s studies is similar to the new oilseed rape study and there is a good correlation between the metabolite structures in the old and new studies, considering the new studies identified conjugated metabolites prior to further extraction whereas the 1980s studies mostly identified metabolites after deconjugation. As the new studies used LC-MS/MS for identification, the exact positions of hydroxylation were uncertain given regioisomers have the same m/z ratio and typical key fragments.

Table 7-86 Comparison of metabolites identified in old and new studies

| Metabolites identified in old peanut (pulses and oilseeds) study | Correlation in new studies (unconjugated) | Correlating metabolites identified in new oilseed rape (pulses and oilseeds) study |
|--|---|--|
| SD 211368  | M684H019                                  | Direct: M684H015<br>One of the possible isomers of M684H007 and M684H008           |
| SD 211647  | M684H018                                  | One of the possible isomers of M684H007 and M684H008                               |
| SD 211648  | M684H017                                  | Direct: M684H016<br>One of the possible isomers of M684H007 and M684H008           |
| SD 207856  | M684H002                                  | Direct: M684H005 and M684H006  |
| SD 207850  | -----                                     | One of the possible isomers of M684H046 and M684H055                               |
| SD 205588  | M684H004                                  | One of the possible isomers of M684H046 and M684H055                               |
| SD 207430  | M684H024                                  | One of the possible isomers of M684H046 and M684H055                               |
| SD 211731  | M684H039                                  | One of the possible isomers of M684H051  |
| SD 207855  | M684H044                                  | One of the possible isomers of M684H051  |
| SD 213323  | M684H039                                  | One of the possible isomers of M684H051  |

#### ***B.7.2.1.6. Plants – Rice***

The metabolism of BAS 684 H in rice has been investigated in two parallel reports: outdoor-grown and indoor-grown rice. The reports are from 1988 and 1989 and has major deviations to current OECD guidelines (see conclusions section). Therefore the study is seen as supporting information.

Report: CA 6.2.1/12  
Edwards, V.T., 1988a  
Metabolism of 14C w195481 in rice outdoors  
CI-640-011  
Guidelines: EPA 171-4  
GLP: Yes

#### **Materials:**

##### 1. C-label BAS 684 H

**Description:**

Phenyl-U- $^{14}\text{C}$  and  $^{13}\text{C}$  in the bridge methylene  
(spec. activity of a.s. 32.4  $\mu\text{Ci/mg}$ )

**Lot/Batch #:**

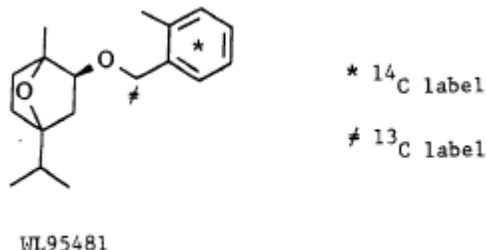
S1006/4

**Radiochemical Purity:**

98%

**Chemical Purity:**

99%

**Structure:****Method**

A metabolism study in rice plants (*onda*) grown outdoors was carried out in 1988 at Shell Chimie, Berre L'étang, near Marseilles, France. Transplanted rice was grown under paddy conditions in tanks housed in enclosure for work with radio-chemicals. They were transplanted at the 3-4 leaf stage and treated 11 days later with  $^{14}\text{C}$ -ring BAS 648 H formulated as an emulsifiable concentrate and at a dose rate equivalent to 200 g a.s./ha.

**Preparation of the treatment solution:** The radiolabelled test compound as formulated as an emulsifiable concentrate by dissolving 25 mg radioactive BAS 684 H in 224  $\mu\text{L}$  of formulation based on SF06767 but without active ingredient. On the treatment day 220  $\mu\text{L}$  of the formulation concentrate was added to 23 mL of tap water and thoroughly mixed.

**Treatment:** The rice was sprayed with the treatment solution from a few centimetres above the top of the rice seedlings to give an even cover to the surface of the paddy. Each tank (area 0.25  $\text{m}^2$ ) was sprayed with 5 mL of the treatment solution (concentration 1 mg/mL) giving a dose rate equivalent to 200 g a.s./ha and a spray volume of 200 L/ha.

**Plant growth conditions:** The experiment was carried out with rice plants growing under paddy conditions in tanks (50 x 50 x 50 cm) housed in a radiochemical enclosure at Shell Chimie, France. The enclosure was open to the weather except that it was covered by a wire mesh and was in a small pit so that the plants were not sitting above normal ground level.

Rice seedlings sown were transplanted into the tanks containing soil (33 cm) and water (4 cm), when the rice was at 3-4 leaf stage. They were taken from a paddy field in Arles and were transplanted at 240-260 pairs of plants per  $\text{m}^2$  on the same day.

**Sampling**

**Water:** Samples of paddy water were taken for analysis immediately after spraying (approximately 30 minutes), 4 hours, 1, 2, 4, 8, 12 and 14 weeks after treatment. The first samples was a composite sample (16 mL) made of 2 mL sub-samples taken from two places in each treated tank. Thereafter, composite samples (10 mL) were prepared for each tank separately by taking samples from about 9 different places in the tank. The samples were stored in frozen and returned to SRC for analysis by liquid scintillation counting (LSC).

**Plants:** Three plants from each treated tank (i.e. 12 in all) were sampled for analysis 4 hours after treatment. They were deep frozen and sent to SRC for analysis by combustion. At crop maturity the ears were removed from the stalks and the total weight of ears from each tank was recorded. The straw was cut off above soil level and the weights harvested recorded. The samples were sent deep frozen to SRC.

**Soil:** Core samples of soil (0.5 cm and 5-10 cm) were removed from the tanks after the plants had been harvested. The weights were recorded and the samples were sent deep frozen to SRC for analysis.

**Sample processing**

Rice leaves after treatment: The leaves sampled on the treatment day were thawed, chopped finely with scissors, air-dried and divided into three portions for combustion.

Grain: The grain was removed from the ears by hand and the weight from each tank was recorded. All the treated samples (tanks 1-4) were combined and processed to a powder using a Glen Creston mill. The control grain was processed in the same way. The grain was kept cold during milling by mixing it with solid CO<sub>2</sub> to aid the processing. The powdered grain was weighed and stored frozen after removing a sub-sample for analysis.

Straw: The treated straw samples (tanks 1-4) were combined and chopped in a Hobart bowl chopper while still frozen. The control straw was processed in the same way. The treated straw was weighed, stored frozen after sub-samples were removed for analysis.

Soil: Treated soil from tanks 1-4 were combined before processing to provide one sample for 0-5 cm and one sample for 5-10 cm. The soils were allowed to air dry to enable them to be ground in a pestle and mortar for sub-sampling. Representative sub-samples of treated and control soil were ground further for sub-sampling (-0.5g) for combustion. The moisture content on combustion was measured by oven drying 20-30g sub-samples of soil.

#### *Description of analytical procedures*

Water: This was measured by direct liquid scintillation counting of sub-samples (1 mL) in duplicate.

Grain: Total radioactive residues were measured by combustion of triplicate sub-samples (0.23-0.26g) of treated and control grain. The combustion efficiency was measured by combustion of control grain spiked with a standard solution of <sup>14</sup>C-BAS 684 H (4229 dpm in 50 µL).

Straw: Total radioactive residues were measured by combustion of triplicate sub-samples (0.23-0.26g) of chopped straw (treated and control). The combustion efficiency was measured with control straw as described for grain.

#### Extraction

Grain: Due to the low total radioactive residues, treated and control grain were extracted in parallel experiments. Three successive extractions were carried out with:

1. H<sub>2</sub>O
2. H<sub>2</sub>O/ CH<sub>3</sub>CN, 1:1 by volume
3. CH<sub>3</sub>CN

In each case 10g of powdered grain was homogenised with 50 mL of solvent and the mixture filtered through paper under suction and washed with a further 10 mL of extraction solvent. The residue was replaced in the same extraction vessel for the next extraction. After the final extraction the residue was dried at room temperature before sub-sampling for combustion analysis. The extracts were analysed separately by liquid scintillation counting (2 x 1 mL).

Straw: As for grain, 10g of treated and control straw were extracted in parallel experiments. For the treated straw five successive extractions were carried out. For the treated straw five successive extractions were carried out. For the first three extractions, the chopped straw was tumbled for 2 hours with the extraction solvent and for the last two extractions, the tumbling (1 hour) was preceded by homogenisation in the extraction solvent. The extraction solvents were:

1. H<sub>2</sub>O/ CH<sub>3</sub>CN, 1:9 by volume 100 mL
2. H<sub>2</sub>O/ CH<sub>3</sub>, 1:1 by volume 100 mL
3. H<sub>2</sub>O
4. H<sub>2</sub>O/CH<sub>3</sub>CN, 1:9 by volume 50 mL
5. H<sub>2</sub>O 50 mL

Liquid scintillation counting (LSC): Solutions were radioassayed using a Packard liquid scintillation counter with Packard plastic vials and Optiphase 'safe' scintillation fluid 10 mL. Analyses were carried out routinely in duplicate and the average value (after the appropriate background value had been subtracted) was calculated. Counting efficiency was measured using the spectral index of the internal standard and the results corrected



automatically. The machine calibrates were checked monthly by counting a set of quenched standards, commercially prepared in sealed glass vials.

Combustion analysis: Radioassay of solid samples was achieved by combustion in a Packard 306 sample oxidiser followed by LSC. For plant material 200-300 mg sub-samples and for soils 400 – 500 mg sub-samples were analysed normally in triplicate. The  $^{14}\text{CO}_2$  produced was trapped in Carbosorb 8 mL and mixed with Permaflor scintillation fluid (13 mL) for LSC. The machine efficiency was measured by combustion of control samples spiked with  $^{14}\text{C}$ -BAS 684 H.

Liquid-liquid partition: Organosoluble-metabolites were extracted from aqueous mixtures by partition with methyl tertiary butyl ether (MTBE) or hexane. The volumes used varied but as a general rule an equal volume of organic solvents were used. The partition was repeated with two further volumes of organic solvent and the combined organic phase was dried over sodium sulphate. Both phases were radiophased by any concentration carried out.

Thin layer chromatography: Merck silica gel F<sub>254</sub> plates (0.25 mm) were used with the following elution systems:

1. Hexane/diethyl ether, 6:1 by volume
2. Toluene/ethyl acetate, 85:15 by volume
3. Toluene/isopropyl alcohol/ acetic acid, 150:20:1.5

Unlabelled reference compounds were visualised with UV light. Quantification of radioactive zones was performed using an Isomess 3000 linear analyser. Location of radioactive zones was achieved by autoradiography with X-ray film. Typically plates were exposed for 20-30 days.

Hydrolysis with enzymes: The enzymes used were :

1. Cellulase
2. B-glucuronidase
3. Almond meal, containing  $\beta$ -D-galactosidase,  $\alpha$ -D mannosidase and  $\beta$ -glucosidase activities

Each small-scale incubation consisted of 25 mL of crop extract (equivalent to approximately 5g of crop), 10 mL of sodium acetate buffer (0.2 M, pH 4.8). The pH of this mixture was adjusted to pH 5 with 0.1M sodium hydroxide. Before adding the enzymes the samples were pre-incubated at 37°C for 20 hours.

#### High performance liquid chromatography (HPLC)

Reverse phase HPLC was carried out under the following conditions.

Column packing: S5 ODS

Column dimensions: 25 x 0.9cm

Mobile phase A: water/ formic acid, 100 : 0.1

Mobile phase B: acetonitrile/ formic acid, 100: 0.1

Gradient: 0.5 min, 20% B in A, 5-20 min, 20% to 50% B in A, 20-60 min, 50% B followed by wash cycle

Flow rate: 2.0 mL/min

#### Metabolite Characterisation

A large sample of straw (200 g) was extracted as described in the “extraction” sections above. Extract 3 (acetonitrile) was concentrated by rotary evaporation and partitioned first with hexane and then with methyl tertiary butyl ether (MTBE). The organic phases were combined and analysed by thin-layer chromatography (system 3). The aqueous phase was combined with the two aqueous extracts to provide a combined water-soluble metabolite fraction.

The first approach involved partition with MTBE of a sub-sample (equivalent to 10g crop) of the combined aqueous fractions followed by enzyme hydrolysis of the aqueous phase. The hydrolysate was then partitioned with MTBE. Both MTBE phases were analysed by TLC system 3.

The second approach was an attempt to isolate sufficient quantities of material for analysis by gas chromatography – mass spectrometry (GC-MS). A larger portion of the aqueous extract (equivalent to 106 g of crop) was subjected to enzyme hydrolysis without prior partition with organic solvent. The hydrolysate was partitioned with MTBE and the organic phase was chromatographed preparatively on TLC with toluene/ethyl acetate 85:15. Two zones were excised from the plate and eluted from the silica with acetone/hexane 1:1 and



these zones were further purified by TLC in ethyl acetate/toluene 1:1. Chromatography on HPLC was the final separation.

#### Distribution of applied radioactivity

##### Rice

A small number of rice plants were sampled immediately after spraying to enable an estimate of distribution of the applied radioactivity between plants and surrounding water to be made. The leaf residue was measured by combustion as 2.1 mg eq/kg fresh weight. By estimating the portion of plants analysed it was possible to estimate the total amount of  $^{14}\text{C}$ -BAS 684 H intercepted by the leaves to be 133  $\mu\text{g}$ . This represents less than 1% of applied radioactivity.

##### Water

The concentration of radioactivity measured in the paddy immediately after the applications of  $^{14}\text{C}$ -BAS 684 H was complete was 0.4  $\mu\text{g/mL}$ . Four hours later the concentration had dropped to 0.2  $\mu\text{g/mL}$ . This rapidly changing concentration makes precise measurement of the initial dose in the water difficult. However, using an estimate of 8 L of paddy water in each tank a maximum theoretical concentration of 0.61 mg/L can be calculated. Therefore, at least 66% of the nominally applied radioactivity reached the paddy but this is likely to be an underestimate given in the time between spraying the first tank and sampling the water (about 30 minutes).

The concentration of radioactivity in the water was measured at 6 more intervals during the experiment and the results summarised in Table 7-87. By eight weeks after treatment only negligible concentrations of radioactivity were measured.

Table 7-87 Radioactivity in water of a simulated rice paddy following treatment with  $^{14}\text{C}$ -BAS 684 H at a dose rate equivalent to 200 g/ha

| Time after application | dpm/mL             | $\mu\text{g/mL}$ |
|------------------------|--------------------|------------------|
| 0-1 hours              | 28814 <sup>a</sup> | 0.40             |
| 4 hours                | 14459              | 0.20             |
| 1 week                 | 7650               | 0.11             |
| 2 weeks                | 2111               | 0.03             |
| 4 weeks                | 871                | 0.01             |
| 8 weeks                | 43                 | <0.001           |
| 12 weeks               | 38                 | <0.001           |
| 14 weeks               | 55                 | <0.001           |

a) Mean for tanks 1 to 4

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### Distribution of radioactivity at harvest

#### Rice grain

The total yield of rice grain from the four treated rice tanks was 939g. The total radioactive residue was 0.025 mg eq/kg measured by combustion of the processed grain. Control grain was obtained from tank 5 and was processed and analysed in the same way as the treated grain. This provided the relevant background values and enabled the limit of reliable measurement to be calculated.

#### Rice straw

The total yield of straw from the four treated rice tanks was 2537g. The total radioactive residue was 0.094 mg eq/kg of straw measured by combustion of the processed straw. Control straw was obtained from tank 4 and was processed and analysed in the same way as the treated straw. This provided the appropriate background values and enabled the limit of reliable measurement to be calculated.

Of the total crop residue at harvest, 90.6% was present in the straw.

#### Soil

The soil from treated tanks 1-4 were processed and analysed by combustion. Control soil was obtained from tank 5 and provided the appropriate background values. The combustion efficiency was measured by spiking control soil with a solution of  $^{14}\text{C}$ -BAS 684 H. The radioactivity in the 0-5 cm later was measured at 0.12 mg eq/kg and in the 5-10 cm later as 0.03 mg eq/kg, expressed on a dry weight basis.

### Extractability of residues at harvest

#### Grain

Grain was extracted sequentially with three extraction solvents. Each extract was analysed separately by LSC (1 mL in triplicate) in comparison with the appropriate controls. The aqueous extract obtained 11.6% of the total grain residue, the acetonitrile/water extract contained a further 7.5% and the final acetonitrile extract contained no measurable radioactivity. These results were substantiated by combustion analysis of the residue after extraction which contained 79% giving a total recovery of more than 98% through the extraction procedure.

Thus, the grain residue is largely unextracted by thorough extraction with unheated solvents. The traces of radioactivity which were liberated were of a water-soluble nature but too low in concentration (0.005 mg eq/kg) to attempt any further characterisation.

#### Straw

Straw was extracted sequentially five times by two parallel sequences A and B. Each extract was analysed by LSC (1 mL in triplicate) using the appropriate control; the results are presented in

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Table 7-88. Both extraction sequences were equally effective at 50.8% and 52.7% extraction efficiency respectively.

The final two extractions which involved homogenisation as well as tumbling with the solvent together released only 3-4% of the total radioactivity in the straw. The residues after the final extraction were measured by combustion to be 42.5% and 41.7% of the total straw residue for methods A and B, respectively. Therefore, the recovery through both extraction procedures was >90%. In

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Table 7-88 the calculated results for extracted radioactivity are also presented in mg eq/kg. At 0.049 mg eq/kg, the extracted radioactivity is just below the trigger value of 0.05 mg eq/kg set in the protocol. However, because of the absence of mature plant fraction with levels of  $^{14}\text{C}$  above the trigger value, characterisation of these straw residues was attempted.

It was not possible to produce significantly higher residue levels by repeating the experiment at a dose rate higher than 200g/ha as such an application would have caused severe phytotoxicity.

Table 7-88 Extraction of straw

| Method A        |                                  | Method B        |                                  | Average |          |
|-----------------|----------------------------------|-----------------|----------------------------------|---------|----------|
| Extraction code | % of total straw <sup>14</sup> C | Extraction code | % of total straw <sup>14</sup> C | %       | mg eq/kg |
| A <sub>1</sub>  | 33.3                             | B <sub>1</sub>  | 32.0                             |         |          |
| A <sub>2</sub>  | 10.6                             | B <sub>2</sub>  | 14.6                             |         |          |
| A <sub>3</sub>  | 3.3                              | B <sub>3</sub>  | 2.5                              |         |          |
| A <sub>4</sub>  | 1.5                              | B <sub>4</sub>  | 1.5                              |         |          |
| A <sub>5</sub>  | 2.1                              | B <sub>5</sub>  | 2.1                              |         |          |
| Total extracted | 50.8                             |                 | 52.8                             | 51.8    | 0.049    |
| Unextracted     | 42.5                             |                 | 41.7                             | 42.1    | 0.040    |
| Total recovered | 93.9                             |                 | 94.5%                            | 93.9%   | 0.089    |

Characterisation of the extracted radioactive residue in straw

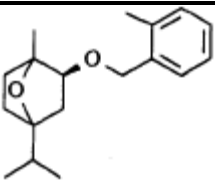
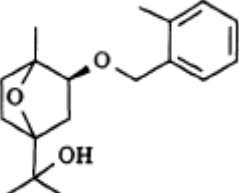
Despite the low levels of extractable radioactivity residues in straw characterisation was attempted by extracting a large quantity of straw (200g). The percentage extracted (51%) was equivalent to that obtained on the smaller scale.

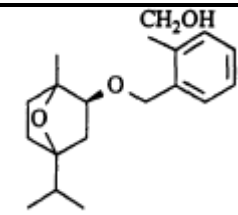
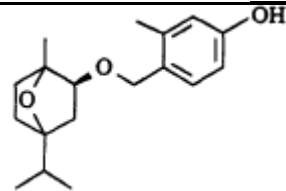
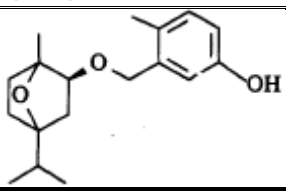
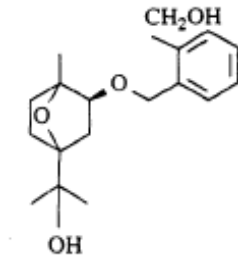
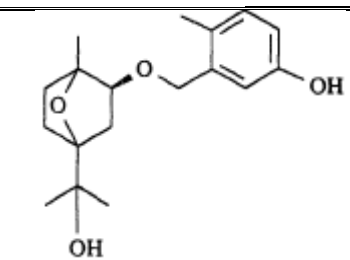
Partition of the acetonitrile extract with hexane released very little radioactivity (1% of the total straw residue) and subsequent partition with MTBE released little more. The two organic phases were combined for analysis by TLC using system 3.

Autoradiography revealed a complex profile of at least ten components. As the total fraction represented less than 0.002 mg eq/kg of straw no further characterisation was attempted.

Partition of the combined water-soluble fraction with MTBE gave a fraction containing 15% (equivalent to 0.014 mg eq/kg) of the total straw residue. This was chromatographed using solvent system 3 and once more a complex profile of at least 10 components were observed by autoradiography. The reference compounds were chromatographed alongside the extracts but gave rather large spots because their poor chromophores demand high loading. It was not possible to co-chromatograph the reference compounds with the extracts as the co-extractives would have prevented visualisation. Thus, although a large portion of the radioactive compounds were monohydroxylated (e.g. M684H019 (211368), M684H002 and M684H004 (205588)) and dihydroxylated (211733 and M684H044 (207855)) derivatives of BAS 684 H, see Table 7-89, identification by co-chromatography was not possible.

Table 7-89 Structures of reference compounds

| Metabolite           | Chemical Structure  |
|----------------------|---|
| BAS 684 H (WL 95481) |  |
| M684H004 (WL 205588) |  |

|                      |   |
|----------------------|---|
| M684H002 (WL 207856) |    |
| M684H019 (WL 211368) |    |
| M684H018 (WL 211647) |    |
| M684H044 (WL 207855) |   |
| WL 211733            |  |

Enzyme hydrolysis of the aqueous phase was carried out on two parallel sub-samples as the cellulase/  $\beta$ -glucuronidase preparation at releasing organosoluble radioactivity, it was not an overall successful as the cellulose/ $\beta$ -glucuronidase preparation at releasing organosoluble radioactivity, it was not an overall success as the organic extract contained too much co-extracted material for chromatographic analysis. A further 11% of the total radioactivity in the straw was released as MTBE-soluble metabolites by enzyme hydrolysis with cellulase/ $\beta$ -glucuronidase. Thin layer chromatography of this fraction revealed that it was similar qualitatively and quantitatively to the 'free' metabolite fraction (i.e. that partitioned before enzyme hydrolysis). Thus, a large portion of the water-soluble metabolites were characterised as conjugates of metabolites also present in their 'free' form. Previous studies with other plant species (Woodward et al., 1985) had demonstrated that a cellulase/ $\beta$ -glucosidase mixtures was more effective at cleaving the conjugates than  $\beta$ -glucosidase and a number of other enzymes. Acid hydrolysis was also shown to be ineffective. Therefore, no further work was attempted with the remaining aqueous fraction.

An alternative approach to characterisation was performed by direct enzyme hydrolysis of a larger portion of the combined water-soluble fraction. An MTBE fraction containing 25% of the total straw residue was obtained in this way. Two zones (A and B) were isolated with chromatographic conditions metabolites in two solvent systems consistent with mono- and di-hydroxylated derivatives of BAS 684 H respectively. Examination of fraction A by HPLC revealed three peaks, probably individual components with retention times of 35.6, 40.7, and 42.0 minutes. The retention times of the mono-hydroxylated derivatives of BAS 684 H using the same gradient elution system were: M684H002 (36.4 min), M684H004 (WL205588) (39.2 min) and M684H019 (WL 211368) (39.2 min). The dihydroxy standards M684H044 (WL207855) and WL211733 had retention times of 24.4 and 26.0 minutes respectively. Fraction B when chromatographed under these conditions gave four poorly

resolved peaks with retention times from 24.6 to 26.0 minutes. Thus, it was confirmed by HPLC that fractions A and B contained mixtures of mono- and di-hydroxylated metabolites of BAS 684 H respectively.

Identification by co-chromatography was not possible as the samples still contained too much co-extracted plant material. GC-MS was unsuccessful for the same reason.

### Conclusion

No significant radioactive residues were observed in rice grain at harvest (<0.05 mg eq/kg). In rice straw the radioactive residues were very low (<0.1 mg eq/kg). Characterisation of this residue by extraction, enzyme hydrolysis and chromatography yielded results which were consistent with the pattern of metabolism in other crop foliage.

It can be concluded that BAS 684 H is extensively metabolised in rice mainly by hydroxylation to form a wide range of mono- and di-hydroxylated metabolites. These primary metabolites are further metabolised by conjugation with naturally occurring plant materials to yield water-soluble metabolites which can be in part be cleaved by enzymes in vitro. Further metabolism leads to a bound residue which may arise from small fragments of the herbicide, i.e.  $^{14}\text{CO}_2$  being incorporated into natural plant constituents as well as by conjugation of metabolites to the plant matrix.

No one extractable metabolite was present in concentrations significantly greater than another and at least ten primary metabolites were observed in the straw. The structures were postulated to be mono-hydroxylated and di-hydroxylated derivatives of BAS 684 H but identification was not definitive due to poor visualisation during co-chromatography (due to co-extracted plant material). The balance between mono- and di-hydroxylated metabolites was approximately 1:1.

|             |  |
|-------------|--|
| Report:     | CA 6.2.1/13<br>Courcher A., Edwards V.T, 1989<br>The Distribution and Metabolism of $^{14}\text{C}$ -WL95481 in Rice Under Controlled Environmental Conditions<br>CI-640-012 |
| Guidelines: | Japanese MAFF Guideline pg 53<br>US EPA Pesticide Assessment Guideline pg 171-174  |
| GLP:        | Yes  |

### **Materials:**

#### 1. C-label BAS 684 H

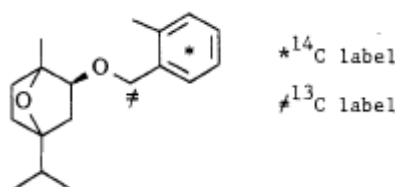
**Description:** Phenyl-U- $^{14}\text{C}$  and  $^{13}\text{C}$  in the bridge methylene (spec. activity of a.s. 32.4  $\mu\text{Ci/mg}$ )

**Lot/Batch #:** S1006/4

**Radiochemical Purity:** >98%

**Chemical Purity:** 99%

**Structure:**



### *Method*

A metabolism study in rice plants (*Oryza sativa*) grown indoors was carried out in 1989 in Sittingbourne, Kent, UK. The test item was applied at a single application rate of approximately 200 g/ha (there is no intended uses proposed on rice) to transplanted rice plants growing in pots under paddy conditions.

Preparation of the treatment solution: The radiolabelled test compound was formulated as an emulsifiable concentrate by dissolving 12 mg radioactive BAS 684 H in 110 mL of formulation based on SF06867 but without active ingredient. The formulation concentration was thoroughly mixed with 12 mL of tap water to form the treatment solution. The concentration of the treatment solution was measured by liquid scintillation counting to be 0.94 mg/mL.

Plant growth and treatment: Rice plants were grown from seed in a glass house and transplanted into pots (13 cm x 13 cm), 6 plants per pot in 3 hills of 2 plants, 12 days after sowing. The soil in the pots was maintained at normal moisture for 5 days after transplanting, when the soil was flooded to a depth of 1-2 cm to simulate paddy conditions. At 7 days after transplanting, when the plants were at the 2-3 leaf stage, they were moved to the Laboratory for the treatment. Pots were treated with 350 µL of the treatment solution applied evenly over the surface of the pot using a precision sprayer fitted with a 500 µL micro syringe. After treatment all plants were placed in a controlled environment chamber maintained during the day at 28°C and 20°C at night with a 12 hour light cycle.

### *Sampling*

Intermediate sampling: At 0, 9 and 40 days after treatment, 3 pots were sampled and analysed separately. The plants were cut off at soil level and the fresh weight recorded. Any water on top of the soil was removed by decanting, the volume measured and the solution radioassayed. The soil was tipped out onto aluminium foil covered trays, allowed to air dry and broken into small lumps by rolling with a glass jar. Sub-samples were removed, combined and ground into a mortar and pestle.

Sampling at harvest (138 days after treatment): Ears of rice were removed from all remaining 17 pots with scissors and the grains separated from the ears by hand. Straw was cut off at soil level and a combined sample prepared. The straw from one pot was divided into 4 sections of approximately equal length. This was to establish the distribution of the radioactivity in the straw with section 1 being nearest the base of the plant.

Soil from 3 treated pots were analysed separately in the same way as the intermediate samples. The root systems of the plants at harvest were quite extensive and were cut into small fragments and mixed with the soil for analysis.

### *Description of analytical procedures*

Homogenised solid plant samples were weighed and combusted by means of an automatic sample oxidizer. Quantitation of metabolites was carried out using thin-layer chromatography followed by autoradiography and liquid scintillation counting.

Processing plant material: At time 0, plants were not extracted but were dried and analysed by combustion/LSC. At 9 days after treatment all the plant material was cut into small fragments and extracted using the systems outlined below. Day 40 plant material was similarly treated but only 10g sub-samples were analysed.

At harvest, rice grains were ground using a Glen Creston milling machine, and the straw was chopped into very small fragments using a mechanical straw cutter. Both rice and straw were analysed by combustion/LSC of sub-samples and a sub-sample of the straw (100g) was extracted.

Plant extraction: Plant material was extracted 4 times using a macerator. The extract was separated from the solid by vacuum filtration and each extract was collected separately. The first two extractions were with acetonitrile/water (9:1), the third was with acetonitrile/water (1:1) and the final extraction was with water (except for Day 9 when only 3 extractions were carried out).

All extracts were radioassayed separately and the remaining residue after extraction was radioassayed by combustion/LSC.

Soil extraction: Soil was extracted by placing a 200g sub-sample of the air-dried soil into a glass jar and tumbling with 300 mL acetonitrile/water (7:3) for 2 hours. The extraction mixture was filtered through a sintered glass funnel and washed with 50 mL of diethyl ether. All extracts were combined and radioassayed by direct LSC. The extracted residue was radioassayed by combustion/LSC.



Liquid/liquid partition: As a first step in the analysis of the plant extracts, partition with dichloromethane or methyl tert-butyl ether (MTBE) was carried out. Most of the acetonitrile in the extracts was removed by rotary evaporation before a portion of the extract was portioned at least 3x with equal volumes of the organic solvent. The organic phases were combined and radioassayed by LSC, as was the remaining aqueous phase.

Liquid scintillation counting (LSC): Solutions were radioassayed using a Packard liquid scintillation counter with Packard plastic vials and Optiphase scintillation fluid (10 mL). Analyses were carried out routinely in duplicate and the average value (after the appropriate background value had been subtracted) was calculated. Counting efficiency was measured using the spectral index of the external standard and the results corrected automatically. The machine calibrations were checked monthly by counting a set of quenched standards, commercially prepared in sealed glass vials.

Combustion analysis: Radioactivity in solid material was quantified by combustion analysis using a Packard 306 sample oxidiser. Small sub-samples (up to 500 mg) of plant material were weighed into paper thimbles and capped with a small cellulose pad for combustion. Extracted soil samples, however, after being ground to a fine powder were weighed (~ 500 mg) into paper thimbles, mixed with an equal volume of cellulose powder, wrapped in a small amount of paper and compressed into a pellet before combustion.

The Packard 306 oxidiser automatically traps the  $^{14}\text{CO}_2$  in Carbosorb and blends it with Permafluor scintillation fluid in a scintillation vial ready for liquid scintillation counting. The machine efficiency was measured by combustion of control samples spiked with  $^{14}\text{C}$ -BAS 684 H.

Thin Layer Chromatography (TLC): Silica gel-coated glass TLC plates were used (0.25 mm Merk 5715) for both radiochemical purity and analysis of extracts. Three solvent systems were used:

1. Hexane/diethyl ether 6:1
2. Toluene/ethyl acetate 10:1
3. Toluene/isopropanol/acetic acid 150:20:1

High performance liquid chromatography (HPLC): This technique was used to obtain metabolite profiles of the plant extracts. A reverse-phase semi-preparative column was used (25 cm x 95 mm, Spherisorb 5  $\mu\text{m}$  ODS) and was eluted with mixtures acetonitrile and water, at 2 mL/min. The elution system was as follows:

0-5 min : 20%  $\text{CH}_3\text{CN}$  in  $\text{H}_2\text{O}$   
5-20 min 20-50%  $\text{CH}_3\text{CN}$  in  $\text{H}_2\text{O}$  linear gradient  
20-40 min 50%  $\text{CH}_3\text{CN}$  in  $\text{H}_2\text{O}$

Radioactivity was detected by collecting 0.5 minute fractions of the eluate which were radioassayed by LSC. The results were plotted out as a line graph to form a chromatogram.

## Results

Plant growth: All treated plants suffered some phytotoxicity with all plants in one pot dying completely. The level of phytotoxicity, duration and timing is not reported, however the plants in the other pots made a good recovery and all matured and produced an average yield of grain equivalent to 1.44 kg/m<sup>2</sup>, a very similar yield of grain to the control plants. The control plants remained healthy (excluding one pot which became over grown with a weed) and produced an average yield of grain of 1.37 kg/m<sup>2</sup>. Therefore the phytotoxicity is not considered to impact significantly on the results of the study. Several times during the experiment the plants became infested with aphids and were sprayed with a commercial aphicide.

Distribution of radioactivity between plants, soil and water: The amount of radioactivity recovered in the plants, soil and water at each sampling time is shown in Table 7-90, expressed in  $\mu\text{g}$  equivalents and as percentage of the total recovered. The average total recovered radioactivity was fairly consistent at days 0, 9 and 40, although considerable variations between replicate pots were observed. At harvest, the total recovered radioactivity was somewhat lower but there was insufficient information to attach any significant to this.

Most of the radioactivity at day 0 was recovered in the water, but by day 9 most has transferred to the soil fraction. As the plants grew, approximately 12% of the applied radioactivity moved from the soil and water into the plants.

In order to quantify the radioactivity in the soil it was necessary to extract the soil and combust the extracted soil residue. The sum of the extracted plus un-extracted radioactivity was used to provide the total. It was not possible to measure the radioactivity in the soil by direct combustion because replication between sub-samples was poor. Therefore, the extractability of the soil radioactive residues in acetonitrile/water (7:3) was measured incidentally and the results are presented in

Table 7-91. The extractability decreased from day 0 (94%) to day 138 (16%).

The radioactive residues present in the plants at each sampling interval, expressed as mg eq/kg are given in Table 7-90. The residues in the straw decreased from 3 mg eq/kg at day 0 to 0.7 mg eq/kg at day 40 and then to 0.3 mg eq/kg at harvest. Although the total radioactive residues in the plant as a percentage of applied radioactivity (see table 2) increased up to day 40, the concentration of radioactivity decreased because of the rapid increase in plant fresh weight during this period of vegetative growth.

#### Distribution of radioactivity in the straw and grain

The total radioactive residues in the straw and grain at harvest are given in Table 7-92. These results are the mean of all harvested pots with control grain and straw providing the appropriate background values. More than 95% of the total plant residue at harvest was recovered in the straw. The level in the grain <0.02mg eq/kg was well below the LOQ. The observed phytotoxicity demonstrates that it would not have been possible to use a higher dose to provide a higher grain residue to facilitate characterisation.

The distribution of radioactivity in the straw could not be determined by autoradiography because of the low levels and was measured by combustion. The results of the analysis of the four sections are presented in Table 7-92 and Table 7-93. The radioactive residues found in the three lower sections were similar but the concentration in the top section was approximately 2-fold lower than in the rest of the straw.

Table 7-90 Distribution of radioactivity between plant, soil and water

| Days after treatment | Expressed as µg BAS 684 H equivalents |       |       |                  |       |
|----------------------|---------------------------------------|-------|-------|------------------|-------|
|                      | Pot No.                               | Plant | Soil  | Water            | Total |
| 0                    | 8                                     | 1.9   | 28.2  | 133.6            | 163.7 |
|                      | 19                                    | 4.3   | 28.0  | 114.7            | 146.9 |
| Mean distribution    | 30                                    | 2.9   | 27.2  | 132.8            | 162.9 |
|                      |                                       | 2.0%  | 17.6% | 80.4%            | 100%  |
| 9                    | 9                                     | 3.4   | 99.3  | 5.6              | 106.7 |
|                      | 20                                    | 3.2   | 190.4 | 14.1             | 206.0 |
| Mean distribution    | 31                                    | 4.4   | 139.5 | 10.5             | 152.2 |
|                      |                                       | 2.5%  | 91.2% | 6.3%             | 100%  |
| 40                   | 10                                    | 24.3  | 78.7  | 3.2              | 106.3 |
|                      | 22                                    | 10.9  | 119.9 | 8.1              | 138.8 |
| Mean distribution    | 32                                    | 22.0  | 150.1 | 4.8              | 176.9 |
|                      |                                       | 14.4% | 81.8% | 3.9%             | 100%  |
| 138 (harvest)        | 14                                    | *1    | 95.2  | N/A <sup>2</sup> | -     |
|                      | 18                                    | *     | 94.6  | N/A              | -     |
|                      | 27                                    | *     | 107.6 | N/A              | -     |
| Average              |                                       | 12.4  | 99.2  |                  | 11.6  |
|                      |                                       | 11.1% | 88.9% |                  | -     |

1. Composite plant sample analysed from soil remaining treated pots

2. Not applicable – no water phase at harvest

Table 7-91 Extractability of radioactivity in soil with acetonitrile/water following treatment with  $^{14}\text{C}$ -BAS 684 H

| Day     | Pot No.        | Extracted radioactivity ( $\mu\text{g}$ ) | Un-extracted radioactivity ( $\mu\text{g}$ ) | Extractability (%)                | Mean |
|---------|----------------|---|--|-----------------------------------|------|
| 0       | 8<br>6<br>9    | 26.6 <sup>1</sup><br>26.3<br>25.6         | 1.6 <sup>2</sup><br>1.7<br>1.6               | 94.4 <sup>3</sup><br>94.0<br>94.2 | 94.2 |
| 9       | 9<br>20<br>31  | 83.0<br>161.3<br>116.4                    | 16.3<br>29.1<br>23.1                         | 83.6<br>84.7<br>83.4              | 83.9 |
| 40      | 10<br>22<br>32 | 36.1<br>90.6<br>100.9                     | 42.7<br>29.3<br>49.2                         | 45.8<br>75.6<br>67.2              | 62.9 |
| 138     | 14<br>17       | 14.0<br>11.9                              | 81.2<br>82.7                                 | 14.7<br>12.6                      | 16.4 |
| Harvest | 28             | 23.5                                      | 84.1   | 21.8                              |      |

1. Measured by liquid scintillation counting

2. Measured by combustion analysis

3. Percentage extracted of total recovered radioactivity

Table 7-92 Radioactivity in plant material following treatment with  $^{14}\text{C}$ -BAS 684 H

| Days after treatment | Total recovered radioactivity (% of recovered) | mg eq/kg fresh weight    |
|----------------------|--|--------------------------|
| 0                    | 2.0  | 3.0                      |
| 9                    | 2.5  | 1.5                      |
| 40                   | 14.4   | 0.7                      |
| 138                  | 11.1   | 0.26 straw<br>0.02 grain |

Characterisation of the residuesGrain

The levels of radioactivity in the grain were <0.02 mg eq/kg below the LOQ.

Straw

The levels of radioactivity in the straw were 0.26 mg eq/kg. This was approximately three times the value obtained when  $^{14}\text{C}$ -BAS 684 H was applied at the same dose rate to plants grown in tanks under outdoor paddy conditions. However, much of that difference was accounted for by the difference in moisture content of the harvested straw (indoor – 35.7%, outdoor – 67.4%). Therefore, the straw produced under more realistic growing conditions in the outdoor experiment (Edwards, 1988) was chosen for detailed characterisation work. Another reason for this decision was that in the controlled environment chamber, metabolism to  $^{14}\text{CO}_2$  followed by reincorporation into the plant matrix via photosynthesis is possible but less likely to be important under outdoor conditions. However, some information on the extractability and polarity of the radioactivity in the intermediate plant and straw samples was obtained and this will be described below.

Table 7-93 Distribution of radioactivity within straw samples from 1 pot analysed 138 days after treatment with [ $^{14}\text{C}$ -ring] BAS 684 H

| Plant section | mg eq/kg fresh weight |
|---------------|-----------------------|
| 1 base        | 0.44                  |
| 2             | 0.38                  |
| 3             | 0.43                  |
| 4 top         | 0.21                  |

Extraction of plant residues

Intermediate samples (9 and 40 days after treatment) were extracted and analysed. A summary of the extractability of plant residues at each sampling interval is presented in Table 7-94. The extractability in all samples was about 60%. These values are broadly in line with that obtained for the harvest straw sample in the outdoor rice metabolism study (Edwards, 1988) and in line with metabolism in other crops (Woodward et al., 1985).

Table 7-94 Sequential extraction of plant material

| Days after treatment | Pot no. | Extract % total |      |      |     | Total % extractable |
|----------------------|---------|-----------------|------|------|-----|---------------------|
|                      |         | 1               | 2    | 3    | 4   |                     |
| 9                    | 9       | 34.1            | 20.3 | 7.2  | ND  | 61.6                |
|                      | 20      | 44.4            | 9.5  | 15.1 | ND  | 69.0                |
|                      | 31      | 42.4            | 8.5  | 14.3 | ND  | 65.1                |
| 40                   | 10      | 46.9            | 3.7  | 10.7 | 1.7 | 63.0                |
|                      | 22      | 44.4            | 2.7  | 6.5  | 1.1 | 54.7                |
|                      | 32      | 43.7            | 2.4  | 7.1  | 0.9 | 54.0                |
| 138                  | -       | 33.6            | ND   | 24.2 | 3.0 | 60.8                |
| Straw at harvest     |         |                 |      |      |     |                     |

Chromatography of plant extracts

The first step in the characterisation of the extracted radioactivity was to combine all extracts and partition with organic solvent. It was known that BAS 684 H would partition quantitatively from the extract into the organic solvent under these conditions. The results of these partitions for each sampling interval are given in Table 7-95. In each case the major portion of the residue was water-soluble indicating rapid metabolism. Even at day 9 more than 77% was water-soluble. The organic phase from the day 9 extract was examined by TLC system 3 and approximately 17% co-chromatography with BAS 684 H (i.e. 4% of extracted and <2% of recovered radioactivity is BAS 684 H). The major portion of the organosoluble radioactivity (74%) i.e. 17% of extracted was present as components which co-chromatographed as mono- and di-hydroxylated derivatives of BAS 684 H. It was known from previous work (Edwards, 1988) that it was not possible to achieve sufficient resolution of potential metabolites B.7.2.1.6-3 by one-dimensional TLC. In addition, the poor UV chromophore of the reference compounds and the high ratio of co-extracted material to radioactivity made co-chromatography by two-dimensional TLC impractical.

Although the Day 40, total composite extract and dichloromethane phase were chromatographed, little additional information was obtained, because of the low concentration of radioactivity. In TLC solvent system 3, at least 9 metabolites were visible by autoradiography.

An attempt was made to examine the MTBE phase of the terminal straw extract which represented 10% of the total residue. Several components were separated by TLC (toluene/ethyl acetate, 85:15) but it was not possible to quantify or characterise them further.

The aqueous phase representing 50% of the total terminal residue was examined by HPLC using two gradient-elution systems. The levels of radioactivity which could be successfully chromatographed were so low that no peaks were visible on the radio-trace. However, by collecting 0.5 min fractions and plotting the output as a line graph it was possible to establish that the aqueous phase consisted of components more polar than BAS 684 H and its mono-hydroxylated derivatives (M684H019 (WL 211368), M684H004 (WL205588) and M684H002). Only 2 di-hydroxylated reference compounds were available (WL211733 and M684H044 (WL207855)). There were at least eight peaks visible in the sample and one was in the correct position for M684H044 (WL207855), the major di-hydroxylated metabolite of BAS 684 H, observed previously in other plant metabolism studies (Woodward et al., 1985). The remainder of the components eluted before M684H044 (WL207855) in this reversed-phase system. It was not possible to co-chromatograph the standards with the sample because of the poor UV chromophores of the standards and because of the high ratio of co-extracted material to radioactivity made visualisation of the standard impractical.

Table 7-95 Partition of plants extracts

| Sampling Interval (days) | Organic Solvent   | Organic phase recovered % | Aqueous phase recovered % |
|--------------------------|-------------------|---------------------------|---------------------------|
| 9                        | Dichloromethane   | 23                        | 77                        |
| 40                       | Dichloromethane   | 34                        | 66                        |
| 138                      | MTBE <sup>1</sup> | 17                        | 83                        |

1. Methyl tert-butyl ether

### Conclusion

BAS 684 H when applied to rice growing in pots under glasshouse conditions was rapidly metabolised to a variety of more polar components. The total radioactive residue in plants analysed nine days after treatment was 1.5 mg eq/kg of which less than 5% was unchanged BAS 684 H.

The total radioactive residues in the grain at harvest were very low (0.02 mg eq/kg) and were not characterised. It was not possible to produce grain with higher residues as the dose in this experiment (200 g/ha) produced severe phytotoxicity in treated plants.

The straw residue was slightly higher (0.26 mg eq/kg) of which approximately 60% was extractable. More than 80% of the extracted radioactivity (0.12 mg eq/kg) comprised water-soluble products, at least eight components being separable by reversed-phased HPLC. Further work on the characterisation of these water-soluble products achieved by enzyme hydrolysis in the parallel study where rice were grown outdoors under paddy conditions (Edwards, 1988).

**HSE comments:** The metabolism of BAS 684 H in rice was investigated in two parallel reports: Edwards, V.T, 1988 and Edwards, V.T, 1989 and were published in 1988 and 1988, respectively. Both reports were not performed to current OECD guidelines. Both metabolism studies applied radioactive BAS 684 H, labelled only on a single ring (Phenyl-U-<sup>14</sup>C), at total nominal application rate of approximately 2.0 kg a.s./ha (there are no intended uses proposed on rice). All samples (rice leaves, grain and straw) were stored under freezer conditions until analysed but the storage period is not stated and no additional data to address the stability was provided in the study.

In the 1988 report, characterisation of metabolites was by TLC using 3 different solvents and identification methods used were co-chromatography with reference standards along with GC-MS. Due to samples containing too much co-extracted plant material identification of the metabolites was not possible in this report, however the structures were postulated to be mono-hydroxylated and di-hydroxylated derivatives of BAS 684 H.

The 1989 report characterised metabolites by TLC using 3 different solvents and identification methods used were co-chromatography with reference standards along with HPLC. The TRR in grains at harvest were very low (0.02 mg eq/kg) and therefore, residues were not characterised. The applicant explained it was not possible to produce grain with higher residues as the dose in this experiment (200 g/ha) produced severe phytotoxicity in treated plants, although the yield of grain was acceptable despite phytotoxicity. The straw residue was slightly higher (0.26 mg eq/kg) of which approximately 60% was extractable. More than 80% of the extracted radioactivity (0.12 mg eq/kg) comprised water-soluble products, at least eight components being separable by reversed-phased HPLC and one tentatively identified as M684H044 (WL207855) by a retention time match only; it was not possible to co-chromatograph the standards with the sample because the poor UV chromophores of the standards and because the high ratio of co-extracted material to radioactivity made visualisation of the standard impractical. The remainder of the components eluted before M684H044 (WL207855) in this reversed-phase system.

Owing to the issues raised the rice studies cannot be relied upon but can be considered supportive information. Additionally, the study was conducted paddy conditions and only tentative identification of metabolites was achieved, hence the studies are of limited use. However, the tentative identification of metabolites as mono- and di-hydroxy derivatives of parent BAS 684 H is consistent with the identification of metabolites in the other primary crop metabolism studies available.

### B.7.2.1.7. Overall conclusion on metabolism in plants

Metabolism was investigated using two radiolabels (BAS 684 H labelled in the phenyl ring and the cyclohexane ring). Investigations were done in three plant species: wheat (cereal crop group), oilseed rape (pulses and oilseed crop group) and carrot (root and tuber vegetable crop group); foliar spray-applied with BAS 684 H reflecting the cGAP.

In most matrices the metabolites M684H005 and M684H006 were the predominate residues notably in wheat straw (cyclohexane label) at 14.8% TRR (1.440 mg eq/kg) and 18.5% TRR (1.798 mg eq/kg) respectively. The sugar conjugates M684H005 and M684H006 occurred in major amounts (>10% TRR) in wheat forage, wheat straw and oilseed rape straw. The parent compound BAS 684 H was found in major amounts in oilseed rape seeds (phenyl label) and carrot leaves (both labels) up to 0.159 mg eq/kg (27.9% TRR). In oilseed rape hulls and wheat grain, no major compounds were identified.

M684H002 is proposed as a key metabolite in the pathway however it has not been identified in the plant metabolism studies. It has been identified by LC-MS/MS as the main deconjugated form of metabolites M684H005 and M684H006 in the cleavage experiments. The process of conjugation to form M684H005 and M684H006 is concluded to take place quicker than the initial hydroxylation such that at sampling no significant amounts of the unconjugated M684H002 are detected.

Metabolites occurring in major amounts (>10% TRR) and in minor amounts (<10% TRR) are listed in Table 7-96. This table groups the metabolites according to their chemical structure

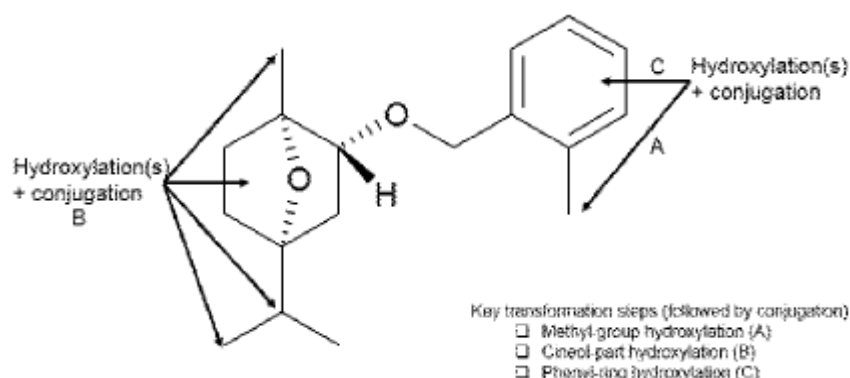
The major compounds (>10% TRR) found in plants were M684H005 and M684H006 (A-branch). The metabolic routes are shown in Figure 7-12 and the transformation reactions summarised in Figure 7-11.

In plants, the metabolic pathway is largely based on:

- hydroxylation of the parent compound at various positions
- subsequent conjugation of these hydroxyl groups with glycoside and malonyl glycoside

The parent BAS 684 H was applied as a racemic mixture of two enantiomers (a ratio of the (-) and (+) enantiomers of approximately 51:49 in the application solution). Chiral analysis of BAS 684 H revealed a ratio of the (-) and (+) enantiomers was approximately 40.6:59.4 and 43.0:57.0 in carrot leaves (phenyl- and cyclohexane labels respectively) demonstrating that the changes in the stereoisomeric excess (SE) are >10%, therefore, these ratios are not considered comparable and further consideration is required. Whereas, BAS 684 H was not detected at sufficient levels (<0.01 mg eq/kg) in all wheat matrices and oilseed rape matrices; therefore, chiral analysis of its main metabolite M684H005 was investigated. Chiral analysis of M684H005 revealed a ratio of the diastereomers was approximately 36:64 and 34:66 in wheat forage and straw respectively (both cyclohexane label) and 26:74 in oilseed rape straw (phenyl-label). There is a significant change in stereoisomeric ratio upon metabolism in wheat, oilseed rape and carrot. The enantiomers of parent BAS 684 H and M584H005 are considered toxicologically equivalent (Vol 1 Section 2.12.3).

Figure 7-11 BAS 684 H transformation reactions in plant



Metabolites occurring in major amounts (>10% TRR) and in minor amounts (<10% TRR) are listed in Table 7-96. This table groups the metabolites according to their chemical structure

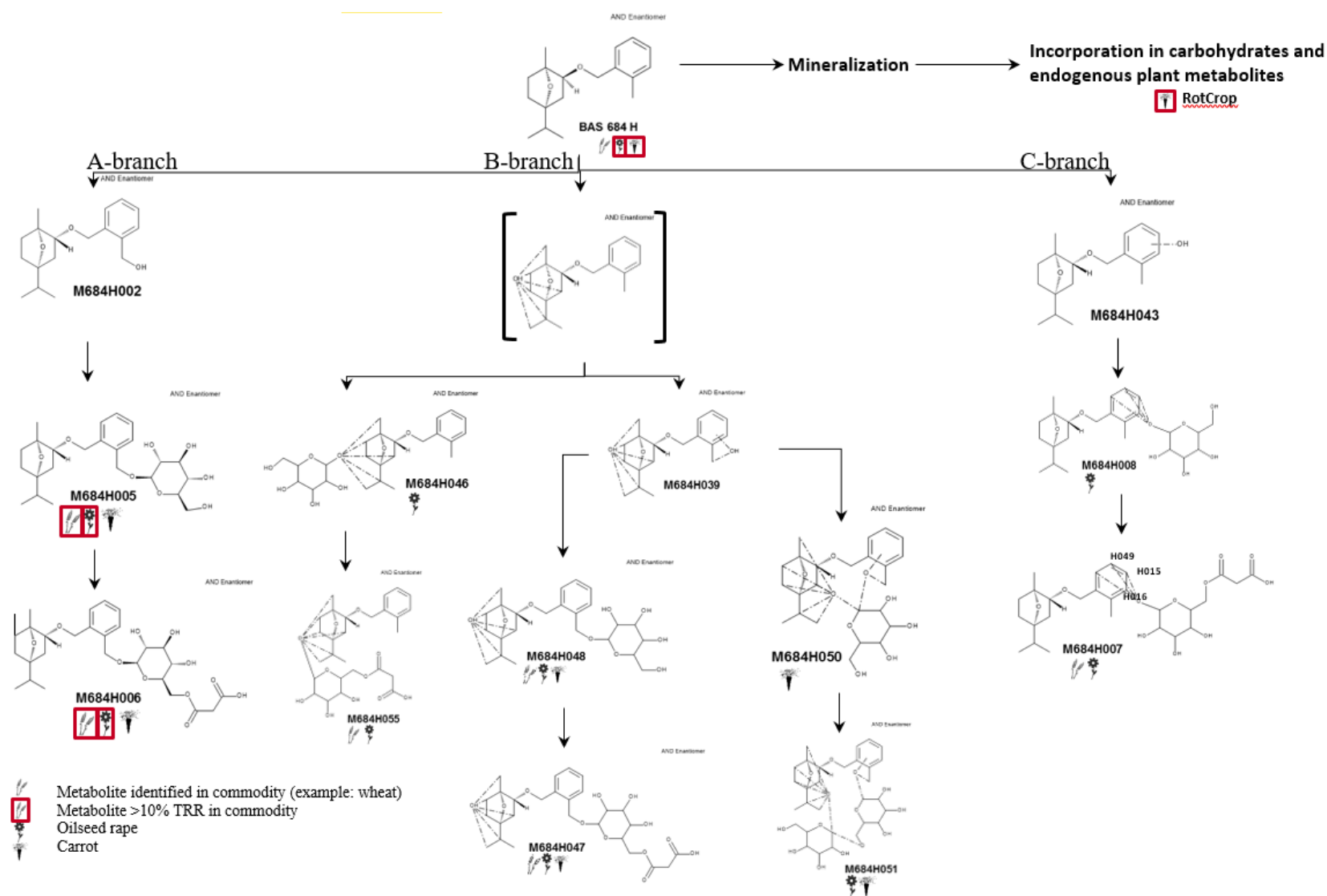
Table 7-96 Metabolites in plant matrices

| <b>A-branch</b><br>(hydroxylation at the methyl group and further phase I and phase II metabolites) | <b>B-branch</b><br>(hydroxylation at the cineol-part and further phase I and phase II metabolites) | <b>C-branch</b><br>(hydroxylation at the phenyl-ring and further phase I and phase II metabolites) |
|---|--|--|
| <b>M684H005</b>   | M684H046   | M684H007   |
| <b>M684H006</b>   | M684H047   | M684H008   |
|   | M684H048   |  |
|   | M684H050   |  |
|   | M684H051   |  |
|   | M684H055   |  |

Metabolites with a content of >10% TRR are indicated in **bold** font



Figure 7-12 BAS 684 H metabolic routes in plant



**B.7.2.2. Poultry**

|                    |  |
|--------------------|--|
| <b>Report:</b>     | CA 6.2.2/1<br>[REDACTED], 2018 a<br>The metabolism of (14C)-Reg. No 900202 (BAS 684 H) in laying hens<br>2017/1068568  |
| <b>Guidelines:</b> | EPA 860.1000, EPA 860.1300, EEC 91/414 (7030(VI/95 Rev. 3), JMAFF No 59<br>NohSan No 4200, OECD Test Guideline 503 - Metabolism in livestock, PMRA<br>Residue Chemistry Guidelines Section 98.2 Nature of the Residue - Plants - Livestock<br>(Canada) |
| <b>GLP:</b>        | yes  |

***Materials and methods******Materials*****1. Phenyl-label BAS 684 H (CAS No. 87818-31-3)**

|                     |   |
|---------------------|---|
| <b>Description:</b> | Phenyl-U- <sup>14</sup> C, 17.1 MBq/mg (specific activity of a.s.), included in a 1:4:4 mixture of <sup>14</sup> C: <sup>13</sup> C: <sup>12</sup> C test item (phenyl-U- <sup>14</sup> C : benzyl- <sup>13</sup> C : unlabelled <sup>12</sup> C) |
| <b>Lot/Batch #:</b> | 1147-2001 (phenyl-U- <sup>14</sup> C)<br>1159-1012 (benzyl- <sup>13</sup> C)<br>L87-84 (unlabelled)   |
| <b>Purity:</b>      | Phenyl label: 97.0 % (98.9 % radiochemical purity)<br>Benzyl label: 99.6 %<br>Unlabelled: 99.0 %  |

**2. Cyclohexane-label BAS 684 H (CAS No. 87818-31-3)**

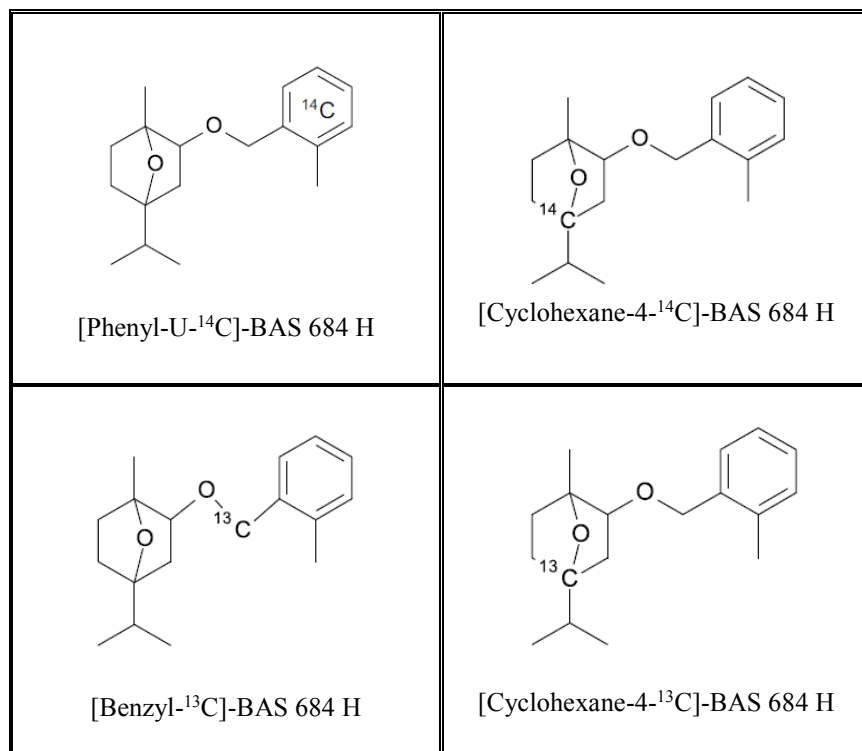
|                     |  |
|---------------------|--|
| <b>Description:</b> | Cyclohexane-4- <sup>14</sup> C, 7.75 MBq/mg (specific activity of a.s.), included in a 0.7:1:1 mixture of <sup>14</sup> C: <sup>13</sup> C: <sup>12</sup> C test item (cyclohexane-4- <sup>14</sup> C : cyclohexane-4- <sup>13</sup> C : unlabelled <sup>12</sup> C) |
| <b>Lot/Batch #:</b> | 1146-1001 (cyclohexane-4- <sup>14</sup> C)<br>1165-2001 (cyclohexane-4- <sup>13</sup> C)<br>L87-84 (unlabelled)  |
| <b>Purity:</b>      | Cyclohexane label ( <sup>14</sup> C): 99.4 % (99.3 % radiochemical purity)<br>Cyclohexane label ( <sup>13</sup> C): 98.1 %<br>Unlabelled: 99.0%  |

***Methods***

The metabolism of BAS 684 H was investigated in hens (breed *Isa Brown*) following repeated oral administration of <sup>14</sup>C-BAS 684 H, labelled either in the phenyl ring (phenyl label) or the cyclohexane ring (cyclohexane label). See

Figure 7-13.

Figure 7-13 Structures of radiolabelled BAS 684 H



A total of 20 hens were dosed with radiolabelled BAS 684 H at a nominal dose of 12 mg/kg feed (0.90 – 0.94 mg/kg bw/day, 900 - 940 N for poultry) for 11 consecutive days (10 hens per label). Each <sup>14</sup>C-labelled test item was mixed with <sup>13</sup>C-labelled test item and unlabelled test item (ratios are given in Table 7-97). The test items were prepared in gelatine capsules, orally administered (once daily) using a dosing gun. The actual dose was based on the food consumption (dry weight) on the previous day. Dose capsules were prepared daily from formulations stored at -20 °C, the capsules were sealed and used immediately for dosing each hen. Prior to the preparation of the actual dose formulation, extensive trial stability tests were carried out on the dose formulation over a 15-day period to ensure the dose solutions remained stable over the daily dosing period (completed as part of the lactating ruminant study). The test item was found to be stable in a solution of PEG 400 for a period of 9 days when stored at -20 °C. For this reason, two dose formulations were prepared for each radiolabel, the first to cover the day 1 to day 9 dosing and the second to cover the remaining dosing days (days 10 and 11). Prior to the preparation of the first day dose capsule and at the end of dosing, the radiochemical purity of the dose formulation was determined by HPLC. The mean achieved daily dose administered was 12.03 and 11.99 mg eq/kg food consumer (dry weight equivalent) corresponding to 0.90 and 0.94 mg/kg bw/d. Details of the study outline are summarized in Table 7-97.

Table 7-97 Dosing of lactating hens with BAS 684 H

| Animal No's                  | Treatment period (days) | Isotope ratio   | Nominal daily dose | Actual daily dose <sup>1, 2</sup> |              | Time of sacrifice <sup>3</sup> (hours) |
|------------------------------|-------------------------|---|--------------------|-----------------------------------|--------------|--|
|                              |                         |   | [mg/kg feed]       | [mg/kg feed]                      | [mg/kg bw/d] |  |
| Phenyl label<br>10 hens      | 11                      | 1:4:4<br>( <sup>14</sup> C: <sup>13</sup> C: <sup>12</sup> C)   | 12                 | 12.03                             | 0.90         | 3 – 6                                  |
| Cyclohexane label<br>10 hens | 11                      | 0.7:1:1<br>( <sup>14</sup> C: <sup>13</sup> C: <sup>12</sup> C) | 12                 | 11.99                             | 0.94         | 3 – 6                                  |

1 Mean value of day 1 – 11

2 Using the mean animal weight at application day 1

3 Hours after last dose

Excreta and cage wash (using ethanol and water) samples were collected prior to dose administration and for each 24 h period until sacrifice. The daily excreta samples were pooled for each dose group and the total weight recorded. The daily cage wash and case rinse samples were pooled for each dose group and the total weight of each recorded. Daily excreta samples were stored in a freezer at *ca.* -20 °C following collection.

Eggs were collected pre-dose and then twice daily until sacrifice (am and pm) during the dosing period. Eggs were separated into yolk and white. A composite egg yolk sample was prepared for each dose group by combining the yolk samples from each hen. Similarly, a composite white sample for each dosing group was prepared by combining the individual egg white samples. Egg shells were retained but were not analysed.

The hens were sacrificed approximately 3 – 6 hours after the final dose. Edible tissues (liver, kidney, muscle and fat), bile, blood, carcass and the GI tract were removed *post mortem*. Any whole eggs still in the oviduct at termination were collected.

Tissues were homogenized prior to analysis. Faeces samples were homogenised to produce a slurry using methanol. Tissues were grated with the aid of dry ice then further homogenised to a fine powder. Blood samples were mixed by vortexing to ensure homogeneity. Composite daily egg yolk and egg white samples were stirred until homogenous. Following processing (and radioactive content determination) each tissue sample was stored at *ca.* -20 °C.

Generally, samples obtained from ten hens were pooled to generate a single sample per label. For muscle, the breast and leg (including thigh) muscle were combined in a ratio of 1/1 (w/w). Similarly, a composite fat sample per label was prepared by combining the omental and subcutaneous fat (including skin) in a ratio of 1/1 (w/w). Excreta sampled within 168 h to 240 h (7 – 10 days) and egg yolk and egg white collected from 168 h to 240 h (7 – 10 days) were used for pooling.

No storage stability investigations were conducted within the study report. The storage period from sampling to analysis is in the range of 263 – 467 days (for initial analyses). As this exceeds the six months acceptable limit further consideration is required, this is provided in the results and discussion section below.

Prior to extraction, samples were combusted to verify the total radioactive residues (TRR). Aliquots of homogenized egg yolk, egg white, muscle and liver (all both labels) were extracted three times with methanol and two times with water. Aliquots of fat were extracted three times with iso-hexane/acetonitrile (1/1; V/V) and two times with water. After each extraction step, solid material was separated from the extract by centrifugation and the iso-hexane/acetonitrile extracts (fat) were separated with a separating funnel. The respective methanol and water extracts (egg yolk, egg white, muscle and liver) or iso-hexane, acetonitrile and water extracts (fat) were combined, adjusted to a defined volume with appropriate solvents and aliquots were radio-assayed. The residue after solvent extraction was subjected to combustion analysis. HPLC analysis was carried out after clean-up (partition and/or SPE fractionation) of samples containing sufficient levels of radioactive residues.

Solubilization of the RRR: As high levels of radioactive residues were detected in the residual radioactive residues (RRR) after solvent extraction of egg yolk (phenyl label) and liver (both labels), these RRRs were further subjected to solubilization. Subsamples of egg yolk (phenyl label) and liver (both labels) RRRs were resuspended in tris/hydrochloric acid buffer at pH 7.0, protease was added, and the mixtures were incubated at 37 °C for 3.5 h. Afterwards, another portion of protease was added and incubated at 37 °C for 24 h. Aliquots of the supernatant and the remaining solid were radio-assayed. The protease solubilizates were cleaned-up by SPE-fractionation. The flow through and the eluate of the washing step was designated as SPE-eluate 1. Elution was performed in two steps with water/methanol (1:1 v:v) and designated as SPE-eluate 2 and methanol designated as SPE-eluate 3. The aliquots of all eluates were radio-assayed. The SPE-eluates 2 of liver were concentrated, diluted with an acetonitrile/water mixture or the corresponding mobile phase and analysed by HPLC.

Isolation and identification of metabolites: Structure elucidation of metabolites was based on HPLC-MS analysis of fractions obtained from extracts of egg white and liver, and peak assignment was primarily based on co-chromatography experiments with reference samples (urine from the related rat metabolism study), the diluted application formulation (for identity of parent BAS 684 H) or MS identified metabolites isolated from egg white. Peak assignment within individual matrices was conducted by comparison of the metabolite patterns and retention times with those of MS-identified samples or the reference items. Specific information is provided for each metabolite below:

Table 7-98 How identification of metabolites was achieved

| Metabolite                     | Initial identification   |
|--------------------------------|--|
| BAS 684 H:                     | Co-chromatography with the diluted application formulation using HPLC-UV method LC02 – as this is a known structure it is considered acceptable to have only used one analytical technique.                  |
| M684H001:                      | HPLC-UV co-chromatography with rat urine (from rat metabolism study ID 741154) using two dissimilar HPLC methods (LC02 and LC04)   |
| M684H010:                      | HPLC-UV co-chromatography with rat urine (from rat metabolism study ID 741154) using two dissimilar HPLC methods (LC02 and LC04)   |
| M684H011:                      | HPLC-UV co-chromatography with rat urine (from rat metabolism study ID 741154) using two dissimilar HPLC methods (LC02 and LC04)   |
| M684H021 22.1_LC02 / M684H058: | HPLC-MS (isolated and cleaned up sub-fractions from egg white and liver)<br>HPLC-UV co-chromatography with rat urine (from rat metabolism study ID 741154) using two dissimilar HPLC methods (LC02 and LC04) |
| M684H021 24.6_LC02:            | HPLC-MS (isolated and cleaned up sub-fractions from egg white and liver)   |
| M684H021 32.5_LC02:            | HPLC-MS (isolated and cleaned up sub-fractions from egg white and liver)   |
| M684H021 33.5_LC02:            | HPLC-MS (isolated and cleaned up sub-fractions from egg white and liver)   |
| M684H026:                      | HPLC-UV co-chromatography with rat urine (from rat metabolism study ID 741154) using two dissimilar HPLC methods (LC02 and LC04)   |
| M684H027:                      | HPLC-MS (isolated and cleaned up sub-fractions from egg white and liver) (co-eluting with MS-characterised compound M306 at trace levels)  |
| M684H039:                      | HPLC-MS (isolated and cleaned up sub-fractions from egg white and liver)   |
| M684H059:                      | HPLC-MS (isolated and cleaned up sub-fractions from egg white and liver)   |

The following details are provided for HPLC methods LC02 and LC04, these details confirm the two methods are dissimilar:

| Method | Column  | Eluents  |   | Flow rate (mL/min) | Gradient  |     |
|--------|---|--|---|--------------------|-----------|-----|
|        |   | A  | B   |                    | Tim (min) | % B |
| LC02   | Restek<br>Raptor Biphenyl<br>5 µm<br>(250 x 4.6 mm)<br>at 25 °C | H <sub>2</sub> O/<br>HCOOH<br><br>1000/1 (v/v) | CH <sub>3</sub> OH/<br>CH <sub>3</sub> CN/<br>HCOOH<br><br>600/400/1<br>(v/v/v) | 1.0                | 0         | 10  |
|        |   |  |   |                    | 15        | 30  |
|        |   |  |   |                    | 75        | 45  |
|        |   |  |   |                    | 85        | 100 |
|        |   |  |   |                    | 95        | 100 |
|        |   |  |   |                    | 95.1      | 10  |
|        |   |  |   |                    | 105       | 10  |
| LC04   | Phenomenex<br>Luna PFP<br>5 µm<br>(250 x 4.6 mm)<br>at 25 °C    | H <sub>2</sub> O/<br>HCOOH<br><br>1000/2 (v/v) | CH <sub>3</sub> OH/<br>CH <sub>3</sub> CN/<br>HCOOH<br><br>300/700/2<br>(v/v/v) | 1.0                | 0         | 10  |
|        |   |  |   |                    | 20        | 10  |
|        |   |  |   |                    | 75        | 50  |
|        |   |  |   |                    | 85        | 100 |
|        |   |  |   |                    | 95        | 100 |
|        |   |  |   |                    | 95.1      | 10  |
|        |   |  |   |                    | 105       | 10  |

For metabolite M684H021 four isomers have been identified, each has been specified based on the retention time from HPLC method LC02; M684H021\_22.1\_LC02, M684H021\_24.6\_LC02, M684H021\_32.5\_LC02 and M684H021\_33.5\_LC02. In addition, metabolite M684H021\_22.1\_LC02 co-elutes with M684H058. The applicant has provided the following additional information for these metabolites:

*The detected isomers can represent regio-isomers (differing in their position of the hydroxy group(s)) and in case of M684H022 additionally diastereomers (due to the conjugation). However, it is not possible to assign a specific isomeric form to a specific retention time/peak via MS/MS, since the m/z ratio is the same and the fragmentation pattern in case of cinmethylin did not allow a clear structural differentiation of the possible isomers. Thus, they were differentiated by their retention time.*

This explanation is considered acceptable. In accordance with OECD 503 metabolism in livestock guidance document, if the metabolite is detected at > 10 %, and 0.01 – 0.05 mg eq/kg significant attempts to identify these metabolites should be made. However, as this metabolism study has been conducted with a highly exaggerated feeding level (900 – 940 N), and the toxicity of these metabolites is considered as the sum of the isomers, full identification of each individual isomer is not considered necessary.

Metabolite M684H027 co-eluted with a MS-characterised compound M306. Within the study report M306 is stated to be parent (BAS 684 H) plus 2xO with a nominal mass of 306 u. As this compound is only present at trace levels no additional data are required.

As the precise position of hydroxylation/conjugation at the cyclohexane moiety is not known for metabolites M684H021, M684H039, M684H027, these metabolites are shown as generic structures (indicated by a ‘dotted’ line). Further details about these structures (‘Markush system’) are shown in Table 7-1.

Enzymatic cleavage of phase II metabolites: For investigation of the label specific metabolite eluting at approximately 7.5 min aliquots of fractions obtained from methanol extracts of egg white, muscle and liver (all cyclohexane label) were subjected to enzyme treatment. Samples were mixed with sodium acetate buffer and  $\beta$ -glucuronidase/arylsulfatase and the mixtures were incubated at 37 °C for 24 h. The samples obtained after incubation were investigated by HPLC.

Enantiomer specific analysis: In order to analyse whether one isomer of BAS 684 H was preferably metabolised in hens, enantiomer-specific analyses of the parent compound, isolated from fat (cyclohexane label), was performed representatively. A subsample of the combined iso-hexane extract of fat was partitioned against acetonitrile and fractionated by HPLC to isolate the parent compound BAS 684 H. The collected fraction and the application solution of the phenyl label were analysed by an enantiomer-specific HPLC method.

## ***Results and discussion***

### ***Total radioactive residue***

The overall recovery of radioactive residues is provided in Table 7-99 as % administered dose and

Table 7-100 expressed as mg eq/kg BAS 684 H. The recoveries are good (> 95 %) and similar for each of the two labels.

Approximately 98.3 % and 96.9 % of the administered dose was recovered in total for the phenyl and cyclohexane label, respectively. The main fraction was excreted via excreta accounting to approximately 91.3 % (phenyl label) and 87.3 % (cyclohexane label). Radioactive residues recovered in the cage wash and rinse accounted for up to 3.8 % (phenyl label) and 4.6 % (cyclohexane label). Radioactive residues associated with edible portions (egg and tissues) accounted for up to 0.1 % (phenyl label) and 0.2 % of the administered dose (cyclohexane label).

Table 7-99 Distribution of radioactive residues in tissue, excreta and egg of laying hens after administration of BAS 684 H for 11 days

| Matrix                    | [% dose]     |                   |
|---------------------------|--------------|-------------------|
|                           | Phenyl label | Cyclohexane label |
| <b>Excreta</b>            | 91.3         | 87.3              |
| <b>Cagewash</b>           | 3.8          | 4.6               |
| <b>Cage rinse</b>         | 2.8          | 4.5               |
| <b>Egg yolk</b>           | <0.1         | 0.1               |
| <b>Egg white</b>          | 0.1          | 0.2               |
| <b>Partly formed eggs</b> | 0.1          | <0.1              |
| <b>Liver</b>              | 0.1          | 0.1               |
| <b>Fat<sup>1</sup></b>    | <0.1         | <0.1              |
| <b>Muscle<sup>1</sup></b> | 0.1          | 0.1               |
| <b>Total</b>              | 98.3         | 96.9              |

<sup>1</sup> Calculated from weight of tissue collected at necropsy

In the present study, the TRR was calculated by summarising the extractable radioactive residue (ERR) and the residual radioactive residue (RRR) after solvent extraction. In general, the TRR measured was comparable the TRR calculated, the exception to this are the muscle, liver and fat matrices in the phenyl labelled study. The results are summarized in



Table 7-100. For the differences in TRR measured and TRR calculated in muscle, liver and fat the following reasoning has been provided by the applicant:

*In this study the in-life phase had been performed at a different test facility (██████). During this in-life phase the total radioactive residues were determined in the tissues, before shipment of samples to ██████ for further analysis. These measured TRR values by ██████ are shown in Appendix 5 of the final study report (see pp. 243 +249 of the final report, Tables 6 and 12 of the in-life phase report). After seeing the obvious discrepancies between the measured TRR (at ██████) and the calculated TRR for muscle, liver and fat of the phenyl label, we compared both values with the measured TRRs provided by ██████. All values are shown in the following table:*

| <b>Matrix</b> | <b>TRR measured ██████<sup>1)</sup></b> | <b>TRR calculated ██████<sup>2)</sup></b> | <b>TRR measured ██████<sup>3)</sup></b> |
|---------------|---|---|---|
|               | [mg eq/kg]                              | [mg eq/kg]                                | [mg eq/kg]                              |
|               | <b>Phenyl label</b>                     |   |   |
| <b>Muscle</b> | 0.036                                   | 0.051                                     | 0.051                                   |
| <b>Liver</b>  | 0.183                                   | 0.223                                     | 0.228                                   |
| <b>Fat</b>    | 0.058                                   | 0.083                                     | 0.081                                   |

1) TRR determined by combustion at ██████

2) TRR calculated as sum of measured residues in extracts plus non-extracted residue at ██████

3) TRR determined by combustion at in-life facility ██████

*As can be seen, the calculated TRR is in good agreement with the measured TRR provided by the in-life test facility ██████.*

*Looking into the data, an obvious reason for the significant deviation of the TRR values at ██████ is difficult to retrieve. The standard deviation between the individual replicates of the combustion were within the admissible range specified by our SOPs. One possible explanation could be that the combustion process due to the rather large amount (0.9g, which is an amount at the upper end for the combustion equipment) was incomplete, which has sometimes been observed with fat containing matrices like muscle and fat.*

This explanation is considered sufficient, as the TRR measured values are not used further in the evaluation and risk assessment (% TRR values are calculated from the 'TRR calculated') no additional information is required. A good agreement is seen between the TRR calculated and the TRR measured at the in-life facility ██████.

Unless otherwise stated, % TRR values are calculated using the TRR calculated (sum of ERR + RRR). The main portions of radioactive residues were recovered in excreta (6.397 – 7.010 mg eq/kg). In the edible matrices, the highest TRR concentrations were calculated for liver (0.221 – 0.223 mg eq/kg). For all other matrices, the calculated TRR was in a range from 0.051 mg eq/kg to 0.083 mg eq/kg (phenyl label) and from 0.078 mg eq/kg to 0.115 mg eq/kg (cyclohexane label).

Table 7-100 Total radioactive residues in samples from laying hens following treatment with BAS 684 H for 11 days

| Matrix                  | Sampling time           | TRR measured<br>[mg eq/kg] | TRR calculated<br>[mg eq/kg] | TRR measured<br>[mg eq/kg] | TRR calculated<br>[mg eq/kg] |
|-------------------------|-------------------------|----------------------------|------------------------------|----------------------------|------------------------------|
|                         |                         | Phenyl label               |                              | Cyclohexane label          |                              |
| Excreta                 | Day 7-10<br>(168-240 h) | 7.010                      | Not determined               | 6.397                      | Not determined               |
| Egg yolk                |                         | 0.051                      | 0.058                        | 0.071                      | 0.078                        |
| Egg white<br>(workup 1) |                         | 0.063                      | 0.065                        | 0.112                      | 0.115                        |
| Egg white<br>(workup 2) |                         | 0.063                      | 0.063                        | Not applied                |                              |
| Muscle                  | Terminal                | 0.036                      | 0.051                        | 0.079                      | 0.096                        |
| Liver                   |                         | 0.183                      | 0.223                        | 0.202                      | 0.221                        |
| Fat                     |                         | 0.058                      | 0.083                        | 0.071                      | 0.079                        |

The concentration of radioactive residues in eggs are provided in Table 7-101. Daily egg samples were obtained on ten consecutive days. Only very low proportions of the administered dose were found. For the phenyl label, the level of radioactive residues increased to a plateau at 7 and 9 days with a concentration of 0.076 mg eq/kg (egg white) and 0.053 mg eq/kg (egg yolk), respectively. For the cyclohexane label, a plateau of 0.122 mg eq/kg (egg white) and 0.070 mg eq/kg (egg yolk) was reached after 7 days. There was then a very gradual increase in concentration in the egg yolk up to a maximum of 0.075 mg eq/kg at day 10.

Table 7-101 Concentration of radioactive residues in egg following oral administration of BAS 684 H to laying hens

| Time<br>[hours]   | Egg white<br>[mg eq/kg] | Egg yolk<br>[mg eq/kg] | Whole egg equivalent<br>[mg eq/kg] <sup>1</sup> |
|-------------------|-------------------------|------------------------|---|
| Phenyl label      |                         |                        |   |
| 24                | 0.047                   | 0.010                  | 0.035   |
| 48                | 0.060                   | 0.019                  | 0.048   |
| 72                | 0.054                   | 0.023                  | 0.045   |
| 96                | 0.055                   | 0.033                  | 0.048   |
| 120               | 0.058                   | 0.039                  | 0.052   |
| 144               | 0.064                   | 0.047                  | 0.059   |
| 168               | 0.076                   | 0.049                  | 0.069   |
| 192               | 0.063                   | 0.051                  | 0.061   |
| 216               | 0.064                   | 0.053                  | 0.060   |
| 240               | 0.063                   | 0.050                  | 0.058   |
| Cyclohexane label |                         |                        |   |
| 24                | 0.044                   | 0.009                  | 0.032   |
| 48                | 0.120                   | 0.033                  | 0.090   |
| 72                | 0.106                   | 0.039                  | 0.083   |
| 96                | 0.115                   | 0.051                  | 0.094   |
| 120               | 0.100                   | 0.055                  | 0.084   |
| 144               | 0.110                   | 0.063                  | 0.095   |
| 168               | 0.122                   | 0.070                  | 0.105   |
| 192               | 0.113                   | 0.072                  | 0.100   |
| 216               | 0.119                   | 0.073                  | 0.104   |
| 240               | 0.114                   | 0.075                  | 0.102   |

<sup>1</sup> Radioactive residues (in mg eq/kg) were determined by LSC measurement separately for egg white and egg yolk. Then the absolute amount of residue (in mg) that is present in the entire egg white or egg yolk sample was calculated by multiplying the mg eq/kg residue with the sample weight (e.g. for egg white Day 1: 0.047 mg eq/kg x 0.368 kg = 0.017 mg total residue). For the whole egg, the sample weight was calculated as sum of weight of yolk and white. Then the absolute amount present in this entire yolk + white sample was calculated. From the total sample weight and the total absolute residue in this sample weight, the relative residue in mg eq/kg was calculated; e.g. whole egg Day 1 0.019 mg eq/kg / (545 g/1000) = 0.035 mg eq/kg. Table 4 from the

in-life phase report (can be found on pp.241+242 of the final report DocID 2017/1068568) shows the numbers from which the way of calculation can be derived. Whole eggs are not considered further in the evaluation – separate analysis of the egg white and egg yolk have been provided.

Overall, low levels (< 0.1 mg eq/kg) were found in egg yolk, egg white (phenyl label only), muscle and fat. Higher residues (> 0.1 mg eq/kg) were found in egg white (cyclohexane label) and liver. Both the phenyl and cyclohexane label in general showed similar results.

For muscle, the TRR (measured) was 0.036 mg eq/kg for the phenyl label and 0.079 mg eq/kg for the cyclohexane label. Similarly, for fat the TRR (measured) was 0.058 mg eq/kg for the phenyl label and 0.071 mg eq/kg for the cyclohexane label. For liver, the TRR (measured) was 0.183 mg eq/kg for the phenyl label and 0.202 mg eq/kg for the cyclohexane label. Egg yolk showed lower levels of radioactive residues compared to egg white. For egg yolk the TRR (measured) was 0.051 mg eq/kg for the phenyl label and 0.071 mg eq/kg for the cyclohexane label. For egg white the TRR (measured) was 0.063 mg eq/kg for the phenyl label and 0.112 mg eq/kg for the cyclohexane label.

For excreta, the phenyl label showed higher residue levels than the cyclohexane label with TRR (measured) values of 7.010 mg eq/kg and 6.397 mg eq/kg respectively.

#### *Extractability*

The extractabilities (ERR – extractable radioactive residues) of <sup>14</sup>C residue from egg yolk, egg white, muscle, liver, and fat are summarized in

Table 7-102.

Egg yolk, egg white, muscle and liver samples were extracted with methanol and water. The main fraction of the radioactive residue was detected in the methanol extracts (up to 97.4 % TRR and 98.4 % TRR for the phenyl and cyclohexane label, respectively). Somewhat lower portions were extracted with methanol from liver and egg yolk samples (68.0 – 88.0 % TRR). Minor amounts were recovered in the water extracts accounting for up to 4.1 % TRR and 3.2 % TRR for the phenyl and cyclohexane label, respectively.

Fat samples were extracted with a mixture of acetonitrile and iso-hexane, and subsequently with water. In the acetonitrile extract 70.6 – 71.2 % TRR were recovered. The portions of radioactive residues extracted with iso-hexane were 15.6 – 23.2 % TRR. Finally, radioactive residues extracted with water amounted to 4.1 – 10.6 % TRR.

In general, the extractability was high ranging from 93.0 % TRR to 99.3 % TRR, except for egg yolk (phenyl label; 77.0% TRR) and liver (71.4% TRR and 82.5% TRR for the phenyl and cyclohexane labels, respectively). Radioactive residues in the RRR obtained after extraction of egg yolk (phenyl label) and liver amounted to 17.5 – 28.6 % TRR, which were further investigated. The RRR of all other relevant matrices were below or equal to 7.0 % TRR (0.004 mg eq/kg, phenyl label) and 8.9 % TRR (0.007 mg eq/kg, cyclohexane label).

Table 7-102 Extractability of radioactive residues from laying hens' matrices following treatment with BAS 684 H for 11 days

| Matrix                  | Methanol extract /<br>acetonitrile extract <sup>1</sup> |         | Iso-hexane<br>extract <sup>2</sup> |         | Water extract <sup>2</sup> |         | ERR <sup>3</sup> |         | RRR <sup>4</sup> |         | TRR <sup>5</sup> |
|-------------------------|---|---------|------------------------------------|---------|----------------------------|---------|------------------|---------|------------------|---------|------------------|
|                         | [mg eq/kg]  | [% TRR] | [mg<br>eq/kg]                      | [% TRR] | [mg<br>eq/kg]              | [% TRR] | [mg<br>eq/kg]    | [% TRR] | [mg<br>eq/kg]    | [% TRR] | [mg<br>eq/kg]    |
| Phenyl label            |   |         |                                    |         |                            |         |                  |         |                  |         |                  |
| Egg yolk                | 0.042   | 72.9    | Not applied                        |         | 0.002                      | 4.1     | 0.045            | 77.0    | 0.013            | 23.0    | 0.058            |
| Egg white<br>(workup 1) | 0.063   | 97.4    | Not applied                        |         | 0.001                      | 1.5     | 0.064            | 98.9    | 0.001            | 1.1     | 0.065            |
| Egg white<br>(workup 2) | 0.063   | 100.0   | Not applied                        |         | Not applied                |         | 0.063            | 100.0   | Not applied      |         | 0.065            |
| Muscle                  | 0.046   | 91.3    | Not applied                        |         | 0.001                      | 1.7     | 0.047            | 93.0    | 0.004            | 7.0     | 0.051            |
| Liver                   | 0.152   | 68.0    | Not applied                        |         | 0.008                      | 3.4     | 0.160            | 71.4    | 0.064            | 28.6    | 0.223            |
| Fat                     | 0.059   | 70.6    | 0.013                              | 15.6    | 10.6                       | 15.5    | 96.7             | 139.7   | 0.003            | 3.3     | 0.083            |
| Cyclohexane label       |   |         |                                    |         |                            |         |                  |         |                  |         |                  |
| Egg yolk                | 0.068   | 88.0    | Not applied                        |         | 0.002                      | 3.2     | 0.071            | 91.1    | 0.007            | 8.9     | 0.078            |
| Egg white               | 0.113   | 98.4    | Not applied                        |         | 0.001                      | 1.0     | 0.114            | 99.3    | 0.001            | 0.7     | 0.118            |
| Muscle                  | 0.093   | 97.3    | Not applied                        |         | 0.001                      | 0.7     | 0.094            | 98.0    | 0.002            | 2.0     | 0.096            |
| Liver                   | 0.177   | 80.3    | Not applied                        |         | 0.005                      | 2.2     | 0.182            | 82.5    | 0.039            | 17.5    | 0.221            |
| Fat                     | 0.057   | 71.2    | 0.018                              | 23.2    | 0.003                      | 4.1     | 0.078            | 98.5    | 0.001            | 1.5     | 0.079            |

1 Egg yolk, egg white, muscle and liver were extracted with methanol; fat was extracted with acetonitrile; values measured from pooled extracts

2 Values measured from pooled extracts

3 Extractable radioactive residues

4 Residual radioactive residues (after solvent extraction)

5 Sum of ERR + RRR

Minor variations are due to differing precision in numbers

#### *Solubilisation of radioactive residues*

The residue after solvent extraction of egg yolk (phenyl label) and liver (both labels) was further investigated and the results are summarized in Table 7-103.

Protease incubation released 9.5 % TRR in liver (cyclohexane label), 19.8 % TRR in liver (phenyl label) and 16.2 % TRR for egg yolk (phenyl label). The final residues after protease solubilization were each below or equal to 0.018 mg eq/kg (liver, phenyl label) or 8.9 % TRR (egg yolk, phenyl label).

Table 7-103 Characterization of the radioactive residues after solvent extraction in laying hen samples

| Fraction / Solubilize  | Egg yolk            |             | Liver        |             | Liver                    |             |
|--|---------------------|-------------|--------------|-------------|--------------------------|-------------|
|  | [mg eq/kg]          | [% TRR]     | [mg eq/kg]   | [% TRR]     | [mg eq/kg]               | [% TRR]     |
|  | <b>Phenyl label</b> |             |              |             | <b>Cyclohexane label</b> |             |
| <i>Residue after solvent extraction</i>                        | 0.013               | 23.0        | 0.064        | 28.6        | 0.039                    | 17.5        |
| Protease solubilize  | 0.009               | 16.2        | 0.044        | 19.8        | 0.021                    | 9.5         |
| Final residue  | 0.005               | 8.9         | 0.018        | 8.1         | 0.007                    | 3.1         |
| <b>Sum of solubilized radioactive residues + final residue</b> | <b>0.015</b>        | <b>25.1</b> | <b>0.062</b> | <b>27.9</b> | <b>0.028</b>             | <b>12.6</b> |

#### *Characterisation and Identification*

Identification of metabolites was based on HPLC-MS analysis of fractions obtained from extracts of egg white and liver (phenyl label) and co-chromatography experiments with reference samples, diluted application solution (phenyl label) and isolated, MS identified metabolites from egg white. In some cases, peaks were assigned by comparison of the retention times and metabolite patterns. A summary of identified and characterized radioactive residues is compiled in

Table 7-104 and

Table 7-105.

In each cyclohexane label sample, an unidentified peak at approximately 7.5 minutes was detected. As this peak was only detected in samples of the cyclohexane label, it seems to be a label specific metabolite. Additional attempts were applied for investigation of this peak, however no unambiguous peak assignment using co-chromatography experiments was possible for this compound. The application has provided the following additional information (edited by HSE for the evaluation):

*Co-chromatography experiments conducted for these matrices show a direct comparison of the metabolite pattern of the cyclohexane label poultry matrices with the pattern observed in urine from female rats (urine from dose group CF, 0-120h, see Volume 3, Section 6). At first, it seemed that the peak at 7.5 min could be identical with the peak at 7.7 min in rat urine, which was identified in the rat study as M684H029, the glucuronide conjugate of M684H026 (label specific for the cyclohexane label), but co-chromatography on two HPLC systems was not conclusive on that. The subsequent experiments with  $\beta$ -glucuronidase however showed no cleavage of the peak at 7.5 min, thus it cannot be the glucuronide M684H029. Another HPLC system (LC07) was especially developed to further investigate this peak, which seems to have high polarity, and gain a longer column retention. The obtained chromatograms show that the "peak at 7.5 min" is obviously no single compound, but contains at least two components. On this LC system, the peak at 7.5 min split up to the following individual peaks:*

*Egg white: 0.011 mg eq/kg 11%TRR and 0.003 mg eq/kg 2.3% TRR*

*Muscle: 0.011 mg eq/kg 11% TRR and 0.004 mg eq/kg 4.3 %TRR*

*Liver: 0.019 mg eq/kg 8.4% TRR and 0.005 mg eq/kg 2.4% TRR*

*In light of the quite low amounts, no clean-up and MS/MS analysis nor NMR analysis has been undertaken. It was assumed that in light of these low amounts and the fact that the other major peaks in the metabolite patterns have been identified, characterization of this peak might be acceptable. It has been characterized as being composed of at least two components, both polar in nature, more polar than M684H026, not being phase II conjugates. Structures similar to M684H026, but with additional functional groups arising from phase I metabolism (additional OH group or further oxidation to carboxyl group) are deemed plausible from a metabolism point of view. The unidentified nature of this peak is considered to not pose any risk to the consumer, since the feedburden for poultry (considering cereals and oilseeds) is very low (even below the trigger of 0.004 mg/kg bw/d). The dose level of the study represents a 900N overdosing, thus no relevant residues are expected for the consumer. The feedburden will also in future not increase, since cereals and oilseed rape are the only possible uses for BAS 684 H from a biology point of view.*

This explanation is considered sufficient, based on the low levels of this unidentified peak, and the overdosing level in the study it is acceptable to have characterised the metabolites rather than fully identified.

#### Egg yolk

Five (phenyl label) or four (cyclohexane label) metabolites and the unchanged parent compound were identified in egg yolk. For the phenyl label, the metabolites M684H039 and M684H059 accounted for the main portions with 0.006 mg eq/kg or 10.9% TRR and 0.007 mg eq/kg or 12.5% TRR, respectively. The parent compound BAS 684 H and the metabolites M684H001, M684H010 and M684H021\_24.6\_LC02 ranged from 0.001 mg eq/kg to 0.005 mg eq/kg (1.6 – 8.3% TRR). For the cyclohexane label, the metabolite M684H026 accounted for the main portion with 0.027 mg eq/kg or 34.7% TRR. The parent compound BAS 684 H and the metabolites M684H001, M684H021\_24.6\_LC02 and M684H039 ranged from 0.001 mg eq/kg to 0.005 mg eq/kg (1.4 – 6.2% TRR). The remaining peaks not assigned by HPLC were at a maximum of 0.002 mg eq/kg or 4.3 % TRR (phenyl label) and 0.002 mg eq/kg or 3.0 % TRR (cyclohexane label). One label specific peak (cyclohexane label) eluting at approximately 7.5 min (0.005 mg eq/kg or 7.0% TRR) remains unable to be unambiguously identified. This peak was further characterized by HPLC and enzyme treatment. In addition, small portions (up to 0.007 mg eq/kg or 12.6 % TRR (phenyl label) and 0.006 mg eq/kg or 7.5 % TRR (cyclohexane label)) were recovered in the combined water extract, the isohehexane phase and other SPE-eluates and were hence characterized by their extraction or distribution properties.

In summary, 0.054 mg eq/kg or 93.5 % TRR (phenyl label) and 0.070 mg eq/kg or 96.0 % TRR (cyclohexane label) were identified and characterized in the ERR. The RRR of the phenyl label was further examined and 0.009 mg eq/kg or 16.2 % TRR were released by protease solubilization leaving a final residue of 0.005 mg

eq/kg or 8.9 % TRR. The “grand total” accounted for 0.059 mg eq/kg or 102.4 % TRR (phenyl label) and 0.077 mg eq/kg or 99.3 % TRR (cyclohexane label).

#### Egg white

Six (phenyl label) or four (cyclohexane label) metabolites and the unchanged parent compound (only cyclohexane label) were identified in egg white. For the phenyl label, the metabolites M684H001, M684H021\_22.1\_LC02 / M684H058 and M684H039 accounted for the main portions with 0.014 mg eq/kg or 22.4 % TRR, 0.013 mg eq/kg or 19.8 % TRR and 0.012 mg eq/kg or 18.8 % TRR, respectively. The metabolites M684H021\_24.6\_LC02, M684H021\_32.5\_LC02 and M684H021\_33.5\_LC02 ranged from 0.003 mg eq/kg to 0.005 mg eq/kg (4.9 – 7.6 % TRR). For the cyclohexane label, the metabolite M684H026 accounted for the main portion with 0.038 mg eq/kg or 33.4 % TRR. The parent compound BAS 684 H and the metabolites M684H001, M684H021\_24.6\_LC02 and M684H039 ranged from 0.001 mg eq/kg to 0.016 mg eq/kg (1.0 – 13.7 % TRR). The remaining peaks not assigned by HPLC were at a maximum of 0.002 mg eq/kg or 3.3 % TRR (phenyl label) and 0.004 mg eq/kg or 3.6 % TRR (cyclohexane label). One label specific peak (cyclohexane label) eluting at approximately 7.5 min (0.015 mg eq/kg or 13.1 % TRR) remains unable to be unambiguously identified. This peak was further characterized by HPLC and enzyme treatment. In addition, small portions (up to 0.001 mg eq/kg or 1.5 % TRR (phenyl label) and 0.001 mg eq/kg or 1.0 % TRR (cyclohexane label)) were recovered in the combined water extract and other SPE-eluates and were hence characterized by their extraction or distribution properties.

In summary, 0.059 mg eq/kg or 91.6 % TRR (phenyl label) and 0.110 mg eq/kg or 95.7 % TRR (cyclohexane label) were identified and characterized in the ERR. For both labels, the final residue was below or equal to 0.001 mg eq/kg or 1.1 % TRR and was not further investigated. The “grand total” accounted for 0.060 mg eq/kg or 92.7 % TRR (phenyl label) and 0.110 mg eq/kg or 96.4 % TRR (cyclohexane label).

#### Muscle

Five (phenyl label) or three (cyclohexane label) metabolites were identified in muscle. For the phenyl label, the metabolite M684H010 accounted for the main portion with 0.021 mg eq/kg or 40.8 % TRR. The metabolites M684H001, M684H021\_24.6\_LC02, M684H039 and M684H059 ranged from 0.001 mg eq/kg to 0.007 mg eq/kg (2.9 – 14.7 % TRR). For the cyclohexane label, the metabolite M684H026 accounted for the main portion with 0.054 mg eq/kg or 56.5 % TRR. The metabolites M684H001 and M684H021\_24.6\_LC02 ranged from 0.001 mg eq/kg to 0.006 mg eq/kg (1.3 – 5.8 % TRR). The remaining peaks not assigned by HPLC were at a maximum of 0.003 mg eq/kg or 5.3 % TRR (phenyl label) and 0.004 mg eq/kg or 3.9 % TRR (cyclohexane label). One label specific peak (cyclohexane label) eluting at approximately 7.5 min (0.015 mg eq/kg or 15.3 % TRR) remains unable to be unambiguously identified. This peak was further characterized by HPLC and enzyme treatment. In addition, small portions (up to 0.001 mg eq/kg or 2.9 % TRR (phenyl label) and 0.003 mg eq/kg or 3.0 % TRR (cyclohexane label)) were recovered in the combined water extract, the combined isohexane phase and another SPE-eluate and were hence characterized by their extraction or distribution properties.

In summary, 0.046 mg eq/kg or 89.6 % TRR (phenyl label) and 0.095 mg eq/kg or 99.3 % TRR (cyclohexane label) were identified and characterized in the ERR. For both labels, the final residue was below or equal to 0.004 mg eq/kg or 7.0 % TRR and was not further investigated. The “grand total” accounted for 0.049 mg eq/kg or 96.6 % TRR (phenyl label) and 0.097 mg eq/kg or 101.4 % TRR (cyclohexane label).

#### Liver

Six (phenyl label) or five (cyclohexane label) metabolites were identified in liver. For the phenyl label, the metabolite M684H059 accounted for the main portion with 0.043 mg eq/kg or 19.3 % TRR. The metabolites M684H001, M684H010, M684H011, M684H021\_24.6\_LC02, and M684H027 (M306 (a MS-characterised metabolite that co-elutes with M684H027, not further considered as only detected at trace levels) was additionally detected at trace levels by MS analysis) ranged from 0.007 mg eq/kg to 0.015 mg eq/kg (3.3 – 6.9 % TRR). For the cyclohexane label, the metabolite M684H026 accounted for the main portion with 0.094 mg eq/kg or 42.5 % TRR. The metabolites M684H001, M684H011, M684H021\_24.6\_LC02 and M684H027 ranged from 0.003 mg eq/kg to 0.012 mg eq/kg (1.3 – 5.4 % TRR). The remaining peaks not assigned by HPLC were at a maximum of 0.009 mg eq/kg or 3.8 % TRR (phenyl label) and 0.007 mg eq/kg or 3.3 % TRR (cyclohexane label). One label specific peak (cyclohexane label) eluting at approximately 7.5 min (0.026 mg eq/kg or 11.7 % TRR) remains unable to be unambiguously identified. This peak was further characterized by HPLC and enzyme treatment. In addition, small portions (up to 0.010 mg eq/kg or 4.3 % TRR (phenyl label) and 0.005 mg eq/kg or 2.2 % TRR (cyclohexane label)) were recovered in the combined water extract, the



combined isohexane phase and other SPE-eluates and were hence characterized by their extraction or distribution properties.

In summary, 0.151 mg eq/kg or 67.6 % TRR (phenyl label) and 0.195 mg eq/kg or 89.0 % TRR (cyclohexane label) were identified and characterized in the ERR.

The RRR of both labels was incubated with protease. The resulting cleaned-up solubilizates were analysed by HPLC, whereby each peak was below or equal to 0.003 mg eq/kg or 1.3% TRR (25 peaks (phenyl label) and 18 peaks (cyclohexane label) in total). In sum, 0.044 mg eq/kg or 19.8 % TRR (phenyl label) and 0.021 mg eq/kg or 9.5 % TRR (cyclohexane label) were characterized by their chromatographic properties.

The final residue was 0.018 mg eq/kg or 8.1 % TRR and 0.007 mg eq/kg or 3.1 % TRR for the phenyl and the cyclohexane label, respectively. The “grand total” accounted for 0.213 mg eq/kg or 95.5 % TRR (phenyl label) and 0.225 mg eq/kg or 101.6 % TRR (cyclohexane label).

#### Fat

Five (phenyl label) or four (cyclohexane label) metabolites and the unchanged parent compound were identified in fat. For the phenyl label, the metabolites M684H010 and M684H059 accounted for the main portions with 0.021 mg eq/kg or 24.7 % TRR and 0.017 mg eq/kg or 20.8 % TRR. The parent compound BAS 684 H and the metabolites M684H001, M684H011 and M684H021\_24.6\_LC02 ranged from 0.002 mg eq/kg to 0.011 mg eq/kg (2.0 – 13.4 % TRR). For the cyclohexane label, the metabolite M684H026 accounted for the main portion with 0.022 mg eq/kg or 27.3 % TRR. The parent compound BAS 684 H and the metabolites M684H001, M684H011 and M684H021\_24.6\_LC02 ranged from 0.001 mg eq/kg to 0.014 mg eq/kg (1.2 – 18.0 % TRR). The remaining peaks not assigned by HPLC were at a maximum of 0.001 mg eq/kg or 1.6 % TRR (phenyl label) and 0.001 mg eq/kg or 1.7 % TRR (cyclohexane label). One label specific peak (cyclohexane label) eluting at approximately 7.5 min (0.006 mg eq/kg or 7.9 % TRR) remains unable to be unambiguously identified. This peak was further characterized by HPLC and enzyme treatment. In addition, small portions (up to 0.009 mg eq/kg or 10.6 % TRR (phenyl label) and 0.007 mg eq/kg or 8.3 % TRR (cyclohexane label)) were recovered in the combined water extract and in the isohexane phases and were hence characterized by their extraction or distribution properties.

In summary, 0.083 mg eq/kg or 99.5 % TRR (phenyl label) and 0.073 mg eq/kg or 92.3 % TRR (cyclohexane label) were identified and characterized in the ERR. For both labels, the final residue was below or equal to 0.003 mg eq/kg or 3.3 % TRR and was not further investigated. The “grand total” accounted for 0.086 mg eq/kg or 102.8 % TRR (phenyl label) and 0.075 mg eq/kg or 93.8 % TRR (cyclohexane label).

Table 7-104 Summary of identified and characterized radioactive residues in edible matrices from laying hens – phenyl label

| Designation  | Egg yolk     |              | Egg white <sup>1</sup> |             | Muscle       |             | Liver              |                  | Fat          |              |
|--|--------------|--------------|------------------------|-------------|--------------|-------------|--------------------|------------------|--------------|--------------|
|  | [mg eq/kg]   | [% TRR]      | [mg eq/kg]             | [% TRR]     | [mg eq/kg]   | [% TRR]     | [mg eq/kg]         | [% TRR]          | [mg eq/kg]   | [% TRR]      |
| <b>Phenyl label</b>                                  |              |              |                        |             |              |             |                    |                  |              |              |
| BAS 684 H  | 0.001        | 1.6          | Not detected           |             | Not detected |             | Not detected       |                  | 0.011        | 13.4         |
| M684H001   | 0.001        | 2.1          | 0.014                  | 22.4        | 0.003        | 6.0         | 0.015              | 6.9              | 0.007        | 8.3          |
| M684H010   | 0.002        | 2.7          | Not detected           |             | 0.021        | 40.8        | 0.014              | 6.2              | 0.021        | 24.7         |
| M684H011   | Not detected |              | Not detected           |             | Not detected |             | 0.007              | 3.3              | 0.002        | 2.0          |
| M684H021 22.1 LC02 / M684H058 <sup>2</sup>           | Not detected |              | 0.013                  | 19.8        | Not detected |             | Not detected       |                  | Not detected |              |
| M684H021 24.6 LC02                                   | 0.005        | 8.3          | 0.004                  | 6.9         | 0.007        | 13.9        | 0.015              | 6.7              | 0.002        | 2.7          |
| M684H021 32.5 LC02                                   | Not detected |              | 0.003                  | 4.9         | Not detected |             | Not detected       |                  | Not detected |              |
| M684H021 33.5 LC02                                   | Not detected |              | 0.005                  | 7.6         | Not detected |             | Not detected       |                  | Not detected |              |
| M684H021, sum of isomers                             | 0.005        | 8.3          | 0.025                  | 39.1        | 0.007        | 13.9        | 0.015              | 6.7              | 0.002        | 2.7          |
| M684H027   | Not detected |              | Not detected           |             | Not detected |             | 0.012 <sup>3</sup> | 5.3 <sup>3</sup> | Not detected |              |
| M684H039   | 0.006        | 10.9         | 0.012                  | 18.8        | 0.001        | 2.9         | Not detected       |                  | Not detected |              |
| M684H059   | 0.007        | 12.5         | Not detected           |             | 0.007        | 14.7        | 0.043              | 19.3             | 0.017        | 20.8         |
| <b>Total identified from ERR</b>                     | <b>0.022</b> | <b>38.2</b>  | <b>0.052</b>           | <b>80.3</b> | <b>0.040</b> | <b>78.2</b> | <b>0.107</b>       | <b>47.7</b>      | <b>0.060</b> | <b>72.1</b>  |
| Maximum other peak (number of other peaks)           | 0.002 (5)    | 4.3 (5)      | 0.002 (7)              | 3.3 (7)     | 0.003 (1)    | 5.3 (1)     | 0.009 (4)          | 3.8 (4)          | 0.001 (4)    | 1.6 (4)      |
| Maximum from precipitation/ partition/ fractionation | 0.007        | 12.6         | 0.001                  | 1.5         | 0.001        | 2.9         | 0.010              | 4.3              | 0.009        | 10.6         |
| <b>Total characterized from ERR</b>                  | <b>0.023</b> | <b>39.2</b>  | <b>0.007</b>           | <b>11.3</b> | <b>0.006</b> | <b>11.4</b> | <b>0.044</b>       | <b>19.9</b>      | <b>0.023</b> | <b>27.4</b>  |
| <b>Total characterized from RRR</b>                  | <b>0.009</b> | <b>16.2</b>  | Not applied            |             | Not applied  |             | <b>0.044</b>       | <b>24.0</b>      | Not applied  |              |
| <b>Total identified and Characterized</b>            | <b>0.054</b> | <b>93.5</b>  | <b>0.059</b>           | <b>91.6</b> | <b>0.046</b> | <b>89.6</b> | <b>0.195</b>       | <b>87.4</b>      | <b>0.083</b> | <b>99.5</b>  |
| Final residue  | 0.005        | 8.9          | 0.001                  | 1.1         | 0.004        | 7.0         | 0.018              | 8.1              | 0.003        | 3.3          |
| <b>Grand total</b>                                   | <b>0.059</b> | <b>102.4</b> | <b>0.060</b>           | <b>92.7</b> | <b>0.049</b> | <b>96.6</b> | <b>0.213</b>       | <b>95.5</b>      | <b>0.086</b> | <b>102.8</b> |

% TRR based on combustion

1 The first extraction procedure was used

2 Peak shared between M684H021 22.1 LC02 and M684H058

3 MS analysis of an isolated sub-fraction of liver (phenyl label) additional detected M306 at trace levels

Minor variations are due to differing precision in numbers

Table 7-105 Summary of identified and characterized radioactive residues in edible matrices from laying hens – cyclohexane label

| Designation  | Egg yolk     |             | Egg white <sup>1</sup> |             | Muscle       |              | Liver        |              | Fat          |             |
|--|--------------|-------------|------------------------|-------------|--------------|--------------|--------------|--------------|--------------|-------------|
|  | [mg eq/kg]   | [% TRR]     | [mg eq/kg]             | [% TRR]     | [mg eq/kg]   | [% TRR]      | [mg eq/kg]   | [% TRR]      | [mg eq/kg]   | [% TRR]     |
| <b>Cyclohexane label</b>                             |              |             |                        |             |              |              |              |              |              |             |
| BAS 684 H  | 0.001        | 1.8         | 0.001                  | 1.0         | Not detected |              | Not detected |              | 0.014        | 18.2        |
| M684H001   | 0.001        | 1.4         | 0.016                  | 13.7        | 0.001        | 1.3          | 0.010        | 4.5          | 0.006        | 7.8         |
| M684H011   | Not detected |             | Not detected           |             | Not detected |              | 0.003        | 1.3          | 0.001        | 1.2         |
| M684H021 24.6 LC02                                   | 0.005        | 6.2         | 0.004                  | 3.9         | 0.006        | 5.8          | 0.012        | 5.4          | 0.003        | 3.4         |
| M684H026   | 0.027        | 34.7        | 0.038                  | 33.4        | 0.054        | 56.5         | 0.094        | 42.5         | 0.022        | 27.3        |
| M684H027   | Not detected |             | Not detected           |             | Not detected |              | 0.009        | 3.9          | Not detected |             |
| M684H039   | 0.003        | 4.1         | 0.011                  | 9.4         | Not detected |              | Not detected |              | Not detected |             |
| <b>Total identified from ERR</b>                     | <b>0.038</b> | <b>48.2</b> | <b>0.070</b>           | <b>61.4</b> | <b>0.061</b> | <b>63.6</b>  | <b>0.127</b> | <b>57.6</b>  | <b>0.046</b> | <b>57.9</b> |
| Maximum other peak (number of other peaks)           | 0.002 (11)   | 3.0 (11)    | 0.004 (15)             | 3.6 (15)    | 0.004 (8)    | 3.9 (8)      | 0.007 (8)    | 3.3 (8)      | 0.001 (8)    | 1.7 (8)     |
| Maximum from precipitation/ partition/ fractionation | 0.006        | 7.5         | 0.001                  | 1.0         | 0.003        | 3.0          | 0.005        | 2.2          | 0.007        | 8.3         |
| Unidentified peak at 7.5 min                         | 0.005        | 7.0         | 0.015                  | 13.1        | 0.015        | 15.3         | 0.026        | 11.7         | 0.006        | 7.9         |
| <b>Total characterized from ERR</b>                  | <b>0.033</b> | <b>42.2</b> | <b>0.039</b>           | <b>34.3</b> | <b>0.034</b> | <b>35.7</b>  | <b>0.069</b> | <b>31.4</b>  | <b>0.027</b> | <b>34.5</b> |
| <b>Total characterized from RRR</b>                  | Not applied  |             | Not applied            |             | Not applied  |              | <b>0.021</b> | <b>9.5</b>   | Not applied  |             |
| <b>Total identified and characterized</b>            | <b>0.070</b> | <b>90.5</b> | <b>0.110</b>           | <b>95.7</b> | <b>0.095</b> | <b>99.3</b>  | <b>0.218</b> | <b>98.5</b>  | <b>0.073</b> | <b>92.3</b> |
| Final residue  | 0.007        | 8.9         | 0.001                  | 0.7         | 0.002        | 2.0          | 0.007        | 3.1          | 0.001        | 1.5         |
| <b>Grand total</b>                                   | <b>0.077</b> | <b>99.3</b> | <b>0.110</b>           | <b>96.4</b> | <b>0.097</b> | <b>101.4</b> | <b>0.225</b> | <b>101.6</b> | <b>0.075</b> | <b>93.8</b> |

% TRR based on combustion

1 The first extraction procedure was used

Minor variations are due to differing precision in numbers

#### Enantiomer ratio

In order to analyse whether one isomer of BAS 684 H was preferably metabolized in hens, enantiomer specific analysis of the parent compound, isolated from fat (cyclohexane label), was performed representatively.

Investigation of the enantiomer ratio of the parent compound BAS 684 H yielded a ratio of the (-) and (+) enantiomers of approximately 43:57 in the application solution (representatively determined for the phenyl label). In fat of the cyclohexane label, containing high portions of BAS 684 H, the ratio of the (-) and (+) enantiomers was approximately 62:38.

#### Storage stability

No storage stability investigations were conducted within the poultry metabolism study. The overall storage period from sampling to analysis is in the range of 263 – 467 days (for initial analyses) and therefore consideration about the storage stability is required.

An egg white (phenyl label) sample was re-extracted to generate samples for metabolite identification. The storage period from sampling to extraction was 487 days. HPLC analysis of the initial and the re-extract revealed a similar metabolite pattern. The portions of radioactive residues extracted with methanol prior and after storage were comparable for both workups. Albeit the peak being initially detected at approximately 22 min seems to miss after re-extraction, it was recovered in another SPE-eluate (slightly different SPE conditions) at lower levels. Additionally, a prominent peak was detected at 15.6 min representing metabolite M684H010. According to the metabolic pathway of BAS 684 H, it seems that metabolite M684H021 is rapidly converted to

M684H010 (phenyl label). This finding was confirmed by the results obtained for the cyclohexane label, where only the corresponding biotransformation product M684H026 was detected.

Furthermore, for the acetonitrile extract of the phenyl label in fat the clean-up was repeated, and the processed acetonitrile phases was re-analysed. The storage period from extraction to analysis was 413 days. The analysis confirmed the pattern of the initial analysis.

Taken all together, no degradation of components detected in hen matrices was observed except for metabolite M684H021. For the phenyl label, a conversion of M684H021 to M684H010 was observed after storage in matrix. This finding was confirmed by the results obtained for the cyclohexane label, where only the corresponding biotransformation product M684H026 was detected. The obtained results indicate the influence of biological processes in the degradation of M684H021. This is considered acceptable and no additional data are required at this time.

#### *Metabolic pathway*

The proposed metabolic pathway of BAS 684 H in laying hens is shown in Figure 7-14. In total nine metabolites were identified. The main biotransformation steps leading to these metabolites include hydroxylation of the cyclohexane and / or benzyl ring or of the alkyl groups, oxidation of the hydroxylated methyl group at the benzyl ring and cleavage of the ether bridge followed by conjugation with glucuronic acid or ring formation.

#### **Conclusion**

BAS 684 H was administered orally to twenty hens in two radiolabelled forms (phenyl and cyclohexane labels) for eleven consecutive days (nominal dose of 12 mg/kg feed/day).

Approximately 98.3 % and 96.9 % of the administered dose were recovered in total for the phenyl and cyclohexane label, respectively. The main fraction was excreted via excreta accounting for approximately 91.3 % (phenyl label) and 87.3 % (cyclohexane label). Radioactive residues recovered in the cage wash and rinse accounted for up to 3.8 % (phenyl label) and 4.6 % (cyclohexane label). Radioactive residues associated with edible portions (egg and tissues) accounted for up to 0.1 % (phenyl label) and 0.2 % of the administered dose (cyclohexane label).

Residues in eggs of the phenyl label increased to a plateau at 7 and 9 days with a concentration of 0.076 mg eq/kg (egg white) and 0.053 mg eq/kg (egg yolk), respectively. For the cyclohexane label, a plateau of 0.122 mg eq/kg (egg white) and 0.070 mg eq/kg (egg yolk) was reached after 7 days. There was then a very gradual increase in concentration in the egg yolk up to a maximum of 0.075 mg eq/kg at day 10.

The main portions of radioactive residues were recovered in excreta (6.397 – 7.010 mg eq/kg). In the edible matrices, the highest TRR concentrations were calculated for liver (0.221 – 0.223 mg eq/kg). For all other matrices, the TRR was in a range from 0.051 mg eq/kg to 0.083 mg eq/kg (phenyl label) and from 0.078 mg eq/kg to 0.115 mg eq/kg (cyclohexane label).

Egg yolk, egg white, muscle and liver samples were extracted with methanol and water. Fat samples were extracted with a mixture of acetonitrile and iso-hexane, and subsequently with water. In general, the extractability was high ranging from 93.0 % TRR to 99.3 % TRR, except for egg yolk (phenyl label; 77.0 % TRR) and liver (71.4% TRR and 82.5% TRR for the phenyl and cyclohexane labels, respectively). Radioactive residues in the RRR obtained after extraction of egg yolk (phenyl label) and liver amounted to 17.5 – 28.6 % TRR, which were further investigated. The RRR of all other relevant matrices were below or equal to 7.0 % TRR (0.004 mg eq/kg, phenyl label) and 8.9 % TRR (0.007 mg eq/kg, cyclohexane label).

Identification of metabolites was based on HPLC-MS analysis of fractions obtained from extracts of egg white and liver (phenyl label) and co-chromatography experiments with reference samples, diluted application solution (phenyl label) and isolated MS identified metabolites from egg white. In some cases, peaks were assigned by comparison of the retention times and metabolite patterns.

For both labels, the unchanged parent compound BAS 684 H (0.001 – 0.014 mg eq/kg or 1.0-18.0% TRR), one of four isomers of M684H021 and the metabolites M684H001 (0.001 – 0.016 mg eq/kg or 1.3-22.4% TRR), M684H039 (0.001 – 0.012 mg eq/kg or 2.0-18.8% TRR), M684H027 (0.009 – 0.012 mg eq/kg or 3.9 – 5.3% TRR) with a latter co-eluting MS-characterized compound (M306, only present at trace levels) and M684H011 (0.001 – 0.007 mg eq/kg or 1.2 – 3.3% TRR) were identified. For the phenyl label, additionally the label specific metabolites M684H010 (0.002 – 0.021 mg eq/kg or 2.7 – 40.8% TRR) and M684H059 (0.007 – 0.043 mg eq/kg or 12.5 – 20.8% TRR), and all four isomers of M684H021 (sum of M684H021: 0.002 – 0.025 mg eq/kg or 2.7 – 39.1% TRR) were detected. One of the isomers of M684H021 co-eluted with metabolite M684H058. For the cyclohexane label, the label specific metabolite M684H026 (0.022 – 0.094 mg eq/kg or 27.3 – 56.5% TRR) was additionally recorded. Further, another label specific peak was detected in all matrices of this label eluting at approximately 7.5 min, which was further characterized by HPLC and enzyme treatment.

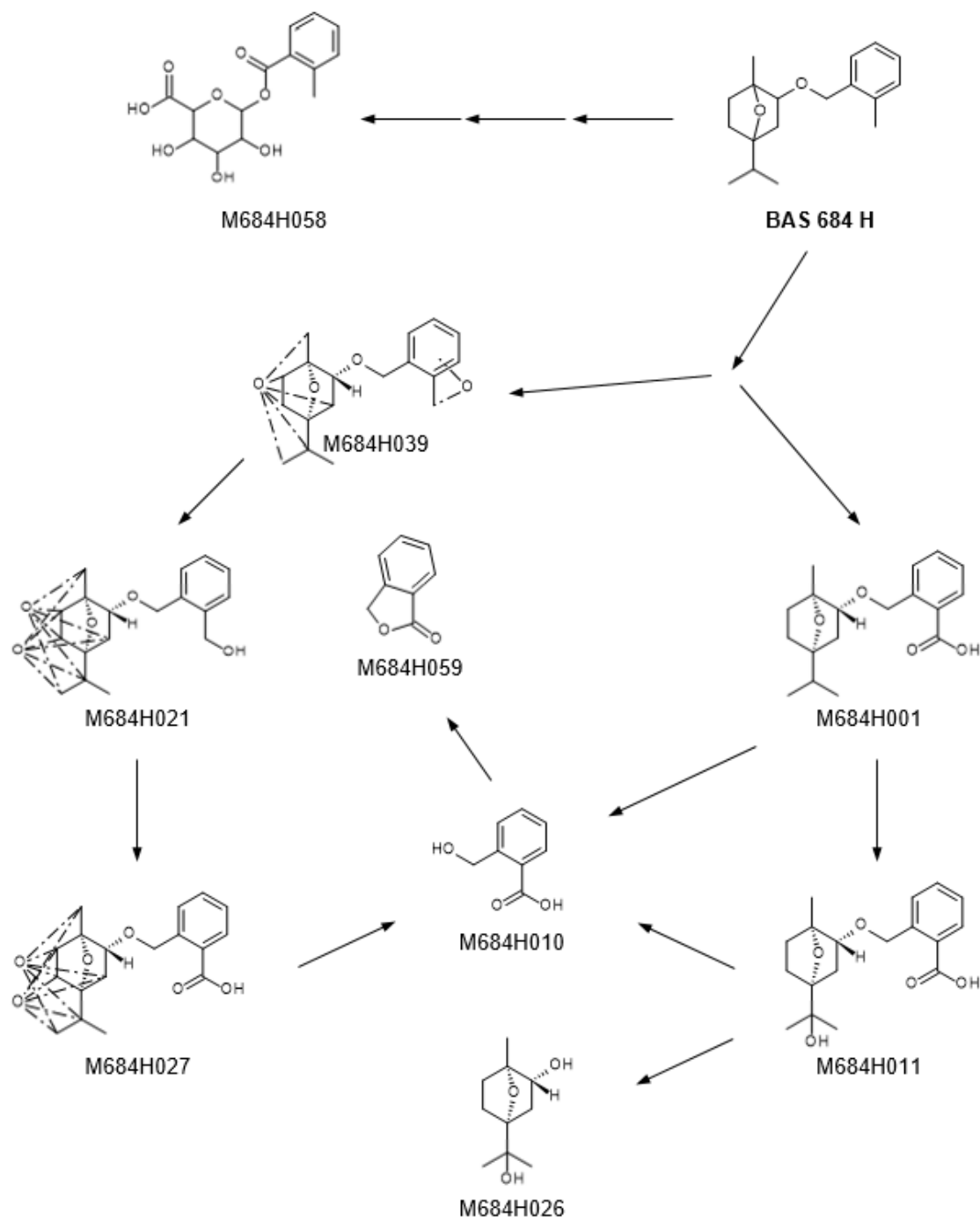
The transformation steps in the metabolic pathway for these are:

- Hydroxylation of the cyclohexane and/or benzyl ring
- Hydroxylation of the alkyl groups at the benzyl and/or cyclohexane ring
- Oxidation of the hydroxylated methyl group at the benzyl ring
- Cleavage of the ether bridge and followed by conjugation with glucuronic acid or run formation

Investigation of the enantiomer ratio of the parent compound BAS 684 H yielded a ratio of the (-) and (+) enantiomers of approximately 43:57 in the application solution (representatively determined for the phenyl label). In fat of the cyclohexane label, containing high portions of BAS 684 H, the ratio of the (-) and (+) enantiomers was approximately 62:38.

No degradation of components detected in hen matrices was observed except for metabolite M684H021. For the phenyl label, a conversion of M684H021 to M684H010 was observed after storage in matrix. This finding was confirmed by the results obtained for the cyclohexane label, where only the corresponding biotransformation product M684H026 was detected. The obtained results indicate the influence of biological processes in the degradation of M684H021. This is considered acceptable and no additional data are required at this time.

Figure 7-14 Proposed metabolic pathway of BAS 684 H in laying hen



The related plant metabolism studies showed only low amounts of the unchanged parent compound, but main portions of metabolites M684H005 and M684H006 after application of BAS 684 H to plants. Hence, livestock animals, being fed with plant material obtained after application of BAS 684 H, are more likely exposed to metabolites M684H005 and M684H006. To avoid additional *in-vivo* studies for investigation of the metabolism of metabolites M684H005 and M684H006 in laying hens, a new alternative *in-vitro* approach was applied to demonstrate suitability of the hen metabolism study, dosed with BAS 684 H. Following information from peer reviewed literature<sup>123</sup>, hen intestine pieces were used to investigate the potential metabolism of both metabolites (M684H005 and M684H006) in a part of the GI tract of hens.

|                    |   |
|--------------------|---|
| <b>Report:</b>     | CA 6.2.2/2<br>Meier M., Bellwon P., 2018 a<br>Metabolism of two metabolites of 14C-BAS 684 H (M684H005 und M684H006) in hen intestine<br>2017/1140183 |
| <b>Guidelines:</b> | OECD Principles of Good Laboratory Practice   |
| <b>GLP:</b>        | yes   |

### **Materials and methods**

#### *Materials*

##### 1. <sup>14</sup>C-labelled M684H005 (Reg. No. 6067256)

**Description:** <sup>14</sup>C-labelled (in the 4C position of the cyclohexane moiety – cyclohexane-4-C14) mixed with unlabelled BAS 684 H. Further solution preparation details provided below.

**Lot/Batch #:** Refers to BAS 684 H (DocID 2017/1004405)

**Purity:** Refers to BAS 684 H (DocID 2017/1004405)

##### 2. 14C-labelled M684H006 (Reg. No. 6067258)

**Description:** <sup>14</sup>C-labelled (in the 4 C position of the cyclohexane moiety – cyclohexane-4-C14) mixed with unlabelled BAS 684 H. Further solution preparation details provided below.

**Lot/Batch #:** Refers to BAS 684 H (DocID 2017/1004405)

**Purity:** Refers to BAS 684 H (DocID 2017/1004405)

##### 3. Polydatin (CAS No. 65914-17-2 (27208-80-6 for the (E)-isomer)

**Description:** 3-hydroxy-5-[(E)-2-(4-hydroxyphenyl)ethenyl]phenylbeta-D-glucopyranoside, used as a positive control.

**Lot/Batch #:** WXBC2301V

**Purity:** 98 %

#### *Methods*

##### Test items

For M684H005 and M684H006, the application solutions had to possess sufficient amounts (dpm) of radioactive material. As the specific activity of each compound was not known, calculations were based on the specific activity of the mixture containing unlabelled and radiolabelled BAS 684 H (cyclohexane-4-C14) used within the wheat metabolism study (Rosenbaum-Stieber C., *et. al.*, 2018, Metabolism of <sup>14</sup>C-BAS 684 H in wheat, 2017/1004405).

For the preparation of the application solutions calculated amounts of each test item or the positive control polydatin were weighed and diluted with medium containing enzymes (potassium phosphate buffer supplemented with casein, yeast, meat extract, bacto soytone, glucose, tween 80 and cysteine HCl).

##### Test system

<sup>1</sup> Amer A. et al; Microbial β-Glucosidase: Sources, Production and Applications, DOI:10.12691/jaem-5-1-4

<sup>2</sup> Mimoza B.-S., et al; Bioconversion of piceid to resveratrol by selected probiotic cell extracts; DOI 10.1007/s00449-016-1662-1

<sup>3</sup> Lee N.-K. et al; Screening of Lactobacilli Derived from Chicken Faeces and Partial Characterization of Lactobacillus acidophilus A12 as Animal Probiotics; J. Microbiol. Biotechnol. (2008), 18(2), 338–342

Hen intestine was obtained from “Hofgut Ochsenschläger” Biblis, Germany. The intestine was dissected from a hen, which was freshly slaughtered, and transported in pre-warmed medium (40 °C). At the Agricultural Centre of BASF SE, the hen intestine was cut into smaller pieces under a nitrogen stream, transferred into vials containing medium and stored gently shaken in an incubator (40 °C) until administration of the test items.

#### In-vitro assay

For investigation of the metabolism of M684H005 and M684H006 incubated with hen intestine, each test item was added to glass vials containing 50 mL medium and hen intestine pieces. Additionally, each test item was incubated in glass vials containing 50 mL medium without hen intestine.

The incubation of polydatin in a glass vial containing 50 mL medium and hen intestine was conducted as positive control.

After addition of the respective test item, glass vials were incubated gently shaken at 40 °C in an incubator.

For experiments performed with M684H005 and M684H006 (with and without hen intestine), samples were collected after 30 min, 4 h, 8 h, 24 h and at the end of incubation. For incubations of M684H005 with hen intestine, additional sampling took place after 1 h and 2 h. For incubations conducted with polydatin, samples were generated 30 min, 45 min, 1 h, 2 h, 4 h, 7 h, 24 h after start of the experiment and at the end of incubation. At each sampling point, 3 mL aliquots were taken, mixed with 0.5 mL acetonitrile and centrifuged. The resulting supernatant was transferred, and the volume was determined. After collection of the last sample, the remaining medium was transferred into a Nalgene bottle and stored. The hen intestine pieces incubated with M684H005 and M684H006 were collected separately, while the hen intestine pieces of the positive control were discarded.

#### Work-up of samples

All samples were stored in a freezer at -18 °C or below until analysis by HPLC. As all analyses were accomplished within a period of less than six months, no storage stability investigations were conducted.

The hen intestine pieces collected after incubation of M684H005 or M684H006 were two times extracted with sufficient amounts of methanol. The resulting extracts were centrifuged, combined and aliquots were subjected to LSC and HPLC analysis. The solid residue after solvent extraction was dried under a fume hood and aliquots were subjected to combustion analysis.

Additionally, a subsample obtained from a previous incubation of M684H005 with hen intestine was cleaned-up for HPLC-MS/MS investigation. The subsample was centrifuged, the resulting supernatant was loaded on a column for solid phase extraction (SPE) and eluted with acetonitrile. The acetonitrile eluate was dried to near dryness using a rotary evaporator and diluted with water / methanol (1/1, v/v).

### **Results and discussion**

#### Metabolite identification

For metabolite identification, a subsample obtained from a previous incubation of M684H005 with hen intestine was subjected to HPLC-MS/MS analysis after SPE clean-up. The detected peak was identified as M684H002. The identity was additionally confirmed by co-chromatography experiments performed respectively with the MS sample as reference item M684H002 and two samples obtained from previous incubations of M684H005 and M684H006 with hen intestine for 30 minutes. Peak assignment was based on comparison of the retention times obtained from HPLC analysis of the reference items. For samples resulting from incubation of polydatin, peak assignment was additionally confirmed by a co-chromatography experiment.

#### Investigation of the potential degradation

##### *Positive control polydatin*

For polydatin, 90.96 % ROI (region of interest) polydatin were detected after incubation for 30 min. The remaining 9.04 % ROI were identified as its cleavage product resveratrol. The degradation continued until polydatin was completely converted to resveratrol after 4 h. This observation confirmed the high metabolic activity of hen intestine.



*Metabolites M684H005 and M684H006*

For M684H005, a fast conversion to metabolite M684H002 was observed in samples collected from incubations with hen intestine. Formation of metabolite M684H002 was already observed after 30 min, which represented the main portion (71.73 % ROI). The remaining 28.27 % ROI were not identified and were not detected anymore after incubation for 1 h. Thereafter, solely M684H002 was detected in the generated samples.

For incubations conducted with M684H006 and hen intestine, formation of metabolite M684H002 was already observed after 30 min accounting for 21.96 % ROI. The amount of M684H002 increased time dependently until the test item M684H006 was completely converted after 24 h.

For incubations of both test items, M684H005 and M684H006, with hen intestine, the recovered applied radioactivity (% AR) in medium samples decreased slightly with incubation time (6.0 % AR to 3.6 % AR for M684H005 and 5.0 % AR to 2.9 % AR for M684H006). However, these radioactive residues were recovered in the methanol extracts of hen intestine. Full details of the radioactive residues recovery are presented in Table 7-106. HPLC analyses of the extracts revealed that the radioactive residues, adsorbed by hen intestine, comprised only M684H002.

Table 7-106 Overview of radioactive residues after incubation of M684H005 and M684H006 with hen intestine

| Sampling timepoint                 | Recovery (dpm) | Recovery (% AR) |
|------------------------------------|----------------|-----------------|
| <b>M684H005 with hen intestine</b> |                |                 |
| 30 min                             | 65945          | 6.01            |
| 1 h                                | 58190          | 4.30            |
| 2 h                                | 51722          | 4.71            |
| 4 h                                | 45617          | 4.16            |
| 8 h                                | 41833          | 3.81            |
| 24 h                               | 39457          | 3.60            |
| End of incubation                  | 582498         | 53.10           |
| Hen intestine (extract)            | 422220         | 38.49           |
| Hen intestine (residue)            | 6574           | 0.60            |
| <b>M684H006 with hen intestine</b> |                |                 |
| 30 min                             | 57233          | 5.01            |
| 4 h                                | 46178          | 4.04            |
| 8 h                                | 41558          | 3.64            |
| 24 h                               | 32637          | 2.86            |
| End of incubation                  | 627181         | 54.93           |
| Hen intestine (extract)            | 405687         | 35.53           |
| Hen intestine (residue)            | 8656           | 0.76            |

In samples obtained from incubations of M684H005 and M684H006 without hen intestine, formation of M684H002 was also observed. For incubation of M684H005 formation of metabolite M684H002 was observed after 30 min, but to a lesser extent (6.16 % ROI). Formation of metabolite M684H002 proceeded and was completed after 24 h. For incubation of M684H006 formation of metabolite M684H002 was noticed after incubation for 4 h (7.39 % ROI). The amount of M684H002 further increased and accounted for 27.45 % ROI at the end of incubation, while the test item M684H006 still represented the main portion amounting to 61.44 % ROI.

These results revealed that both test items, M684H005 and M684H006, are already cleaved to M684H002 under physiological conditions, and that this biotransformation step is catalysed by enzymes present in hen intestine.

In summary, the present metabolism study performed with M684H005 and M684H006 in hen intestine showed a complete degradation to M684H002 within 1 h and 24 h, respectively.

**Conclusion**

Within the present study, a potential degradation of M684H005 and M684H006 was investigated in hen intestine.

For M684H005 and M684H006 incubated with hen intestine, a complete conversion to metabolite M684H002 was observed within 1 h and 24 h, respectively. Furthermore, the % AR in medium samples decreased slightly with incubation time. However, these radioactive residues were recovered in the methanol extracts of hen intestine and consisted only of M684H002.

In samples obtained from incubations of M684H005 and M684H006 without hen intestine, formation of M684H002 was also observed, but much slower and most likely stimulated by the buffer composition.

It can be concluded that both test items, M684H005 and M684H006, are already cleaved to M684H002 under physiological conditions, and that this biotransformation step is catalysed by enzymes present in hen intestine.

Incubations conducted with the positive control polydatin confirmed the metabolic activity of hen intestine.

Overall these data are considered appropriate to support the metabolism in poultry. Metabolites M684H002, M684H005 and M684H006 are hydroxylated or conjugated forms of parent BAS 684 H. The results of the *in-vitro* study are as expected, the metabolites M684H005 and M684H006 are cleaved to form M684H002. From a toxicological perspective, metabolite M684H002 is equivalent to parent BAS 684 H. Even though M684H002 is not found in the hen metabolism study of BAS 684 H, the metabolic pathway indicates that similar metabolites would likely be formed from exposure to M684H002 compared to BAS 684 H. No additional data are required to support the poultry metabolism of the major plant metabolites M684H005 and M684H006.

### B.7.2.3. Lactating ruminants

|                    |  |
|--------------------|--|
| <b>Report:</b>     | CA 6.2.3/1<br>[REDACTED] 2018 a<br>The metabolism of (14C)-Reg.No. 900202 (BAS 684 H) in lactating goats<br>2017/1037602   |
| <b>Guidelines:</b> | 2004/10/EC of 11 February 2004, OECD Test Guideline 503 - Metabolism in livestock, EPA 860.1300: Nature of the Residue in Plants Livestock, EPA 860.1000: EPA Residue Chemistry Test Guidelines, EPA 860.1000: Background - PMRA Section 97.2 (Canada): Residue Chemistry Guidelines: Plants and Lifestock (June 1997), EEC 91/414 (7030(VI/95 Rev. 3), JMAFF No 59 NohSan No 4200 |
| <b>GLP:</b>        | yes  |

### Materials and methods

#### Materials

#### 1. Phenyl-label BAS 684 H (CAS No. 87818-31-3)

|                     |   |
|---------------------|---|
| <b>Description:</b> | Phenyl-U- <sup>14</sup> C, 17.1 MBq/mg (specific activity of a.s.), included in a 1:4:4 mixture of <sup>14</sup> C: <sup>13</sup> C: <sup>12</sup> C test item (phenyl-U- <sup>14</sup> C : benzyl- <sup>13</sup> C : unlabelled <sup>12</sup> C) |
| <b>Lot/Batch #:</b> | 1147-2001 (phenyl-U- <sup>14</sup> C)<br>1159-1012 (benzyl- <sup>13</sup> C)<br>L87-84 (unlabelled)   |
| <b>Purity:</b>      | Phenyl label: 97.0 % (98.9 % radiochemical purity)<br>Benzyl label: 99.6 %<br>Unlabelled: 99.0 %  |

#### 2. Cyclohexane-label BAS 684 H (CAS No. 87818-31-3)

|                     |  |
|---------------------|--|
| <b>Description:</b> | Cyclohexane-4- <sup>14</sup> C, 7.75 MBq/mg (specific activity of a.s.), included in a 0.7:1:1 mixture of <sup>14</sup> C: <sup>13</sup> C: <sup>12</sup> C test item (cyclohexane-4- <sup>14</sup> C : cyclohexane-4- <sup>13</sup> C : unlabelled <sup>12</sup> C) |
| <b>Lot/Batch #:</b> | 1146-1001 (cyclohexane-4- <sup>14</sup> C)<br>1165-2001 (cyclohexane-4- <sup>13</sup> C)<br>L87-84 (unlabelled)  |
| <b>Purity:</b>      | Cyclohexane label ( <sup>14</sup> C): 99.4 % (99.3 % radiochemical purity)<br>Cyclohexane label ( <sup>13</sup> C): 98.1 %<br>Unlabelled: 99.0%  |

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*Methods*

The metabolism of BAS 684 H was investigated in lactating goats (breed *Toggenburg/Saanen/Alpine cross*) following repeated oral administration of  $^{14}\text{C}$ -BAS 684 H, labelled either in the phenyl ring (phenyl label) or the cyclohexane ring (cyclohexane label). See

Figure 7-13.

A total of 4 goats were dosed with radiolabelled BAS 684 H at a nominal dose of 12 mg/kg feed (0.21 – 0.49 mg/kg bw/d, ~300 – 390 N for dairy cattle) for 7 consecutive days (2 goats per label). Each  $^{14}\text{C}$ -labelled test item was mixed with  $^{13}\text{C}$ -labelled test item and unlabelled test item (ratios are given in Table 7-107). The test items were prepared in gelatine capsules, orally administered (once daily). The actual dose was based on the food consumption (dry weight) on the previous day. Dose capsules were prepared daily from formulations stored at -20 °C, the capsules were sealed and used immediately for dosing each goat. Prior to the preparation of the actual dose formulation, extensive trial stability tests were carried out on the dose formulation over a 15-day period to ensure the dose solutions remained stable over the daily dosing period. The test item was found to be stable in a solution of PEG 400 for a period of 9 days when stored at -20 °C. Prior to the preparation of the first day dose capsule and at the end of dosing, the radiochemical purity of the dose formulation was determined by HPLC. The mean achieved daily dose administered was 12.0 – 12.3 mg eq/kg food consumer (dry weight equivalent) corresponding to 0.21 – 0.49 mg/kg bw/d. Details of the study outline are summarized in Table 7-107.

Table 7-107 Dosing of lactating goats with BAS 684 H

| Animal No                  | Treatment period (days) | Isotope ratio  | Nominal daily dose | Actual daily dose <sup>1</sup> |              | Time of sacrifice <sup>2</sup> (hours) |
|----------------------------|-------------------------|--|--------------------|--------------------------------|--------------|--|
|                            |                         |  | [mg/kg feed]       | [mg/kg feed]                   | [mg/kg bw/d] |  |
| Phenyl label:<br>1, 2      | 7                       | 1:4:4<br>( $^{14}\text{C}$ : $^{13}\text{C}$ : $^{12}\text{C}$ )   | 12                 | 12.3, 12.2                     | 0.38, 0.21   | 4                                      |
| Cyclohexane label:<br>3, 4 | 7                       | 0.7:1:1<br>( $^{14}\text{C}$ : $^{13}\text{C}$ : $^{12}\text{C}$ ) | 12                 | 12.0, 12.2                     | 0.28, 0.49   | 5                                      |

1 Based on mean body weights on study day -1

2 Hours after last dose

Blood samples were taken prior to first dose and at 1, 2, 3, 4, 6, 8, 10, 12 and 24 h (immediately prior the second dose) post first dose.

Urine and faeces samples were collected immediately prior to first dose and for each 24 h period until sacrifice. These samples were stored at *ca.* -20 °C prior to analysis. In addition, following removal of the goats for sacrifice, any urine and faeces remaining in the metabolism cages were collected. The total weight of each urine and faeces sample obtained for each goat and collection period was recorded.

Following each urine and faeces collection, a cage wash and a cage rinse was carried out. The washes and rinses were collected separately, and the weights recorded.

Milk was collected and retained pre-dose and twice daily (AM and PM) until sacrifice. For each goat, a representative 24 h milk sample (day 6) from the plateau period was prepared by combining aliquots from the 128 h and 144 h milk samples. Aliquots of this sample were separated by centrifugation into fat and aqueous fractions (cream and skimmed milk). Milk samples (including skimmed milk and cream) were retained at *ca.* +4 °C prior to analysis.

The goats were sacrificed approximately 4 – 5 hours after the final dose and edible tissues (liver, kidney, muscle (loin and flank) and fat (omental, renal and subcutaneous)) as well as bile, blood, carcass and the GI tract and contents were removed *post mortem*. Flank muscle was not collected immediately following sacrifice however was obtained from the carcass of each animal *ca.* one month following sacrifice. During this time the carcasses were stored in a freezer set to -20 °C.

Tissues were homogenised prior to analysis. Faeces samples were homogenised to produce a slurry using methanol. Tissues were grated with the aid of dry ice then further homogenised to a fine powder. Following processing (and radioactive content determination) each tissue sample was stored at *ca.* -20 °C and the dry ice allowed sublime. Each blood sample was mixed with lithium heparin as an anti-coagulant then centrifuged. The resultant plasma was removed from each sample and transferred to a new tube.

Milk, tissue homogenate, urine, bile and faeces samples were pooled before shipping to the analysis laboratory. Generally, samples obtained from two goats of a label were pooled to generate a single sample per label. Muscle types from each goat were pooled in a ratio of 1:2 (flank : loin). Fat types from each goat were pooled in a ratio of 2:1:1 (omental : subcutaneous : renal). Milk collected from day 4 to day 6 (72-144 h) was used for pooling. Urine and faeces sampled within day 4 to day 6 (72-144 h) were combined (4% and 3% by weight, respectively). All samples were stored at -18 °C or below prior to analysis or work up.

The maximum time of frozen storage between sampling and extraction was 162 days. The maximum time of frozen storage between extraction and analysis was 179 days. Further details, including storage stability data to support these time periods are presented in the results section.

Prior to extraction, samples were combusted to verify the total radioactive residues (TRR). The homogenised samples were then extracted and worked up in the following manner:

*Milk, muscle:* Extracted three times with methanol and two times with water. The samples were centrifuged and filtered when necessary. The respective methanol and water extracts were combined, filled up to a defined volume and taken for LSC measurement.

*Liver (workup 1), kidney, faeces:* Extracted three times with methanol and two times with water. The samples were centrifuged and filtered when necessary. The individual extracts were LSC measured.

*Liver (workup 2):* Extracted three times with methanol. The samples were centrifuged and filtered when necessary. The methanol extracts were combined, filled up to a defined volume and taken for LSC measurement.

*Fat:* Extracted three times with a mixture of iso-hexane and acetonitrile (1/1; v/v) and twice with water. The samples were centrifuged and filtered when necessary. The respective iso-hexane, acetonitrile and water extracts were combined, filled up to a defined volume and taken for LSC measurement.

*Urine, bile:* No work up.

HPLC analysis was carried out after clean-up (precipitation with acetone, partition and/or SPE-fractionation) of samples containing sufficient levels of radioactive residues. The combined methanol extracts of liver from workup 2 of both labels were not further cleaned up and after concentration directly analysed with HPLC.

The residues after solvent extraction were dried, homogenized and aliquots were combusted for determination of the radioactive residues. The RRR of fat were mixed with tissue solubilizer and toluene and incubated at 60 °C for 1 hour. Then the samples were cooled down and Hionic Fluor was added prior to combustion analysis.

Solubilization of the RRR (liver): As relatively high levels of radioactive residues were detected in the residual radioactive residues (RRRs) after solvent extraction of liver (both labels), these RRRs were further subjected to enzyme solubilization. Therefore, the RRRs were resuspended in tris/hydrochloric acid buffer at pH 7.0 and protease was added. After about 2 h another portion of protease was added and the sample was incubated at 37 °C for 48 h. Thereafter, the incubation mixture was centrifuged, mixed with acetonitrile and filled up to a defined volume with water. The residues from the protease treatments were resuspended in hydrochloric acid solution (pH 1.0) containing pepsin and incubated at 37 °C for 20 h. Then the residues from the pepsin treatment were resuspended in phosphate buffer (pH 7.5) containing pancreatin and incubated at 37 °C for 23 h. After each solubilization step, the incubation mixtures were centrifuged, filled up to a defined volume with adequate solvent and LSC measured. The residues after the last enzyme treatment were dried and aliquots were subjected to combustion analysis.

Isolation and identification of metabolites: Components of the residue were identified by HPLC-MS, NMR spectroscopy and GC-MS through comparison of metabolite patterns and retention times. Peak assignment within individual matrices was based on co-chromatography experiments with reference items isolated in the current study or reference items from other studies and by comparison of the metabolite patterns and retention times with those of the identified metabolites and the reference items. Specific information is provided for each metabolite below.

Table 7-108 How identification of metabolites was achieved

| Metabolite         | Initial identification  |
|--------------------|---|
| BAS 684 H:         | Co-chromatography with the diluted application formulation using HPLC-UV method LC02 – as this is a known structure it is considered acceptable to have only used one analytical technique.   |
| M684H001           | HPLC-MS (unprocessed goat urine)  |
| M684H002           | HPLC-MS (unprocessed goat urine)  |
| M684H009           | HPLC-MS (isolated and cleaned up sub-fractions from goat urine)<br>GC-MS (isolated and cleaned up subfraction from goat urine, acetylated)<br>HPLC-UV co-chromatography with rat urine using three different HPLC methods (LC07, LC15 and LC01) |
| M684H011           | HPLC-MS (unprocessed goat urine)<br>HPLC-MS/MS (isolated and cleaned up sub-fractions from goat urine)  |
| M684H012a          | HPLC-MS (unprocessed goat urine)  |
| M684H012b          | HPLC-MS (unprocessed goat urine)  |
| M684H022 32.0 LC07 | HPLC-MS/MS (isolated and cleaned up sub-fractions from goat urine)  |
| M684H022 34.0 LC07 | HPLC-MS/MS (isolated and cleaned up sub-fractions from goat urine)  |
| M684H026           | HPLC-MS (isolated and cleaned up sub-fraction of liver)<br>HPLC-UV co-chromatography with rat urine   |
| M684H029           | HPLC-MS/MS (isolated and cleaned up sub-fractions from goat urine)  |
| M684H034           | HPLC-MS/MS (isolated and cleaned up sub-fractions from goat urine)  |
| M684H052           | HPLC-MS/MS (isolated and cleaned up sub-fractions from goat urine)  |
| M684H056           | HPLC-MS/MS (isolated and cleaned up sub-fractions from goat urine)<br>Additional confirmation by enzyme treatment and NMR of cleavage product   |
| M684H057           | HPLC-MS/MS (isolated and cleaned up sub-fractions from goat urine)<br>Additional confirmation by enzyme treatment and NMR of cleavage product   |

The following details are provided for HPLC methods LC01, LC02, LC07 and LC15, these details confirm the co-chromatography methods are dissimilar:

| Method        | Column   | Eluents  |   | Flow rate<br>(mL/min) | Gradient  |     |
|---------------|--|--|---|-----------------------|-----------|-----|
|               |  | A  | B   |                       | Tim (min) | % B |
| LC01<br>LC02† | YMC<br>ProC18 RS<br>5 µm<br>(250 x 4.6 mm)<br>at 25 °C           | H <sub>2</sub> O/<br>HCOOH<br><br>1000/1 (v/v) | CH <sub>3</sub> CN/<br>HCOOH<br><br>1000/1 (v/v)                  | 1.0                   | 0         | 10  |
|               |  |  |   |                       | 15        | 30  |
|               |  |  |   |                       | 35        | 30  |
|               |  |  |   |                       | 60        | 45  |
|               |  |  |   |                       | 75        | 100 |
|               |  |  |   |                       | 85        | 100 |
|               |  |  |   |                       | 85.1      | 10  |
| LC07          | Restec Raptor<br>Biphenyl<br>5 µm<br>(250 x 4.6 mm)<br>at 25 °C  | H <sub>2</sub> O/<br>HCOOH<br><br>1000/1 (v/v) | MeOH/<br>CH <sub>3</sub> CN/<br>HCOOH<br><br>600/400/1<br>(v/v/v) | 1.0                   | 95        | 10  |
|               |  |  |   |                       | 0         | 10  |
|               |  |  |   |                       | 15        | 30  |
|               |  |  |   |                       | 75        | 45  |
|               |  |  |   |                       | 85        | 100 |
|               |  |  |   |                       | 95        | 100 |
| LC15          | Phenomenex<br>Luna PFP (2)<br>5 µm<br>(250 x 4.6 mm)<br>at 25 °C | H <sub>2</sub> O/<br>HCOOH<br><br>1000/1 (v/v) | MeOH/<br>HCOOH<br><br>1000/2 (v/v)                                | 1.0                   | 95.1      | 10  |
|               |  |  |   |                       | 105       | 10  |
|               |  |  |   |                       | 0         | 10  |
|               |  |  |   |                       | 20        | 10  |
|               |  |  |   |                       | 75        | 50  |
|               |  |  |   |                       | 85        | 100 |
|               |  |  |   |                       | 95        | 100 |
|               |  |  |   |                       | 95.1      | 10  |
|               |  |  |   |                       | 105       | 10  |

† Methods LC01 and LC02 are identical in all aspects (column, solvents, gradients), the only difference is that with method LC01 a solid scintillator cell was used for radio detection instead of a liquid scintillator cell.

For metabolite M684H012 two isomers (diastereomers) have been identified. For a better comparison with the related rat metabolism study (Section 6) the two components are designated in accordance with the rat metabolism study as M684H012a (M684H012\_60.0\_LC07 and M684H012b (M684H012\_61.3\_LC07). The applicant has provided the following additional information for these metabolites:

*The metabolite M684H012 was identified in the goat metabolism study and was detected as 2 peaks representing the two possible diastereomers (stereoisomers). The separation of both diastereomers was mainly possible in the HPLC-MS analysis (using method LC07). In the chromatograms generated by radio-HPLC (using method LC05) for quantification, the peaks for the two diastereomers were not in all cases separated. It is not unusual that with MS detection a better separation of such isomers is possible, compared to the radio detection which has to be used for quantification of the peaks. In the radio-HPLC, often not such a good peak separation can be achieved, even when using identical HPLC methods, this is due to the specifics of the radio-detection using scintillator cells.*

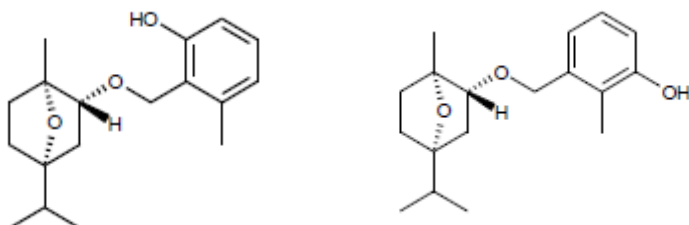
Two isomers of metabolite M684H022 were identified. According to their retention times in the HPLC chromatogram of the MS analysis the two isomers of M684H022 were designated as M684H022\_32.0\_LC07 and M684H022\_34.0\_LC07. However, in the isolated fraction containing M684H022\_32.0\_LC07, two diastereomers or regio-isomers are present, that are co-eluting during analysis with HPLC method LC05 / LC05\_A but can be separated using HPLC method LC22\_A. The applicant has provided the following additional information for these metabolites:

*The detected isomers can represent regio-isomers (differing in their position of the hydroxy group(s)) and in case of M684H022 additionally diastereomers (due to the conjugation). However, it is not possible to assign a specific isomeric form to a specific retention time/peak via MS/MS, since the m/z ratio is the same and the fragmentation pattern in case of cinmethylin did not allow a clear structural differentiation of the possible isomers. Thus, they were differentiated by their retention time.*

These explanations are considered acceptable. In accordance with OECD 503 metabolism in livestock guidance document, if the metabolite is detected at > 10 %, and 0.01 – 0.05 mg eq/kg significant attempts to identify these metabolites should be made, and for metabolites detected at > 10 % and > 0.05 mg eq/kg identify using all possible means. However, as this metabolism study has been conducted with a highly exaggerated feeding level (300 – 390 N), and the toxicity of these metabolites is considered as the sum of the isomers, full identification of each individual isomer is not considered necessary.

For the components M684H022 (both isomers), M684H034, M684H052 and M684H029 the exact position of the OH-group could not be determined by MS analysis. To allow a more detailed analysis, these metabolites were isolated from the respective SPE sub-fractions by HPLC fractionations. The isolated metabolites were treated with a mixture of  $\beta$ -Glucuronidase/arylsulfatase. The samples were analysed by HPLC before and after enzyme treatment and the retention times of the cleaved products were compared with those of selected reference items for which the exact position of the OH-group is known, see Figure 7-15, but no accordance was observed. Therefore the structures of these metabolites are shown as generic structures (indicated by a 'dotted' line). Further details about these structures ('Markush system') are shown in Table 7-1.

Figure 7-15 Structure of reference items used predict exact position of conjugate groups



Enzymatic cleavage of glucuronic acid conjugates: For the components M684H022, M684H034, M684H052 and M684H029 the exact position of the hydroxy-group could not be determined by MS-analysis. To allow a more detailed analysis, these metabolites were isolated. The samples were concentrated, resuspended in ammonium

acetate buffer (pH 5.5) and treated with a mixture of  $\beta$ -glucuronidase/arylsulfatase. The mixture was incubated at 37 °C overnight. The samples were investigated by HPLC before and after enzyme treatment.

**Enantiomer specific analyses:** To analyse if one enantiomer of BAS 684 H was preferably metabolized in goat, the parent compound was isolated from the methanol extract of goat liver (cyclohexane label) and analysed on a chiral HPLC column. For sample preparation, the sample was concentrated and partitioned with ethyl acetate. The ethyl acetate phase was fractionated by HPLC and the isolated parent compound was analysed. To allow an assignment of the peaks to the (+/-)-enantiomers of BAS 684 H, the analyses of the present study were compared to analyses performed in the related rat metabolism study in which the unlabelled (+)- and (-)-enantiomers of BAS 684 H were analysed using the same HPLC method and the same chiral column.

## Results and discussion

### Total radioactive residue

The overall recovery of radioactive residues is provided in Table 7-109 as % administered dose and Table 7-110 expressed as mg eq/kg BAS 684 H. The recoveries are good (> 90 % for each goat) and similar for each of the two labels.

Approximately 94.8 % and 92.0 % of the administered dose were recovered in total for the phenyl and cyclohexane label, respectively. The main fraction was excreted via faeces and urine, accounting in sum for approximately 82.6 % (phenyl label) and 70.6 % (cyclohexane label). Radioactive residues recovered in the GI tract and contents, carcass, cage wash and rinse accounted for up to 6.4 % (phenyl label) and 10.3 % (cyclohexane label). Radioactive residues associated with edible portions (milk and tissues) accounted for up to 0.6 % (phenyl label) and 0.5 % of the administered dose (cyclohexane label).

**Table 7-109 Average distribution of radioactive residues in excreta, tissues and carcass of lactating goats after administration of BAS 684 H for 7 days**

| Matrix              | Phenyl label<br>% of dose |             |                 | Cyclohexane label<br>% of dose |             |                 |
|---------------------|---------------------------|-------------|-----------------|--------------------------------|-------------|-----------------|
|                     | Goat 1                    | Goat 2      | Calculated mean | Goat 3                         | Goat 4      | Calculated mean |
| Faeces              | 20.2                      | 19.7        | 20.0            | 16.9                           | 26.1        | 21.5            |
| Urine               | 63.4                      | 61.8        | 62.6            | 62.2                           | 35.9        | 49.1            |
| Cagewash            | 4.7                       | 2.2         | 3.5             | 3.3                            | 17.3        | 10.3            |
| Cage rinse          | 0.1                       | 0.2         | 0.2             | 0.1                            | 0.6         | 0.4             |
| Milk                | 0.1                       | < 0.1       | 0.1             | 0.1                            | 0.2         | 0.2             |
| Liver               | 0.5                       | 0.6         | 0.6             | 0.5                            | 0.4         | 0.5             |
| Kidney              | < 0.1                     | < 0.1       | <0.1            | 0.1                            | < 0.1       | 0.1             |
| Fat <sup>1</sup>    | < 0.1                     | < 0.1       | <0.1            | < 0.1                          | < 0.1       | <0.1            |
| Muscle <sup>1</sup> | < 0.1                     | < 0.1       | <0.1            | < 0.1                          | < 0.1       | <0.1            |
| GI tract contents   | 6.0                       | 6.8         | 6.4             | 5.8                            | 9.6         | 7.7             |
| GI tract            | 1.6                       | 1.6         | 1.6             | 3.2                            | 1.7         | 2.5             |
| <b>Total</b>        | <b>96.6</b>               | <b>92.9</b> | <b>94.8</b>     | <b>92.2</b>                    | <b>91.8</b> | <b>92.0</b>     |

<sup>1</sup> Calculated from weight of tissue collected at necropsy

In the present study, the TRR was calculated by summing the extractable radioactive residues (ERR) and the residual radioactive residue (RRR) after solvent extraction. In general, the TRR measured was similar to the TRR calculated. The results are summarized in Table 7-110. Unless otherwise stated, % TRR values are calculated using the TRR calculated (sum of ERR + RRR). The lowest concentrations of radioactive residues were determined in milk, muscle and fat for both labels ranging from 0.010 mg eq/kg to 0.022 mg eq/kg. In liver and kidney, the radioactive residues were in the range from 0.361 mg eq/kg to 0.681 mg eq/kg for both labels. Thereby, higher values of radioactive residues were detected in liver (up to 0.681 mg eq/kg) than in kidney (up to 0.472 mg eq/kg). The highest concentrations were detected in bile, urine and faeces for both labels amounting to up to 16.314 mg eq/kg, 13.682 mg eq/kg and 2.429 mg eq/kg, respectively.



Table 7-110 Total radioactive residues in samples from lactating goats following treatment with BAS 684 H for 7 days

| Matrix              | Sampling time | TRR measured<br>[mg eq/kg]    | TRR calculated<br>[mg eq/kg] | TRR measured<br>[mg eq/kg]    | TRR calculated<br>[mg eq/kg] |
|---------------------|---------------|-------------------------------|------------------------------|-------------------------------|------------------------------|
|                     |               | Phenyl label                  |                              | Cyclohexane label             |                              |
|                     |               |                               |                              |                               |                              |
| <b>Milk</b>         | Day 4-6       | 0.011                         | 0.010                        | 0.013                         | 0.013                        |
| <b>Skimmed milk</b> | Day 4-6       | 0.007                         | Not applied                  | 0.010                         | Not applied                  |
| <b>Cream</b>        | Day 4-6       | 0.002                         | Not applied                  | 0.002                         | Not applied                  |
| <b>Muscle</b>       | Terminal      | 0.012                         | 0.011                        | 0.022                         | 0.022                        |
| <b>Fat</b>          | Terminal      | 0.008                         | 0.010                        | 0.019                         | 0.019                        |
| <b>Liver</b>        | Terminal      | 0.690                         | 0.681                        | 0.673                         | 0.656                        |
| <b>Kidney</b>       | Terminal      | 0.372                         | 0.361                        | 0.474                         | 0.472                        |
| <b>Urine</b>        | Day 4-6       | 9.725<br>9.792 <sup>1</sup>   | -                            | 13.754<br>13.682 <sup>1</sup> | -                            |
| <b>Bile</b>         | Terminal      | 11.276<br>11.440 <sup>1</sup> | -                            | 16.257<br>16.314 <sup>1</sup> | -                            |
| <b>Faeces</b>       | Day 4-6       | 2.622                         | 2.429                        | 2.239                         | 2.129                        |

<sup>1</sup> Urine and bile were not extracted, but the TRR was measured once again during the analytical phase of the study

The concentration of radioactive residues in milk are provided in Table 7-111. Daily milk samples were obtained on six consecutive days. Only very low proportions of the administered dose were found. For the phenyl label, the level of radioactive residues reached a maximum of 0.015 mg eq/kg after 4 days for goat 1 and a maximum of 0.008 mg eq/kg after 5 days for goat 2. For the cyclohexane label, the level of radioactive residues increased to an initial maximum of 0.011 mg eq/kg after 2 days for goat 3 and remained consistent from day 3-5 (0.009 - 0.010 mg eq/kg) before a slight increase at day 6 to a maximum of 0.013 mg eq/kg. Residues in the milk increased to a maximum of 0.020 mg eq/kg after 2 days for goat 4. It is noted that a plateau was reached in goats 1 and 4 which had the slightly higher doses of labelled BAS 684 H compared to goats 2 and 3 where no definitive plateau was recorded, based on the 24 hour milk samples (calculated from PM and AM milk collections and as presented in Table 7-111). However, additional PM milk samples are available on the last dosing day 7. The results from these samples (goat 2 0.011 and goat 3 0.014 mg eq/kg) when compared to the values from the previous PM milk samples, as shown in Table 7-112 and Table 7-113, indicate a plateau has been reached. Overall these data are considered acceptable as a plateau is reached at the higher dose rates after 5 days, no additional data are required at this time.

Table 7-111 Radioactive residues in milk of lactating goats following administration of BAS 684 H

| Time<br>[hours]        | TRR<br>[mg eq/kg] |                |                   |                |
|------------------------|-------------------|----------------|-------------------|----------------|
|                        | Phenyl label      |                | Cyclohexane label |                |
|                        | Goat 1            | Goat 2         | Goat 3            | Goat 4         |
| <b>24</b>              | 0.011             | 0.005          | 0.007             | 0.012          |
| <b>48</b>              | 0.013             | 0.006          | 0.011             | 0.020          |
| <b>72</b>              | 0.013             | 0.006          | 0.009             | 0.015          |
| <b>96</b>              | 0.015             | 0.007          | 0.010             | 0.016          |
| <b>120</b>             | 0.012             | 0.008          | 0.010             | 0.016          |
| <b>144</b>             | 0.012             | 0.008          | 0.013             | 0.015          |
| <b>152<sup>1</sup></b> | Not applicable    | Not applicable | Not applicable    | Not applicable |

<sup>1</sup> Goats were sacrificed 4 h (phenyl label) and 5 h (cyclohexane label) after administration of the last dose.

Table 7-112 Radioactive residues in all milk samples of lactating goat 2 following administration of BAS 684 H

| Timepoint<br>(h) | Study<br>Day | Goat 2 Milk Plateau  |       |                          |                      |       |                          |                                 |                    |       |
|------------------|--------------|----------------------|-------|--------------------------|----------------------|-------|--------------------------|---------------------------------|--------------------|-------|
|                  |              | PM Milk              |       |                          | AM Milk              |       |                          | 24 h Milk Sample <sup>(a)</sup> |                    |       |
|                  |              | Sample<br>weight (g) | mg/kg | Total<br>residue<br>(mg) | Sample<br>weight (g) | mg/kg | Total<br>residue<br>(mg) | Sample<br>weight (g)            | Total residue (mg) | mg/kg |
| 24               | 1            | 539                  | 0.01  | 0.005                    | 740                  | 0.003 | 0.002                    | 1279                            | 0.007              | 0.005 |
| 48               | 2            | 491                  | 0.007 | 0.003                    | 693                  | 0.006 | 0.004                    | 1184                            | 0.007              | 0.006 |
| 72               | 3            | 284                  | 0.012 | 0.003                    | 677                  | 0.005 | 0.003                    | 961                             | 0.006              | 0.006 |
| 96               | 4            | 312                  | 0.014 | 0.004                    | 698                  | 0.005 | 0.003                    | 1010                            | 0.007              | 0.007 |
| 120              | 5            | 307                  | 0.012 | 0.004                    | 710                  | 0.006 | 0.004                    | 1017                            | 0.008              | 0.008 |
| 144              | 6            | 341                  | 0.011 | 0.004                    | 697                  | 0.006 | 0.004                    | 1038                            | 0.008              | 0.008 |
| 152              | 7            | 308                  | 0.011 | 0.003                    | NA                   | NA    | NA                       | NA                              | NA                 | NA    |

Table 7-113 Radioactive residues in all milk samples of lactating goat 3 following administration of BAS 684 H

| Timepoint<br>(h) | Study<br>Day | Goat 3 Milk Plateau  |       |                          |                      |       |                          |                                 |                    |       |
|------------------|--------------|----------------------|-------|--------------------------|----------------------|-------|--------------------------|---------------------------------|--------------------|-------|
|                  |              | PM Milk              |       |                          | AM Milk              |       |                          | 24 h Milk Sample <sup>(a)</sup> |                    |       |
|                  |              | Sample<br>weight (g) | mg/kg | Total<br>residue<br>(mg) | Sample<br>weight (g) | mg/kg | Total<br>residue<br>(mg) | Sample<br>weight (g)            | Total residue (mg) | mg/kg |
| 24               | 1            | 739                  | 0.01  | 0.007                    | 1543                 | 0.005 | 0.008                    | 2282                            | 0.015              | 0.007 |
| 48               | 2            | 661                  | 0.014 | 0.009                    | 454                  | 0.006 | 0.003                    | 1115                            | 0.012              | 0.011 |
| 72               | 3            | 1372                 | 0.013 | 0.018                    | 1388                 | 0.006 | 0.008                    | 2760                            | 0.026              | 0.009 |
| 96               | 4            | 611                  | 0.019 | 0.012                    | 1337                 | 0.006 | 0.008                    | 1948                            | 0.020              | 0.010 |
| 120              | 5            | 600                  | 0.017 | 0.01                     | 1107                 | 0.006 | 0.007                    | 1707                            | 0.017              | 0.010 |
| 144              | 6            | 904                  | 0.020 | 0.018                    | 1157                 | 0.007 | 0.008                    | 2061                            | 0.026              | 0.013 |
| 152              | 7            | 675                  | 0.014 | 0.009                    | NA                   | NA    | NA                       | NA                              | NA                 | NA    |

The concentration of radioactive residues in plasma was measured at regular intervals throughout the 24 h period following the first dose administration. Results are provided in Table 7-114. Although these data are not strictly required, they have been presented for completeness. For the phenyl label, the maximum plasma level amounted to 0.079 mg eq/kg (goat 1) and 0.063 mg eq/kg (goat 2) after 1-3 h post first dose. For the cyclohexane label, the maximum plasma level of goat 3 and 4 amounted to 0.091 mg eq/kg after 1-4 h post first dose. The sacrifice time was set to 4 h (phenyl label) and 5 h (cyclohexane label) post final dose.

Table 7-114 Concentration of radioactive residues in plasma following first administration of BAS 684 H to lactating goats

| Time<br>[hours] | TRR<br>[mg eq/kg] |        |       |                   |        |       |
|-----------------|-------------------|--------|-------|-------------------|--------|-------|
|                 | Phenyl label      |        |       | Cyclohexane label |        |       |
|                 | Goat 1            | Goat 2 | Mean  | Goat 3            | Goat 4 | Mean  |
| <b>1</b>        | 0.074             | 0.063  | 0.069 | 0.091             | 0.070  | 0.081 |
| <b>2</b>        | 0.067             | 0.042  | 0.055 | 0.089             | 0.073  | 0.081 |
| <b>3</b>        | 0.079             | 0.044  | 0.062 | 0.082             | 0.083  | 0.083 |
| <b>4</b>        | 0.077             | 0.041  | 0.059 | 0.085             | 0.091  | 0.088 |
| <b>6</b>        | 0.063             | 0.036  | 0.050 | 0.087             | 0.078  | 0.083 |
| <b>8</b>        | 0.048             | 0.024  | 0.036 | 0.052             | 0.066  | 0.059 |
| <b>10</b>       | 0.036             | 0.024  | 0.030 | 0.048             | 0.053  | 0.051 |
| <b>12</b>       | 0.027             | 0.019  | 0.023 | 0.039             | 0.057  | 0.048 |
| <b>24</b>       | 0.010             | 0.007  | 0.009 | 0.012             | 0.016  | 0.014 |

Overall, low residue levels (< 0.1 mg eq/kg) were found in milk, muscle and fat. Higher residues (> 0.1 mg eq/kg) were found in kidney and liver. Both the phenyl and cyclohexane label showed similar results.

For muscle, the TRR (measured) was 0.012 mg eq/kg for the phenyl label and 0.022 mg eq/kg for the cyclohexane label. Similarly, for milk the TRR (measured) was 0.011 mg eq/kg for the phenyl label and 0.013 mg eq/kg for the cyclohexane label. There was predominant portioning into the skimmed milk for both labels. For fat, the TRR (measured) was 0.008 mg eq/kg for the phenyl label and 0.019 for the cyclohexane label.

For both labels the TRR (measured) in liver was 0.673 – 0.690 mg eq/kg and in kidney 0.372 – 0.474 mg eq/kg.

For excreta, the cyclohexane label showed higher levels than the phenyl label in urine and bile, however the levels in faeces were similar (2.239 – 2.622 mg eq/kg). In urine the phenyl label and cyclohexane labels were 9.725 and 13.754 mg eq/kg respectively. In bile the phenyl label and cyclohexane labels were 11.276 mg eq/kg and 16.257 mg eq/kg respectively.

#### *Extractability*

The extractabilities (ERR – extractable radioactive residues) of <sup>14</sup>C residue from milk, muscle, liver, kidney, fat and faeces are summarized in

Table 7-115.

Milk, muscle, liver, kidney and faeces samples were extracted with methanol and water. From milk, muscle and kidney (both labels), high amounts of radioactive residues were recovered in the methanol extracts (87.3 – 97.8 % TRR). The portions of radioactive residues extracted subsequently with water ranged from 1.2 % TRR to 2.9 % TRR.

Somewhat lower portions were extracted with methanol from liver and faeces samples (59.2 – 68.5 % TRR) and extraction with water released 2.9 – 3.1 % TRR (liver) and 8.9 – 9.1 % TRR (faeces). Within a second extraction of liver (both labels) performed only with methanol, the values found in the methanol extracts of liver were similar to the first extraction.

Fat samples were extracted with a mixture of acetonitrile and iso-hexane, and subsequently with water. In the acetonitrile extract 63.5 – 67.5 % TRR were recovered. The portions of radioactive residues extracted with iso-hexane were comparable for both labels and ranged from 16.6 % TRR to 19.0 % TRR. Radioactive residues extracted with water amounted to 12.4 – 14.3 % TRR.

Altogether, high amounts of radioactive residues were extracted by solvent extraction. Without taking the liver samples (62.1 – 65.0 % TRR) and faeces samples (71.7 – 77.6 % TRR) into account, amounts  $\geq 90.2$  % TRR were extracted from milk samples, organs and tissues. Radioactive residues in the RRR obtained after extraction of liver amounted to 0.239 – 0.248 mg eq/kg or 35.0 – 37.9 % TRR, which were further investigated. The RRR of all other relevant matrices (except faeces) were below or equal to 0.009 mg eq/kg or 9.8 % TRR.

Table 7-115 Extractability of radioactive residues from lactating goat matrices following treatment with BAS 684 H for 7 days

| Matrix              | Methanol extract /<br>acetonitrile extract <sup>1</sup> |                   | Iso-hexane<br>extract <sup>2</sup> |         | Water extract <sup>2</sup> |         | ERR <sup>3</sup> |         | RRR <sup>4</sup> |         | TRR <sup>5</sup> |
|---------------------|---|-------------------|------------------------------------|---------|----------------------------|---------|------------------|---------|------------------|---------|------------------|
|                     | [mg eq/kg]  | [% TRR]           | [mg<br>eq/kg]                      | [% TRR] | [mg<br>eq/kg]              | [% TRR] | [mg<br>eq/kg]    | [% TRR] | [mg<br>eq/kg]    | [% TRR] | [mg<br>eq/kg]    |
| Phenyl label        |   |                   |                                    |         |                            |         |                  |         |                  |         |                  |
| Milk                | 0.010   | 97.8              | Not applied                        |         | <0.001                     | 1.3     | 0.010            | 99.0    | <0.001           | 1.0     | 0.010            |
| Muscle              | 0.009   | 87.3              | Not applied                        |         | <0.001                     | 2.9     | 0.010            | 90.2    | 0.001            | 9.8     | 0.011            |
| Fat                 | 0.007   | 67.5              | 0.002                              | 16.6    | 0.001                      | 12.4    | 0.010            | 96.5    | <0.001           | 3.5     | 0.010            |
| Liver<br>(workup 1) | 0.422   | 61.9              | Not applied                        |         | 0.021                      | 3.1     | 0.443            | 65.0    | 0.239            | 35.0    | 0.681            |
| Liver<br>(workup 2) | 0.436   | 64.0              | Not applied                        |         | Not applied                |         | 0.436            | 64.0    | 0.242            | 35.5    | 0.679            |
| Kidney              | 0.347 <sup>2</sup>                                      | 93.3 <sup>2</sup> | Not applied                        |         | 0.005                      | 1.2     | 0.352            | 94.6    | 0.009            | 2.4     | 0.361            |
| Faeces              | 1.524   | 62.7              | Not applied                        |         | 0.216                      | 8.9     | 1.741            | 71.7    | 0.689            | 28.3    | 2.429            |
| Cyclohexane label   |   |                   |                                    |         |                            |         |                  |         |                  |         |                  |
| Milk                | 0.013   | 97.6              | Not applied                        |         | <0.001                     | 1.7     | 0.013            | 99.3    | <0.001           | 0.7     | 0.013            |
| Muscle              | 0.021   | 92.6              | Not applied                        |         | <0.001                     | 2.0     | 0.021            | 94.6    | 0.001            | 5.4     | 0.022            |
| Fat                 | 0.012   | 63.5              | 0.004                              | 19.0    | 0.003                      | 14.3    | 0.018            | 96.5    | <0.001           | 3.2     | 0.019            |
| Liver<br>(workup 1) | 0.388   | 59.2              | Not applied                        |         | 0.019                      | 2.9     | 0.407            | 62.1    | 0.248            | 37.9    | 0.656            |
| Liver<br>(workup 2) | 0.418   | 63.7              | Not applied                        |         | Not applied                |         | 0.418            | 63.7    | 0.245            | 37.3    | 0.662            |
| Kidney              | 0.456 <sup>2</sup>                                      | 96.2 <sup>2</sup> | Not applied                        |         | 0.007                      | 1.5     | 0.463            | 97.7    | 0.009            | 1.9     | 0.472            |
| Faeces              | 1.459   | 68.5              | Not applied                        |         | 0.193                      | 9.1     | 1.652            | 77.6    | 0.477            | 22.4    | 2.129            |

1 Milk, muscle, liver, kidney and faeces were extracted with methanol; fat was extracted with acetonitrile; values measured from pooled extracts

2 Sum of values measured from single extracts

3 Extractable radioactive residues

4 Residual radioactive residues (after solvent extraction)

5 Sum of ERR + RRR

Minor variations are due to differing precision in numbers

#### *Solubilisation of radioactive residues*

The residue after solvent extraction of liver from both labels of workup 1 was further investigated, the results are summarized in

Table 7-116.

The solubilization steps were performed by subsequent enzyme incubations with protease, pepsin and pancreatin. The highest amounts of radioactive residues were released by protease treatment (21.5 – 30.7 % TRR). Incubation with pepsin released up to 8.4 % TRR while pancreatin released  $\leq 1.0$  % TRR. The final RRR accounted for 0.016 mg eq/kg or 2.3 % TRR (phenyl label) and 0.008 mg eq/kg or 1.3 % TRR (cyclohexane label).

Table 7-116 Characterization of the radioactive residues after solvent extraction in lactating goat samples

| Fraction / Solubilizate  | Liver        |             | Liver             |             |
|--|--------------|-------------|-------------------|-------------|
|  | [mg eq/kg]   | [% TRR]     | [mg eq/kg]        | [% TRR]     |
|  | Phenyl label |             | Cyclohexane label |             |
| <i>Residue after solvent extraction</i>                        | 0.239        | 35.0        | 0.248             | 37.9        |
| Protease solubilizate  | 0.146        | 21.5        | 0.202             | 30.7        |
| Pepsin solubilizate  | 0.058        | 8.4         | 0.032             | 4.8         |
| Pancreatin solubilizate  | 0.007        | 1.0         | 0.004             | 0.7         |
| <b>Sum of Enzyme Solubilizates</b>                             | <b>0.210</b> | <b>30.9</b> | <b>0.238</b>      | <b>36.3</b> |
| Final residue  | 0.016        | 2.3         | 0.008             | 1.3         |
| <b>Sum of solubilized radioactive residues + final residue</b> | <b>0.226</b> | <b>33.2</b> | <b>0.246</b>      | <b>37.5</b> |

*Characterisation and Identification*

Identification of metabolites was based on HPLC-MS and NMR analysis of fractions obtained from urine (both labels) and co-chromatography experiments with isolated MS- and NMR-identified metabolites from urine and with reference samples from other studies. In some cases, peaks were assigned by comparison of the retention times and metabolite patterns. A summary of identified and characterized radioactive residues is compiled in

Table 7-117 and



Table 7-118 (edible matrices) and in

Table 7-119 and

Table 7-120 (urine and faeces).

Based on the workup schemes presented in the study report only minor portions of radioactivity were not identified or extracted with each step i.e. none of the sample preparation steps resulted in a loss above the trigger values of 0.010 mg eq/kg and 10 % TRR.

#### Milk

Two metabolites (one for each label) and the unchanged parent compound (only cyclohexane label) were identified in the pooled sample of milk. For the phenyl label, the only relevant component was the label specific metabolite M684H009, accounting for 0.007 mg eq/kg (71.6 % TRR). For the cyclohexane label, the label specific metabolite M684H026 (0.004 mg eq/kg or 27.4 % TRR) and the parent compound BAS 684 H (0.001 mg eq/kg or 8.5 % TRR) were identified. The remaining peaks not assigned by HPLC were at a maximum of 0.001 mg eq/kg or 11.2 % TRR (phenyl label) and 0.001 mg eq/kg or 9.9 % TRR (cyclohexane label). In addition, small portions (up to 0.001 mg eq/kg or 6.7 % TRR (phenyl label) and 0.001 mg eq/kg or 9.3 % TRR (cyclohexane label)) were recovered in the water extracts, the precipitate from the acetone precipitation, and the radioactive residues in the isohexane phase and were hence characterized by their extraction or distribution properties.

Milk of both labels was separated into cream and skimmed milk within the analytical phase of the study. Since the radioactive residues in cream of both labels were low ( $\leq 0.002$  mg eq/kg), these samples were not extracted for analyses. Skimmed milk was also not further investigated, since the metabolite pattern was seen to be sufficiently covered by the composite milk sample.

In summary, 0.010 mg eq/kg or 95.5 % TRR (phenyl label) and 0.012 mg eq/kg or 93.8 % TRR (cyclohexane label) were identified and characterized in the ERR. For both labels, the final residue was below or equal to  $< 0.001$  mg eq/kg or 1.0 % TRR and was not further investigated. The “grand total” accounted for 0.010 mg eq/kg or 96.4 % TRR (phenyl label) and 0.012 mg eq/kg or 94.4 % TRR (cyclohexane label).

#### Muscle

Nine (phenyl label) or six (cyclohexane label) metabolites were identified in muscle. For the phenyl label, the metabolites M684H056, M684H009, M684H022\_32.0\_LC07, M684H012a and / or M684H012b, M684H001, M684H034, M684H022\_34.0\_LC07 and M684H052 were detected, all of them  $\leq 0.001$  mg eq/kg or 5.2 % TRR. For the cyclohexane label, metabolite M684H026 accounted for the main portion with 0.005 mg eq/kg or 23.6 % TRR. The metabolites M684H052, M684H012b, M684H011, M684H012a and M684H056 were each  $\leq 0.001$  mg eq/kg or 3.3 % TRR. The remaining peaks not assigned by HPLC were at a maximum of 0.001 mg eq/kg or 5.2 % TRR (phenyl label) and 0.001 mg eq/kg or 5.8 % TRR (cyclohexane label). In addition, small portions (up to 0.001 mg eq/kg or 11.1 % TRR (phenyl label) and 0.001 mg eq/kg or 5.5 % TRR (cyclohexane label)) were recovered in the water extracts, the isohexane phase and another SPE-eluate and were hence characterized by their extraction or distribution properties.

In summary, 0.008 mg eq/kg or 79.5% TRR (phenyl label) and 0.019 mg eq/kg or 83.7% TRR (cyclohexane label) were identified and characterized in the ERR. For both labels, the final residue was below or equal to 0.001 mg eq/kg or 9.8% TRR and was not further investigated. The “grand total” accounted for 0.010 mg eq/kg or 89.2% TRR (phenyl label) and 0.020 mg eq/kg or 89.1% TRR (cyclohexane label).

#### Fat

One metabolite (cyclohexane label) and the unchanged parent compound (both labels) were identified in fat. For both labels, the parent compound BAS 684 H was the main component, accounting for 0.002 mg eq/kg or 14.9 % TRR (phenyl label) and 0.004 mg eq/kg or 22.3 % TRR (cyclohexane label). The label specific metabolite M684H026 was found in fat of the cyclohexane label and accounted for 0.001 mg eq/kg or 5.7 % TRR. The remaining peaks not assigned by HPLC were at a maximum of 0.0009 mg eq/kg or 8.8 % TRR (phenyl label) and 0.0012 mg eq/kg or 6.4 % TRR (cyclohexane label). In addition, small portions (up to 0.002 mg kg or 16.6 % TRR (phenyl label) and 0.004 mg eq/kg or 19.0 % TRR (cyclohexane label)) were recovered in the isohexane extract, the water extract, the isohexane phase obtained from partition and in the vials after centrifugation and hence were characterized by their extraction or distribution properties.

In summary, 0.009 mg eq/kg or 87.1 % TRR (phenyl label) and 0.018 mg eq/kg or 95.1 % TRR (cyclohexane label) were identified and characterized in the ERR. For both labels, the final residue was below or equal to  $<$

0.001 mg eq/kg or 3.5 % TRR and was not further investigated. The “grand total” accounted for 0.009 mg eq/kg or 90.6 % TRR (phenyl label) and 0.019 mg eq/kg or 98.3 % TRR (cyclohexane label).

#### Liver

Nine (phenyl label) or ten (cyclohexane label) metabolites and the unchanged parent compound were identified in liver. For both labels, the unchanged parent compound BAS 684 H accounted for the main portions with 0.097 mg eq/kg or 14.3 % TRR (phenyl label) and 0.048 mg eq/kg or 7.3 % TRR (cyclohexane label). For the phenyl label, the metabolites M684H012b, M684H001, M684H057, M684H052, M684H022\_34.0\_LC07, M684H011, M684H022\_32.0\_LC07, M684H056 and M584H012a were further identified and ranged from 0.007 mg eq/kg to 0.033 mg eq/kg or from 1.0 % TRR to 4.9 % TRR. In the methanol extract of the cyclohexane label, the label specific metabolite M684H026 represented 0.013 mg eq/kg or 2.0 % TRR. Additionally, M684H026 was assigned in the liver protease and pepsin solubilizate of the RRR of the cyclohexane label, amounting in sum to 0.079 mg eq/kg or 12.1 % TRR. Further metabolites detected in the cyclohexane label were M684H001, M684H052, M684H012b, M684H011, M684H022\_34.0\_LC07, M684H022\_32.0\_LC07, M684H057, M684H056 and M684H012a ranging from 0.006 mg eq/kg to 0.032 mg eq/kg or 1.0% TRR to 4.8 % TRR. The remaining peaks not assigned by HPLC were at a maximum of 0.013 mg eq/kg or 1.8 % TRR (phenyl label) and 0.017 mg eq/kg or 2.6 % TRR (cyclohexane label). Of these unidentified peaks, 6 peaks and 7 peaks were  $\geq 0.01$  mg eq/kg for the phenyl and cyclohexane label respectively. These peaks have been classified as characterised which is considered acceptable based on the levels of residues that have been identified.

In summary, 0.423 mg eq/kg or 62.4 % TRR (phenyl label) and 0.400 mg eq/kg or 61.0 % TRR (cyclohexane label) were identified and characterized in the ERR. The RRR of both labels was incubated with protease, pepsin and pancreatin. The resulting cleaned-up protease and pepsin solubilizates were analysed by HPLC, whereby each characterized peak was below or equal to 0.023 mg eq/kg or 3.5 % TRR. The final residue was 0.016 mg eq/kg or 2.3 % TRR and 0.008 mg eq/kg or 1.3 % TRR for the phenyl and the cyclohexane label, respectively. The “grand total” accounted for 0.649 mg eq/kg or 95.3 % TRR (phenyl label) and 0.646 mg eq/kg or 98.5 % TRR (cyclohexane label).

#### Kidney

Ten (phenyl label) or eleven (cyclohexane label) metabolites were identified in kidney. The metabolite M684H012b accounted for the main portions in kidney of both labels with 0.050 mg eq/kg or 13.4 % TRR (phenyl label) and 0.044 mg eq/kg or 9.3 % TRR (cyclohexane label). The related diastereomer M684H012a was detected at 0.017 mg eq/kg or 4.6 % TRR of the phenyl label and at 0.013 mg eq/kg or 2.8 % TRR for the cyclohexane label. For the phenyl label, the next most prominent compounds were the metabolites M684H009 (0.044 mg eq/kg or 11.9 % TRR), M684H022\_34.0\_LC07 (0.030 mg eq/kg or 8.0 % TRR), M684H057 (0.025 mg eq/kg or 6.7 % TRR) and M684H034 (0.024 mg eq/kg or 6.5 % TRR). The metabolites M684H052, M684H022\_32.0\_LC07, M684H056 and M684H011 were further identified and ranged from 0.004 mg eq/kg to 0.014 mg eq/kg or from 1.2 % TRR to 3.9 % TRR. For the cyclohexane label, the next most prominent compounds were the metabolites M684H022\_34.0\_LC07 (0.041 mg eq/kg or 8.7 % TRR), M684H057 (0.029 mg eq/kg or 6.1 % TRR), M684H022\_32.0\_LC07 (0.027 mg eq/kg or 5.7 % TRR). The metabolites M684H034, M684H052, M684H034, M684H026, M684H011 and M684H001 were further identified and ranged from 0.005 mg eq/kg to 0.022 mg eq/kg or from 1.0 % TRR to 4.7 % TRR. The remaining peaks not assigned by HPLC were at a maximum of 0.011 mg eq/kg or 3.0 % TRR (phenyl label) and 0.018 mg eq/kg or 3.8 % TRR (cyclohexane label). Of these unidentified peaks, 2 peaks and 4 peaks were  $\geq 0.01$  mg eq/kg for the phenyl and cyclohexane label respectively. These peaks have been classified as characterised which is considered acceptable based on the levels of residues that have been identified. In addition, small portions (up to 0.006 mg eq/kg or 1.7 % TRR (phenyl label) and 0.017 mg eq/kg or 3.7 % TRR (cyclohexane label)) were recovered in the water extracts and two additional SPE-eluates and hence were characterized by their extraction or distribution properties.

In summary, 0.318 mg eq/kg or 85.6 % TRR (phenyl label) and 0.420 mg eq/kg or 88.6 % TRR (cyclohexane label) was identified and characterized in the ERR. For both labels, the final residue was below or equal to 0.009 mg eq/kg or 2.4 % TRR and was not further investigated. The “grand total” accounted for 0.327 mg eq/kg or 87.9 % TRR (phenyl label) and 0.429 mg eq/kg or 90.5 % TRR (cyclohexane label).

Table 7-117 Summary of identified and characterized radioactive residues in edible matrices from lactating goats – phenyl label

| Designation  | Milk         |             | Muscle        |                   | Fat          |            | Liver <sup>2</sup>          |                  | Kidney                     |                   |
|--|--------------|-------------|---------------|-------------------|--------------|------------|-----------------------------|------------------|----------------------------|-------------------|
|  | [mg eq/kg]   | [% TRR]     | [mg eq/kg]    | [% TRR]           | [mg eq/kg]   | [% TRR]    | [mg eq/kg]                  | [% TRR]          | [mg eq/kg]                 | [% TRR]           |
| Phenyl label   |              |             |               |                   |              |            |                             |                  |                            |                   |
| BAS 684 H  | Not detected |             | Not detected  |                   | 0.002        | 14.9       | 0.097                       | 14.3             | Not detected               |                   |
| M684H001   | Not detected |             | <0.001        | 2.2               | Not detected |            | 0.032                       | 4.7              | Not detected               |                   |
| M684H002   | Not detected |             | Not detected  |                   | Not detected |            | Not determined <sup>4</sup> |                  | Not detected               |                   |
| M684H009   | 0.007        | 71.6        | <0.001        | 3.3               | Not detected |            | Not detected                |                  | 0.044                      | 11.9              |
| M684H011   | Not detected |             | Not detected  |                   | Not detected |            | 0.015                       | 2.2              | 0.004                      | 1.2               |
| M684H012a  | Not detected |             | <0.001        | 3.1               | Not detected |            | 0.007                       | 1.0              | 0.017                      | 4.6               |
| M684H012b  | Not detected |             |               |                   | Not detected |            | 0.033                       | 4.9              | 0.050                      | 13.4              |
| M684H012, sum of isomers                                   | Not detected |             | <0.001        | 3.1               | Not detected |            | 0.040                       | 5.8              | 0.067                      | 17.9              |
| M684H022 32.0 LC07   | Not detected |             | <0.001        | 1.5               | Not detected |            | 0.011 <sup>1</sup>          | 1.6 <sup>1</sup> | 0.013 <sup>1</sup>         | 3.5 <sup>1</sup>  |
| M684H022 34.0 LC07   | Not detected |             | <0.001        | 3.1               | Not detected |            | 0.020                       | 3.0              | 0.030                      | 8.0               |
| M684H022, sum of isomers                                   | Not detected |             | 0.001         | 4.6               | Not detected |            | 0.031                       | 4.6              | 0.043                      | 11.5              |
| M684H034   | Not detected |             | <0.001        | 1.9               | Not detected |            | Not detected                |                  | 0.024                      | 6.5               |
| M684H052   | Not detected |             | 0.001         | 1.4               | Not detected |            | 0.022 <sup>1</sup>          | 3.3 <sup>1</sup> | 0.014 <sup>1</sup>         | 3.9 <sup>1</sup>  |
| M684H056   | Not detected |             | <0.001        | 5.2               | Not detected |            | 0.011                       | 1.6              | 0.008                      | 2.0               |
| M684H057   | Not detected |             | Not detected  |                   | Not detected |            | 0.029                       | 4.3              | 0.025                      | 6.7               |
| Total identified from ERR                                  | 0.007        | 71.6        | 0.002         | 21.7              | 0.002        | 14.9       | 0.277                       | 40.7             | 0.229                      | 61.7              |
| Maximum other peak<br>(number of other peaks)              | 0.001<br>(1) | 11.2<br>(1) | 0.001<br>(31) | 5.2<br>(31)       | 0.002<br>(9) | 8.8<br>(9) | 0.013<br>(19) <sup>5</sup>  | 1.8<br>(19)      | 0.011<br>(25) <sup>6</sup> | 3.0<br>(25)       |
| Maximum from<br>precipitation/ partition/<br>fractionation | 0.001        | 6.7         | 0.001         | 11.1              | 0.002        | 16.6       | -                           | -                | 0.006                      | 1.7               |
| Total characterized<br>from ERR                            | 0.001        | 23.9        | 0.006         | 57.8              | 0.007        | 72.2       | 0.146                       | 21.4             | 0.089                      | 23.9              |
| Protease solubilizate                                      | -            | -           | -             | -                 | -            | -          | 0.146                       | 21.5             | -                          | -                 |
| Pepsin solubilizate  | -            | -           | -             | -                 | -            | -          | 0.058                       | 8.4              | -                          | -                 |
| Pancreatin solubilizate                                    | -            | -           | -             | -                 | -            | -          | 0.007                       | 1.0              | -                          | -                 |
| Total characterized<br>from RRR                            | Not applied  |             | Not applied   |                   | Not applied  |            | 0.210                       | 30.9             | Not applied                |                   |
| Total identified and<br>characterized                      | 0.010        | 95.5        | 0.008         | 79.5              | 0.009        | 87.1       | 0.634                       | 93.0             | 0.318                      | 85.6              |
| Final residue  | <0.001       | 1.0         | 0.001         | 9.8               | <0.001       | 3.5        | 0.016                       | 2.3              | 0.009                      | 2.4               |
| Grand total  | 0.010        | 96.4        | 0.010         | 89.2 <sup>3</sup> | 0.009        | 90.6       | 0.649                       | 95.3             | 0.327                      | 87.9 <sup>3</sup> |

1 Representing two isomers (diastereomers or regio-isomers)

2 The values of metabolites identified or characterized in the ERR from workup 2 of liver (both labels) were used for quantification, the assigned metabolites were in good accordance between the two workups, however as workup 2 had a higher proportion of total identified from the ERR only these data have been presented. Radioactive residues identified or characterized in the RRR were taken from workup 1

3 The lower “grand total” can be attributed to lower step by step recoveries during the workup steps (solubilization procedure, HPLC sample preparation), whereby none of the sample preparation steps resulted in a loss above the trigger values of 0.010 mg eq/kg and 10% TRR

4 Possibly present but not determined since no relevant peak

5 6 peaks at  $\geq 0.01$  mg eq/kg

6 2 peaks at  $\geq 0.01$  mg eq/kg

Table 7-118 Summary of identified and characterized radioactive residues in edible matrices from lactating goats – cyclohexane label

| Designation  | Milk         |             | Muscle       |                         | Fat                         |             | Liver <sup>1</sup>      |                   | Kidney                      |             |
|--|--------------|-------------|--------------|-------------------------|-----------------------------|-------------|-------------------------|-------------------|-----------------------------|-------------|
|  | [mg eq/kg]   | [% TRR]     | [mg eq/kg]   | [% TRR]                 | [mg eq/kg]                  | [% TRR]     | [mg eq/kg]              | [% TRR]           | [mg eq/kg]                  | [% TRR]     |
| <b>Cyclohexane label</b>                           |              |             |              |                         |                             |             |                         |                   |                             |             |
| BAS 684 H  | 0.001        | 8.5         | Not detected |                         | 0.004                       | 22.3        | 0.048                   | 7.3               | Not detected                |             |
| M684H001   | Not detected |             | Not detected |                         | Not detected                |             | 0.032                   | 4.8               | 0.005                       | 1.0         |
| M684H002   | Not detected |             | Not detected |                         | Not determined <sup>4</sup> |             | Not detected            |                   | Not determined <sup>4</sup> |             |
| M684H011   | Not detected |             | <0.001       | 1.6                     | Not detected                |             | 0.019                   | 2.9               | 0.008                       | 1.7         |
| M684H012a  | Not detected |             | <0.001       | 0.6                     | Not detected                |             | 0.006                   | 1.0               | 0.013                       | 2.8         |
| M684H012b  | Not detected |             | <0.001       | 2.2                     | Not detected                |             | 0.028                   | 4.2               | 0.044                       | 9.3         |
| M684H012, sum of isomers                           | Not detected |             | 0.001        | 2.8                     | Not detected                |             | 0.034                   | 5.2               | 0.057                       | 12.1        |
| M684H022 32.0 LC07                                 | Not detected |             | Not detected |                         | Not detected                |             | 0.016                   | 2.5               | 0.027                       | 5.7         |
| M684H022 34.0 LC07                                 | Not detected |             | Not detected |                         | Not detected                |             | 0.018                   | 2.8               | 0.041                       | 8.7         |
| M684H022, sum of isomers                           | Not detected |             | Not detected |                         | Not detected                |             | 0.034                   | 5.3               | 0.068                       | 14.4        |
| M684H026   | 0.004        | 27.4        | 0.005        | 23.6                    | 0.001                       | 5.7         | 0.092 <sup>2</sup>      | 14.1 <sup>2</sup> | 0.014                       | 2.9         |
| M684H034   | Not detected |             | Not detected |                         | Not detected                |             | Not detected            |                   | 0.022                       | 4.7         |
| M684H052   | Not detected |             | 0.001        | 3.3                     | Not detected                |             | 0.030                   | 4.5               | 0.020                       | 4.2         |
| M684H056   | Not detected |             | <0.001       | 0.4                     | Not detected                |             | 0.010                   | 1.6               | 0.016                       | 3.4         |
| M684H057   | Not detected |             | Not detected |                         | Not detected                |             | 0.015                   | 2.3               | 0.029                       | 6.1         |
| <b>Total identified from ERR</b>                   | <b>0.005</b> | <b>35.9</b> | <b>0.007</b> | <b>31.7</b>             | <b>0.005</b>                | <b>28.0</b> | <b>0.235</b>            | <b>35.8</b>       | <b>0.240</b>                | <b>50.5</b> |
| Maximum other peak (number of other peaks)         | 0.001 (16)   | 9.9 (16)    | 0.001 (34)   | 5.8 (34)                | 0.0012 (5)                  | 6.4 (5)     | 0.017 (19) <sup>5</sup> | 2.6 (19)          | 0.018 (24) <sup>6</sup>     | 3.8 (24)    |
| Maximum from precipitation/partition/fractionation | 0.001        | 9.3         | 0.001        | 5.5                     | 0.004                       | 19.0        | -                       | -                 | 0.017                       | 3.7         |
| <b>Total characterized from ERR</b>                | <b>0.008</b> | <b>57.8</b> | <b>0.012</b> | <b>52.0</b>             | <b>0.013</b>                | <b>67.1</b> | <b>0.165</b>            | <b>25.2</b>       | <b>0.181</b>                | <b>38.1</b> |
| Protease solubilizate                              | -            | -           | -            | -                       | -                           | -           | 0.202                   | 30.7              | -                           | -           |
| Pepsin solubilizate                                | -            | -           | -            | -                       | -                           | -           | 0.032                   | 4.8               | -                           | -           |
| Pancreatin solubilizate                            | -            | -           | -            | -                       | -                           | -           | 0.004                   | 0.7               | -                           | -           |
| <b>Total identified and characterized from RRR</b> | Not applied  |             | Not applied  |                         | Not applied                 |             | <b>0.238</b>            | <b>36.3</b>       | Not applied                 |             |
| <b>Total identified and characterized</b>          | <b>0.012</b> | <b>93.8</b> | <b>0.019</b> | <b>83.7</b>             | <b>0.018</b>                | <b>95.1</b> | <b>0.638</b>            | <b>97.2</b>       | <b>0.420</b>                | <b>88.6</b> |
| Final residue                                      | <0.001       | 0.7         | 0.001        | 5.4                     | <0.001                      | 3.2         | 0.008                   | 1.3               | 0.009                       | 1.9         |
| <b>Grand total</b>                                 | <b>0.012</b> | <b>94.4</b> | <b>0.020</b> | <b>89.1<sup>3</sup></b> | <b>0.019</b>                | <b>98.3</b> | <b>0.646</b>            | <b>98.5</b>       | <b>0.429</b>                | <b>90.5</b> |

1 The values of metabolites identified or characterized in the ERR from workup 2 of liver (both labels) were used for quantification; radioactive residues identified or characterized in the RRR were taken from workup 1

2 Given is the sum of M684H026 detected in the ERR (0.013 mg eq/kg or 2.0 % TRR) and in the solubilizates of the RRR after Protease and Pepsin treatment (together 0.079 mg eq/kg or 12.1 % TRR)

3 The lower “grand total” can be attributed to lower step by step recoveries during the workup steps (solubilization procedure, HPLC sample preparation), whereby none of the sample preparation steps resulted in a loss above the trigger values of 0.010 mg eq/kg and 10% TRR

4 Possibly present but not determined since no relevant peak

5 7 peaks at  $\geq 0.01$  mg eq/kg

6 4 peaks at  $\geq 0.01$  mg eq/kg

### Urine

Twelve (phenyl label) or thirteen (cyclohexane label) metabolites were identified in urine. The label specific metabolite M684H009 was the most prominent metabolite in urine of the phenyl label, accounting for 1.263 mg eq/kg or 12.9 % TRR. The next most prominent compounds were the metabolites M684H012b (1.169 mg eq/kg or 11.9 % TRR), M684H022\_34.0\_LC07 (0.808 mg eq/kg or 8.3 % TRR), M684H034 (0.746 mg eq/kg or 7.6 % TRR), M684H057 (0.697 mg eq/kg or 7.1 % TRR) and M684H022\_32.0\_LC07 (0.513 mg eq/kg or 5.2 % TRR). The metabolites M684H056, M684H012a, M684H052, M684H011, M684H001 and M684H002 were further identified and ranged from 0.012 mg eq/kg to 0.286 mg eq/kg or from 0.1 % TRR to 2.9 % TRR. For the cyclohexane label, the metabolites M684H012b and M684H022\_34.0\_LC07 accounted for the main portions with 1.333 mg eq/kg or 9.7 % TRR and 1.096 mg eq/kg or 8.0 % TRR, respectively. The next most prominent compounds were the metabolites M684H052 (0.863 mg eq/kg or 6.3 % TRR), M684H022\_32.0\_LC07

(0.797 mg eq/kg or 5.8 % TRR) and M684H057 (0.753 mg eq/kg or 5.5 % TRR). The metabolites M684H056, M684H012a, M684H034, M684H011, M684H029, M684H026, M684H002 and M684H001 were further identified and ranged from 0.125 mg eq/kg to 0.505 mg eq/kg or from 0.9 % TRR to 3.7 % TRR. The peaks assigned to M684H022\_32.0\_LC07 and M684H052 were further split up during one HPLC analyses of urine sub-fractions of the phenyl label, indicating the presence of two diastereomers or regio-isomers covered by these metabolite codes. The remaining peaks not assigned by HPLC were at a maximum of 0.473 mg eq/kg or 4.8 % TRR (phenyl label) and 0.612 mg eq/kg or 4.5 % TRR (cyclohexane label).

In summary, 9.120 mg eq/kg or 93.1 % TRR (phenyl label) and 12.812 mg eq/kg or 93.6 % TRR (cyclohexane label) was identified and characterized in the ERR. For both labels, the residue obtained from centrifugation of the sample was below or equal to 0.174 mg eq/kg or 1.3 % TRR. The “grand total” accounted for 9.163 mg eq/kg or 93.6 % TRR (phenyl label) and 12.985 mg eq/kg or 94.9 % TRR (cyclohexane label).

#### Faeces

Nine (phenyl label) or twelve (cyclohexane label) metabolites were identified in faeces. For both labels, M684H001 accounted for the main portion with 0.146 mg eq/kg or 6.0 % TRR (phenyl label) and 0.071 mg eq/kg or 3.3 % TRR (cyclohexane label). The metabolites M684H011, M684H012a, M684H012b, M684H022\_32.0\_LC07, M684H022\_34.0\_LC07, M684H034 (only for the cyclohexane label), M684H052 (only for the cyclohexane label), M684H056 and M684H057 were identified and ranged from 0.010 mg eq/kg to 0.066 mg eq/kg or from 0.4 % TRR to 3.1 % TRR. Furthermore, the label specific metabolites M684H009 (phenyl label), M684H026 (cyclohexane label) and M684H029 (cyclohexane label) and were detected at up to 0.042 mg eq/kg or 2.0 % TRR. The remaining peaks not assigned by HPLC were at a maximum of 0.173 mg eq/kg or 7.1 % TRR (phenyl label) and 0.075 mg eq/kg or 3.5 % TRR (cyclohexane label). In addition, small portions (up to 0.114 mg eq/kg or 4.7 % TRR (phenyl label) and 0.121 mg eq/kg or 5.7 % TRR (cyclohexane label)) were recovered in the water extracts and three additional SPE-eluates and were hence characterized by their extraction or distribution properties

In summary, 1.744 mg eq/kg or 71.8 % TRR (phenyl label) and 1.578 mg eq/kg or 74.1 % TRR (cyclohexane label) was identified and characterized in the ERR. The final residue was 0.689 mg eq/kg or 28.3 % TRR and 0.477 mg eq/kg or 22.4 % TRR for the phenyl and the cyclohexane label, respectively. The RRR was not investigated, since the identification of metabolites in faeces is not required in the respective guidelines of metabolism studies. The “grand total” accounted for 2.433 mg eq/kg or 100.1% TRR (phenyl label) and 2.055 mg eq/kg or 96.7% TRR (cyclohexane label).

#### Bile

Bile samples were investigated using HPLC method LC02 and altogether the composition of the detected components was comparable to that detected in the other matrices. Since the investigation of bile metabolites is not required in the respective guidelines of metabolism studies, and no metabolite isolation was performed from these samples, the chromatograms of bile are not shown in the study report.

Table 7-119 Summary of identified and characterized radioactive residues in urine and faeces of lactating goats – phenyl label

| Designation   | Urine                         |                  | Faeces                        |                 |
|---|-------------------------------|------------------|-------------------------------|-----------------|
|   | [mg eq/kg]                    | [% TRR]          | [mg eq/kg]                    | [% TRR]         |
| <b>Phenyl label</b>   |                               |                  |                               |                 |
| BAS 684 H   | Not detected                  |                  | Not detected                  |                 |
| M684H001  | 0.057                         | 0.6              | 0.146                         | 6.0             |
| M684H002  | 0.012                         | 0.1              | Not determined <sup>3</sup>   |                 |
| M684H009  | 1.263                         | 12.9             | 0.025                         | 1.0             |
| M684H011  | 0.154                         | 1.6              | 0.052                         | 2.2             |
| M684H012a   | 0.286                         | 2.9              | 0.010                         | 0.4             |
| M684H012b   | 1.169                         | 11.9             | 0.038                         | 1.6             |
| M684H012, sum of isomers                                    | 1.454                         | 14.9             | 0.048                         | 2.0             |
| M684H022 32.0 LC07  | 0.513 <sup>1</sup>            | 5.2 <sup>1</sup> | 0.052                         | 2.2             |
| M684H022 34.0 LC07  | 0.808                         | 8.3              | 0.039                         | 1.6             |
| M684H022, sum of isomers                                    | 1.316                         | 13.5             | 0.091                         | 3.8             |
| M684H034  | 0.746                         | 7.6              | Not detected                  |                 |
| M684H052  | 0.155 <sup>1</sup>            | 1.6 <sup>1</sup> | Not detected                  |                 |
| M684H056  | 0.286                         | 2.9              | 0.051                         | 2.1             |
| M684H057  | 0.697                         | 7.1              | 0.050                         | 2.1             |
| <b>Total identified from ERR</b>                            | <b>6.145</b>                  | <b>62.8</b>      | <b>0.465</b>                  | <b>19.1</b>     |
| <i>Maximum other peak (number of other peaks)</i>           | <i>0.473 (30)<sup>4</sup></i> | <i>4.8 (30)</i>  | <i>0.173 (49)<sup>5</sup></i> | <i>7.1 (49)</i> |
| <i>Maximum from precipitation/ partition/ fractionation</i> | -                             | -                | 0.114                         | 4.7             |
| <b>Total characterized from ERR</b>                         | <b>2.974</b>                  | <b>30.4</b>      | <b>1.279</b>                  | <b>52.7</b>     |
| Total characterized from RRR                                | Not applicable                |                  | Not applied                   |                 |
| <b>Total identified and characterized</b>                   | <b>9.120</b>                  | <b>93.1</b>      | <b>1.744</b>                  | <b>71.8</b>     |
| Final residue   | 0.043 <sup>2</sup>            | 0.4 <sup>2</sup> | 0.689                         | 28.3            |
| <b>Grand total</b>  | <b>9.163</b>                  | <b>93.6</b>      | <b>2.433</b>                  | <b>100.1</b>    |

1 Representing two isomers (diastereomers or regio-isomers)

2 Residue after centrifugation

3 Possibly present but not determined since no relevant peak

4 30 peaks at  $\geq 0.01$  mg eq/kg5 36 peaks at  $\geq 0.01$  mg eq/kg



Table 7-120 Summary of identified and characterized radioactive residues in urine and faeces of lactating goats – cyclohexane label

| Designation   | Urine                         |                  | Faeces                        |                 |
|---|-------------------------------|------------------|-------------------------------|-----------------|
|   | [mg eq/kg]                    | [% TRR]          | [mg eq/kg]                    | [% TRR]         |
| <b>Cyclohexane label</b>                                    |                               |                  |                               |                 |
| BAS 684 H   | Not detected                  |                  | Not detected                  |                 |
| M684H001  | 0.125                         | 0.9              | 0.071                         | 3.3             |
| M684H002  | 0.140                         | 1.0              | Not determined <sup>2</sup>   |                 |
| M684H011  | 0.314                         | 2.3              | 0.049                         | 2.3             |
| M684H012a   | 0.493                         | 3.6              | 0.014                         | 0.7             |
| M684H012b   | 1.333                         | 9.7              | 0.056                         | 2.6             |
| M684H012, sum of isomers                                    | 1.826                         | 13.3             | 0.071                         | 3.3             |
| M684H022 32.0 LC07  | 0.797                         | 5.8              | 0.066                         | 3.1             |
| M684H022 34.0 LC07  | 1.096                         | 8.0              | 0.051                         | 2.4             |
| M684H022, sum of isomers                                    | 1.893                         | 13.8             | 0.117                         | 5.5             |
| M684H026  | 0.279                         | 2.0              | 0.042                         | 2.0             |
| M684H029  | 0.297                         | 2.2              | 0.024                         | 1.1             |
| M684H034  | 0.445                         | 3.3              | 0.034                         | 1.6             |
| M684H052  | 0.863                         | 6.3              | 0.024                         | 1.1             |
| M684H056  | 0.503                         | 3.7              | 0.053                         | 2.5             |
| M684H057  | 0.753                         | 5.5              | 0.053                         | 2.5             |
| <b>Total identified from ERR</b>                            | <b>7.438</b>                  | <b>54.4</b>      | <b>0.538</b>                  | <b>25.3</b>     |
| <i>Maximum other peak (number of other peaks)</i>           | <i>0.612 (38)<sup>3</sup></i> | <i>4.5 (38)</i>  | <i>0.075 (49)<sup>4</sup></i> | <i>3.5 (49)</i> |
| <i>Maximum from precipitation/ partition/ fractionation</i> | -                             | -                | <i>0.121</i>                  | <i>5.7</i>      |
| <b>Total characterized from ERR</b>                         | <b>5.374</b>                  | <b>39.3</b>      | <b>1.039</b>                  | <b>48.8</b>     |
| Total characterized from RRR                                | Not applicable                |                  | Not applied                   |                 |
| <b>Total identified and characterized</b>                   | <b>12.812</b>                 | <b>93.6</b>      | <b>1.578</b>                  | <b>74.1</b>     |
| Final residue   | 0.174 <sup>1</sup>            | 1.3 <sup>1</sup> | 0.477                         | 22.4            |
| <b>Grand total</b>  | <b>12.985</b>                 | <b>94.9</b>      | <b>2.055</b>                  | <b>96.7</b>     |

1 Residue after centrifugation

2 Possibly present but not determined since no relevant peak

3 38 peaks at  $\geq 0.01$  mg eq/kg4 26 peaks at  $\geq 0.01$  mg eq/kg

*Enantiomer ratio*

In order to analyse whether one isomer of BAS 684 H was preferably metabolized in goats, enantiomer specific analysis of the parent compound, isolated from liver (cyclohexane label), was performed representatively.

Investigation of the enantiomer ratio of the parent compound BAS 684 H yielded a ratio of the (-) and (+) enantiomers of approximately 49:51 in the application solution (representatively determined for the cyclohexane label). In liver of the cyclohexane label, containing high portions of BAS 684 H, the ratio of the (-) and (+) enantiomers was approximately 53:47.

*Storage stability*

To investigate the storage stability in the extracts and in the matrix, investigations were performed at representative time points covering the specific intervals for quantitative analyses of relevant matrices of both labels.

The stability of metabolites in the (cleaned-up) extracts was investigated by comparing the initial HPLC analyses after extraction with analyses performed at the end of the study (re-analyses of stored extracts). To investigate the stability of the radioactive residues during storage of the matrix, a subsample of liver was extracted at the end of the study. The HPLC profiles of the re-extracted samples were compared with the profiles obtained from the initial extract (stability in matrix). These storage stability investigations were performed for the following selected goat matrices, covering the relevant metabolites:

- Milk from the phenyl and cyclohexane label.
- Muscle, liver, fat and kidney of the cyclohexane label.

A summary of the storage periods, including the storage stability studies is shown in

Table 7-121 and

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Table 7-122. The length of time the extracts and liver sample were stored for support the time periods in the metabolism study.

Re-analysis of the stored extracts confirmed the stability of the extracts in each of the matrix types for up to 324 - 340 days (difference of the time interval from extraction to the initial analysis and extraction to re-analysis of the sample). The metabolite pattern of the re-analysis was qualitatively and quantitatively similar to the initial analysis (with the exception of the liver extract) and therefore confirmed the stability of the metabolites in the extracts. The analysis of the phenyl label in milk confirmed the stability of the only relevant metabolite in the sample, M684H009. It is considered appropriate to use these data to support the stability of M684H009 in all other matrices (it is not detected in the cyclohexane label samples).

It was noted in the liver re-analysis of the cleaned-up methanol extract the peak for the parent compound at *ca.* 88.2 minutes was missing. However, this was explained due to the slightly different work-up procedures and the parent compound being quantitatively brought into the isohexane phase. Apart from these differences the metabolite patterns were in good accordance and hence stability in this matrix extract is confirmed.

Liver of the cyclohexane label was re-extracted 482 days after sampling. The portions of radioactive residue extracted with methanol prior to and after storage were comparable confirming storage stability for up to 379 days (difference of the time interval from sampling to the extraction and sampling to re-extraction) for stored samples.

Table 7-121 Storage intervals for individual HPLC samples – phenyl label

| Matrix       | Extract      | Analysis                     | Date [dd.mm.yyyy] |            |               | Time interval [days]   |                        |                      |
|--------------|--------------|------------------------------|-------------------|------------|---------------|------------------------|------------------------|----------------------|
|              |              |                              | Sampling          | Extraction | HPLC analysis | Sampling to extraction | Extraction to analysis | Sampling to analysis |
| Phenyl label |              |                              |                   |            |               |                        |                        |                      |
| Milk         | Methanol     | Initial analysis             | 17.05.2016        | 26.10.2016 | 11.11.2016    | 162                    | 16                     | 178                  |
|              | Methanol     | Initial analysis             |                   |            | 16.02.2017    | 162                    | 113                    | 275                  |
|              | Methanol     | Stored extract (re-analysis) |                   |            | 13.10.2017    | 162                    | 352                    | 514                  |
| Muscle       | Methanol     | Initial analysis             | 18.05.2016        | 26.10.2016 | 16.11.2016    | 161                    | 21                     | 182                  |
|              | Methanol     | Initial analysis             |                   |            | 07.03.2017    | 161                    | 132                    | 293                  |
| Liver        | Methanol     | Initial analysis             | 18.05.2016        | 29.08.2016 | 10.11.2016    | 103                    | 73                     | 176                  |
|              | Methanol     | Initial analysis             |                   |            | 14.08.2017    | 103                    | 169                    | 272                  |
| Fat          | Acetonitrile | Initial analysis             | 18.05.2016        | 27.09.2016 | 15.11.2016    | 132                    | 49                     | 181                  |
| Kidney       | Methanol     | Initial analysis             | 18.05.2016        | 11.10.2016 | 10.11.2016    | 146                    | 30                     | 176                  |

Table 7-122 Storage intervals for individual HPLC samples – cyclohexane label

| Matrix            | Extract      | Analysis                          | Date [dd.mm.yyyy] |            |               | Time interval [days]   |                        |                      |
|-------------------|--------------|-----------------------------------|-------------------|------------|---------------|------------------------|------------------------|----------------------|
|                   |              |                                   | Sampling          | Extraction | HPLC analysis | Sampling to extraction | Extraction to analysis | Sampling to analysis |
| Cyclohexane label |              |                                   |                   |            |               |                        |                        |                      |
| Milk              | Methanol     | Initial analysis                  | 17.05.2016        | 12.09.2016 | 11.11.2016    | 118                    | 60                     | 178                  |
|                   | Methanol     | Initial analysis                  |                   |            | 15.02.2017    | 118                    | 156                    | 274                  |
|                   | Methanol     | Stored extract (re-analysis)      |                   |            | 06.10.2017    | 118                    | 389                    | 507                  |
| Muscle            | Methanol     | Initial analysis                  | 18.05.2016        | 15.09.2016 | 16.11.2016    | 120                    | 62                     | 182                  |
|                   | Methanol     | Initial analysis                  |                   |            | 13.03.2017    | 120                    | 179                    | 299                  |
|                   | Methanol     | Stored extract (re-analysis)      |                   |            | 06.10.2017    | 120                    | 386                    | 506                  |
| Liver             | Methanol     | Initial analysis                  | 18.05.2016        | 29.08.2016 | 10.11.2016    | 103                    | 73                     | 179                  |
|                   | Methanol     | Initial analysis                  |                   |            | 14.02.2017    | 103                    | 169                    | 272                  |
|                   | Methanol     | Stored extract (re-analysis)      |                   |            | 16.10.2017    | 103                    | 413                    | 516                  |
|                   | Methanol     | Storage stability (re-extraction) |                   | 12.09.2017 | 19.09.2017    | 482                    | 7                      | 489                  |
| Fat               | Acetonitrile | Initial analysis                  | 18.05.2016        | 27.09.2016 | 15.11.2016    | 132                    | 49                     | 181                  |
|                   | Acetonitrile | Initial analysis                  |                   |            | 08.03.2017    | 132                    | 162                    | 294                  |
|                   | Acetonitrile | Stored extract (re-analysis)      |                   |            | 16.10.2017    | 132                    | 384                    | 516                  |
| Kidney            | Methanol     | Initial analysis                  | 18.05.2016        | 11.10.2016 | 10.11.2016    | 146                    | 30                     | 176                  |
|                   | Methanol     | Initial analysis                  |                   |            | 14.02.2017    | 146                    | 126                    | 272                  |
|                   | Methanol     | Stored extract (re-analysis)      |                   |            | 16.10.2017    | 146                    | 370                    | 516                  |

*Metabolic pathway*

The proposed metabolic pathway of BAS 684 H in lactating goats is shown in Figure 7-16. Altogether twelve metabolites were identified. The transformation steps in the metabolic pathway to form these are:

- Hydroxylation (mono-, di- or triple-) of the cyclohexane and/or benzyl ring
- Hydroxylation (mono-, di- or triple-) of the alkyl groups at the benzyl and/or cyclohexane ring
- Oxidation of the hydroxylated methyl group at the benzyl ring
- Cleavage of the ether bridge
- Conjugation with glucuronic acid
- Conjugation with glycine

The initial biotransformation step is either hydroxylation or cleavage of the parent compound.

Hydroxylation of the methyl group at the benzyl ring leads to M684H002, which is subsequently conjugated with glucuronic acid (M684H012) and further hydroxylated (M684H022). Oxidation of M684H002 generates M684H001. Further hydroxylation of metabolite M684H001 at the isopropyl group results in M684H011. A two- or threefold hydroxylation followed by conjugation with glucuronic acid results in the components M684H056 or M684H057. Hydroxylation of the parent compound and subsequent conjugation with glucuronic acid at the cyclohexane or phenyl moiety leads to metabolites M684H052 or M684H034, respectively.

Cleavage of the ether bridge at BAS 684 H results in the phenyl and the cyclohexane moiety, subsequently conjugated with glycine at the methyl group (M684H009) or hydroxylated at the isopropyl group (M684H026), respectively. Metabolite M684H026 or another hydroxylated intermediate is conjugated with glucuronic acid leading to metabolite M684H029.

As the precise position of hydroxylation/conjugation at the cyclohexane moiety is not known for metabolites M684H029, M684H052, M684H057 and M684H022 and at the phenyl moiety for metabolite M684H034, these metabolites are shown as generic structures (indicated by a 'dotted' line). Further details about these structures ('Markush system') are shown in Table 7-1.

### **Conclusion**

BAS 684 H was administered orally to two lactating goats in two radiolabelled forms (phenyl and cyclohexane label) for seven consecutive days (nominal dose of 12 mg/kg feed/day).

Radioactive residues in plasma increased to a maximum of 0.079 mg eq/kg (goat 1) and 0.063 mg eq/kg (goat 2) after 1-3 h post first dose for the phenyl label and to 0.091 mg eq/kg (goat 3 and 4) after 1-4 h post first dose for the cyclohexane label.

Residues in milk from goats of the phenyl label reached a maximum of 0.015 mg eq/kg after 4 days for goat 1 and a maximum of 0.008 mg eq/kg after 5 days for goat 2. For the cyclohexane label, the level of radioactive residues increased to an initial maximum of 0.011 mg eq/kg after 2 days for goat 3 and remained consistent from day 3-5 (0.009 - 0.010 mg eq/kg) before a slight increase at day 6 to a maximum of 0.013 mg eq/kg. Residues in the milk increased to a plateau maximum of 0.020 mg eq/kg after 2 days for goat 4. It is noted that a plateau was reached in goats 1 and 4 which had the slightly higher doses of labelled BAS 684 H compared to goats 2 and 3 where no definitive plateau was recorded, based on the 24 hour milk samples (calculated from PM and AM milk collections). However, additional PM milk samples are available on the last dosing day 7. The results from these samples (goat 2 0.011 and goat 3 0.014 mg eq/kg) when compared to the values from the previous PM milk samples indicate a plateau has been reached. Overall these data are considered acceptable as a plateau is reached at the higher dose rates after 5 days, no additional data are required at this time.

Approximately 94.8 % and 92.0 % of the administered dose were recovered in total for the phenyl and cyclohexane label, respectively. Thereby, the major portion of radioactive residues was determined for faeces, urine, cage wash and rinse, GI tract and contents. Radioactive residues associated with edible portions (milk and tissues) accounted for a maximum of 0.6 % (phenyl label, liver) and 0.5 % of the administered dose (cyclohexane label, liver).

The main portions of radioactive residues were recovered in urine, faeces and bile (2.129 – 16.314 mg eq/kg). In the relevant matrices, the highest TRR concentrations were calculated for liver and kidney (0.361 – 0.681 mg eq/kg). For all other edible matrices, the TRR was in a range from 0.002 mg eq/kg to 0.022 mg eq/kg.

Radioactive residues were extracted with methanol and water (milk, muscle, liver, kidney and faeces), or only methanol (workup 2 of liver), or acetonitrile, iso-hexane and water (fat). In general, the extractability was high ranging from 90.2 % TRR to 99.3 % TRR, except for liver and faeces (both labels) being between 62.1 % TRR and 77.6 % TRR. From liver, aliquots of the TRR of both labels were incubated subsequently with protease, pepsin and pancreatin which released additional 30.9 % TRR and 36.3 % TRR for the phenyl and cyclohexane label, respectively.

Structure elucidation of metabolites was based on HPLC-MS and NMR analysis and co-chromatography experiments with isolated, MS-and NMR-identified metabolites and reference samples. In some cases, peaks were assigned by comparison of the retention times and metabolite patterns. The unchanged parent compound BAS 684 H was detected in milk of the cyclohexane label and in liver and fat of both labels, ranging from 0.001 - 0.097 mg eq/kg (7.3 – 22.3 % TRR). Regarding only the relevant matrices (milk, tissues and organs), further metabolites identified for both labels were M684H001 (< 0.001 – 0.032 mg eq/kg or 1.0 – 4.8 % TRR), M684H011 (< 0.001 – 0.019 mg eq/kg or 1.2 – 2.9 % TRR), two isomers of M684H012 (sum of M684H012: < 0.001 - 0.067 mg eq/kg or 2.8 – 17.9 % TRR) and two isomers of M684H022 (sum of M684H022: < 0.001– 0.068 mg eq/kg or 4.6 – 14.4 % TRR). In addition, metabolites M684H034, M684H052, M684H056 and M684H057 ranging from < 0.001 mg eq/kg to 0.030 mg eq/kg or from 0.4 % TRR to 6.7 % TRR were detected. The label specific metabolites M684H026 (cyclohexane label) and M684H009 (phenyl label) were identified in milk, tissues and organs ranging from < 0.001 mg eq/kg to 0.092 mg eq/kg or from 2.9 % TRR to 71.6 % TRR.

The metabolite M684H002 was identified / assigned only in urine (both labels) and the label specific metabolite M684H029 was only identified / assigned in urine and faeces of the cyclohexane label.

BAS 684 H was metabolized *via* the following reactions:

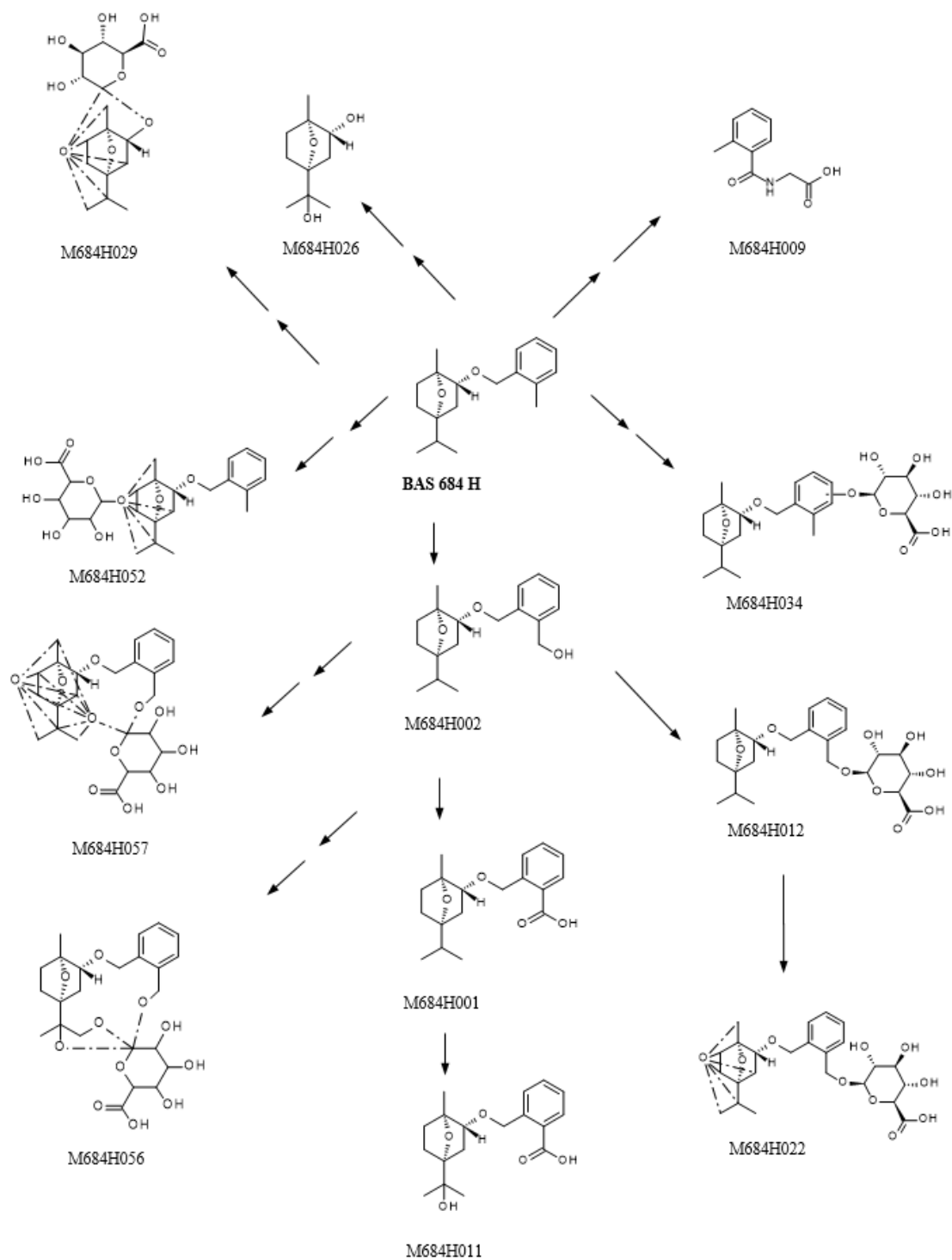
- Hydroxylation (mono-, di- or triple-) of the cyclohexane and/or benzyl ring
- Hydroxylation (mono-, di- or triple-) of the alkyl groups at the benzyl and/or cyclohexane ring
- Oxidation of the hydroxylated methyl group at the benzyl ring
- Cleavage of the ether bridge
- Conjugation with glucuronic acid
- Conjugation with glycine

Investigation of the enantiomer ratio of the parent compound BAS 684 H yielded a ratio of the (-) and (+) enantiomers of approximately 49:51 in the application solution (representatively determined for the cyclohexane label). In liver of the cyclohexane label, containing high portions of BAS 684 H, the ratio of the (-) and (+) enantiomers was approximately 53:47. These two ratios are considered comparable.

Investigations of storage stability were performed for selected goat matrices at the beginning and at the end of the study. Stability investigations in the extracts was representatively performed for milk of both labels and for muscle, fat, liver and kidney of the cyclohexane label. The stability in the extracts was proven for 11 months. The stability in matrix was verified representatively for liver (cyclohexane label) for up to 12 months.



Figure 7-16 Proposed metabolic pathway of BAS 684 H in lactating goats



The related plant metabolism studies showed only low amounts of the unchanged parent compound, but main portions of metabolites M684H005 and M684H006 after application of BAS 684 H to plants. Hence, livestock animals, being fed with plant material obtained after application of BAS 684 H, are more likely exposed to metabolites M684H005 and M684H006. To avoid additional *in-vivo* studies for investigation of the metabolism of metabolites M684H005 and M684H006 in lactating goats, a new alternative *in vitro* approach (RUSITEC<sup>4</sup>) was applied to demonstrate suitability of the goat metabolism study, dosed with BAS 684 H. Thereby, rumen fluid was used to investigate the potential metabolism of both metabolites (M684H005 and M684H006) in a part of the GI tract of ruminants.

|                    |   |
|--------------------|---|
| <b>Report:</b>     | CA 6.2.3/2<br>Meier M., Bellwon P., 2018 b<br>Metabolism of two metabolites of <sup>14</sup> C-BAS 684 H (M684H005 and M684H006) in rumen fluid<br>2017/1140182 |
| <b>Guidelines:</b> | OECD Principles of Good Laboratory Practice, GLP Principles of the German Chemikaliengesetz (Chemicals Act)   |
| <b>GLP:</b>        | yes   |

### Materials and methods

#### Materials

##### 1. <sup>14</sup>C-labelled M684H005 (Reg. No. 6067256)

**Description:** <sup>14</sup>C-labelled (in the 4C position of the cyclohexane moiety – cyclohexane-4-C14) mixed with unlabelled BAS 684 H. Further solution preparation details provided below.

**Lot/Batch #:** Refers to BAS 684 H (DocID 2017/1004405)

**Purity:** Refers to BAS 684 H (DocID 2017/1004405)

##### 2. <sup>14</sup>C-labelled M684H006 (Reg. No. 6067258)

**Description:** <sup>14</sup>C-labelled (in the 4 C position of the cyclohexane moiety – cyclohexane-4-C14) mixed with unlabelled BAS 684 H. Further solution preparation details provided below.

**Lot/Batch #:** Refers to BAS 684 H (DocID 2017/1004405)

**Purity:** Refers to BAS 684 H (DocID 2017/1004405)

##### 3. Polydatin (CAS No. 65914-17-2 (27208-80-6 for the (E)-isomer)

**Description:** 3-hydroxy-5-[(E)-2-(4-hydroxyphenyl)ethenyl]phenylbeta-D-glucopyranoside, used as a positive control.

**Lot/Batch #:** WXBC2301V

**Purity:** 98 %

#### Methods

##### Test items

For the metabolites M684H005 and M684H006, the application solutions had to possess sufficient amounts (dpm) of radioactive material due to the continuous dilution during incubation. As the test items derived from purification of sample material obtained from the wheat metabolism study performed with BAS 684 H, the value of the specific activity was not determined per metabolite, but taken from the related, blended application solution used within the metabolism study (Rosenbaum-Stieber C., *et. al.*, 2018, Metabolism of <sup>14</sup>C-BAS 684 H in wheat, 2017/1004405).

For the preparation of the application solutions, calculated amounts of M684H005 and M684H006, obtained as <sup>14</sup>C-labelled (cyclohexane-4-C14) and unlabelled material dissolved in acetonitrile, were weighed and diluted with buffer (NaCl, KCl, CaCl<sub>2</sub> x 2 H<sub>2</sub>O, MgCl<sub>2</sub> x 6 H<sub>2</sub>O, NaH<sub>2</sub>PO<sub>4</sub> x H<sub>2</sub>O, Na<sub>2</sub>HPO<sub>4</sub> x 12H<sub>2</sub>O, NH<sub>4</sub>Cl, NaHCO<sub>3</sub>, adjusted to pH 7.35 with HCl).

<sup>4</sup> Birk B. et al, Investigation of ruminant xenobiotic metabolism in a modified rumen simulation system (RUSITEC), accepted by ALTEX

Each application solution was analysed by HPLC, using HPLC method LC01, to determine the purity. For the application solution containing M684H005, only the test item was detected. For the application solution containing M684H006, the test item was present at a relative portion of approximately 62 %. However, this has no influence on the study, as the qualitative degradation of M684H006 was the most significant signal.

#### Test system

Rumen fluid was sampled from cow via a rumen fistula. Samples were freshly collected, directly transferred in a Thermos flask on the day of treatment and subsequently shipped to the Agricultural Centre of BASF SE in Limburgerhof, Germany.

The rumen fluid was filtered and the pH value and redox potential were measured using specific electrodes.

The RUSITEC was equipped with a water bath (generally adjusted to 39.5 °C), where three fermenters were incubated. Each fermenter consisted of a gastight container holding the rumen fluid and an inner container for two feeding bags (each containing 2.5 g hay, 1 g feed pellet). The inner container was moved up and down automatically simulating ruminal activity. For simulation of a constant ruminal saliva flow, each fermenter was linked to buffer (pumped at a constant flow rate). The resulting overflow was caught in devices separately for each fermenter.

#### In-vitro assay

At the beginning of the equilibration phase, the fermenters were closed and incubated in the water bath. During the study, each feeding bag was replaced after 48 h. Hence, fresh feeding bags were placed into the fermenters every 24 h.

Simultaneously to the replacement of the feeding bags, samples were taken for determination of the pH and redox potential. Therefore, two aliquots (1 mL each) were taken from each fermenter. The first aliquot (native) was transferred directly into a vial. The second aliquot (acetonitrile) was mixed with 200 µL acetonitrile to stop any enzymatic reactions. The sample was centrifuged subsequently, and the supernatant was transferred into a new vial. All vials were stored in a freezer until analysis.

The equilibration phase lasted for 48 h, and the experimental phase started by addition of the test items.

The three application solutions were taken up in syringes and injected separately into three fermenters through an afflux pipe. For determination of the pH and redox potential as well as for HPLC analyses, samples were taken and exchange of the feeding bags took place, whereby the volume of sampled aliquots and added acetonitrile amounted to 3 mL and 500 µL, respectively.

For the test items M684H005 and M684H006, sample collection was performed 4 h, 8 h, 24 h, 48 h, 72 h after treatment and at the end of the incubation.

For the positive control polydatin, sample collection for HPLC analysis was conducted 5 min, 15 min, 30 min, 45 min, 1 h and 2 h after application.

#### Work-up of samples

All samples were stored in a freezer at -18 °C or below. All analyses were accomplished within a period of less than six months therefore no storage stability investigations are required.

For the metabolites M684H005 and M684H006, aliquots of all samples generated after application of the test items were analysed by HPLC. For polydatin, aliquots of all acetonitrile samples generated during the experimental phase as well as the acetonitrile sample taken directly prior treatment were subjected to HPLC analysis. Peak assignment was based on comparison of the retention times obtained from HPLC analysis of the application solutions and reference items. For samples resulting from incubation of metabolites M684H005 and M684H006, peak assignment was additionally confirmed by a co-chromatography experiment conducted on representative samples.

## Results and discussion

### Determination of the pH and redox potential

The pH values remained stable for the observation period and were within the physiological range of pH 6.09 to 6.71.

The redox potential was -253 mV at start of the equilibration phase and increased thereafter, ranging between -70 mV and -31 mV at the end of the observation period.

### Investigation of the potential degradation

#### *Positive control polydatin*

After incubation for 5 minutes, 34.6 % ROI (region of interest) polydatin and 46.9 % ROI resveratrol, the aglycone of polydatin, were detected. Additionally, a peak accounting for approximately 18.4 % ROI was detected. Albeit the component eluting in this peak was not identified, it can be excluded as another biotransformation/degradation product of polydatin as this peak was also detected in the blank rumen fluid sample. The level of polydatin decreased further and was not recovered in samples obtained after incubation for longer than 30 minutes. Simultaneously, the amount of resveratrol increased time-dependently.

This observation confirmed the cleavage potential of rumen fluid in this approach.

#### *Metabolites M684H005 and M684H006*

For both the native and the acetonitrile sample, a complete degradation of M684H005 by rumen fluid was observed after incubation for 4 h. The detected peak eluting at 70.6 – 70.8 min (native samples) and 71.0 – 71.5 min (acetonitrile samples) was assigned to metabolite M684H002.

For both the native and the acetonitrile sample, the degradation of M684H006 in rumen fluid was already completed after 4 h. Two peaks were detected. The polar peak (retention time = ~6.9 min (native) and ~10.0 min (acetonitrile)) seems to result from biotransformation of the impurities being already detected in the application solution, as the other peak corresponds quantitatively (approximately 64 – 68 % ROI) to the portion of M684H006 applied. Furthermore, the chemical structure of M684H006 gives no indications of potential biotransformation / degradation products being more polar than M684H006 itself. The peak eluting at 71.1 – 71.4 (native samples) and 76.1 – 77.8 min (acetonitrile samples) was assigned to metabolite M684H002.

Co-chromatography experiments were conducted representatively for two native samples obtained from incubations of M684H005 and M684H006 for 4 h. The samples were spiked with the reference item M684H002. The peak assignment was confirmed by these co chromatography experiments, however as only one HPLC-UV method was used this identification is not in accordance with OECD guideline 503.

## Conclusion

Within the present study, a potential degradation of M684H005 and M684H006 was investigated in rumen fluid. For both metabolites, M684H005 and M684H006, a complete degradation was observed in native and acetonitrile samples collected after incubation for 4 h indicating that both metabolites were rapidly cleaved to M684H002. The peak assignment was confirmed by representative co chromatography experiments.

Incubations conducted with the positive control polydatin confirmed the metabolic activity of rumen fluid.

Overall these data are considered appropriate to support the metabolism in lactating ruminants. Metabolites M684H002, M684H005 and M684H006 are hydroxylated or conjugated forms of parent BAS 684 H. The results of the *in-vitro* study are as expected, the metabolites M684H005 and M684H006 are cleaved to form M684H002. From a toxicological perspective, metabolite M684H002 is equivalent to parent BAS 684 H. Metabolite M684H002 is found in the goat metabolism study of BAS 684 H in urine and the metabolic pathway shows it is a key intermediate for several other metabolites. Exposure to M684H002 would likely be comparable to exposure from BAS 684 H. Therefore, no additional data are required to support the lactating ruminant metabolism of the major plant metabolites M684H005 and M684H006.

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**Report:** CA 6.2.3/3  
[REDACTED] 1983 a  
Use of goats for ruminant metabolism studies. part 1 an exploratory study  
CI-440-013

**Guidelines:** <none>

**GLP:** no

#### ***Purpose of study***

This report summarises the exploratory work undertaken for the goat metabolism study with BAS 684 H at [REDACTED] as well as the in-life part, i.e. dosing, sacrifice, sampling, and determination of the total radioactive residues in milk and tissues.

It is stated in the report that the intent of the exploratory study was not to determine the structures of metabolites but to gain confidence in handling goats and gain familiarity with methods to isolate metabolites from ruminant tissue.

#### ***Materials and methods***

##### ***Dosing***

A single goat was orally treated with  $^{14}\text{C}$ -BAS 684 H at a dose rate of 3.64 mg/kg bw/d (102 mg/kg feed), once daily administered in a gelatin capsule (containing 204 mg  $^{14}\text{C}$ -BAS 684 H) with a balling gun for 4 days.

The molecule was labelled in the phenyl ring (phenyl- $\text{U-}^{14}\text{C}$ ). Radiochemical purity was determined by TLC straight after preparation of the material fed to the goat (98.5%) and again five months after preparation (95%). The study uses a value of 97% (via interpolation) as the goat was administered with test material 2 months after preparation. The specific activity of  $^{14}\text{C}$ -BAS 684 H was 4.09  $\mu\text{Ci/mg}$  (0.151 MBq/mg).

##### ***Sampling and analysis***

The goat was milked twice daily and the afternoon and following morning milk pooled.

The goat was sacrificed 6 hours after last dose and the following tissues were sampled: liver, kidney, back and leg muscle, subcutaneous and mesenteric fat. All samples were stored at  $-20\text{ }^{\circ}\text{C}$  prior to analysis. TRR was also determined in urine, faeces, blood (plasma/cells), milk, skimmed milk and cream.

Radioactivity was measured by combustion followed by LSC.

##### ***Extraction and characterisation***

Extraction was performed for skimmed milk and liver only.

For skimmed milk, trichloroacetic acid (TCA) was added to precipitate proteinaceous material. The supernatant following centrifugation was removed and the pellet (residual residue) was washed four times with aqueous TCA and centrifuged. The TCA washes of the pellet were combined with the original TCA supernatant and extracted three times with chloroform. The pellet was extracted twice with methanol. The chloroform extract was further processed by evaporation and subjection to silica gel chromatography; a gradient elution was used (mobile phase A: hexane; mobile phase B: ethyl acetate). The fractions resulting from silica gel chromatography were subjected to two-dimensional TLC (mobile phase 1: toluene:propan-2-ol:acetic acid (150:20:1.5 v:v); mobile phase 2: hexane:propan-2-ol:acetic acid (120:30:1 v:v)).

For liver, TCA was added. The supernatant following centrifugation was removed and the pellet washed by suspending in 0.01 M phosphate buffer followed by centrifugation. The supernatants were combined with the original TCA supernatant and extracted three times with chloroform. The aqueous phase was extracted three times with ethyl acetate. The ethyl acetate phases were combined with the chloroform phase and subjected to TLC (mobile phase: toluene:propan-2-ol:acetic acid 150:20:1.5 v:v). The aqueous phase (resulting from the chloroform and ethyl acetate extraction) was acid hydrolysed (1H HCl) at  $95\text{ }^{\circ}\text{C}$  for 2 hours. The hydrolysate was extracted three times with chloroform. The aqueous phase was extracted three times with ethyl acetate. The buffer-washed pellet was extracted twice with methanol and four times with acetone. The acetone was evaporated, and water added to the remaining aqueous residue. This aqueous phase was extracted three times with ethyl acetate and the ethyl acetate phase chromatographed.

The initial step of using TCA to precipitate proteinaceous material is not in accordance with OECD 503, in which solvents/solvent systems of varying polarities are initially used to extract samples. The harsh nature of this initial extraction may have hindered characterisation and identification of residues.

The study notes that the extraction method could be improved by not using ethyl acetate, given its polarity which may extract water-soluble conjugates from the aqueous phase, especially given the supernatant was acidic.

## Results

### TRR

Table 7-123 gives the TRR in each matrix six hours after the fourth dose. The TRR in milk, muscle and fat were < 0.5 mg eq/kg; whereas in liver and kidney, the TRR amounted to approximately 3 mg eq/kg. The total residues in tissues represented <1% of the administered dose.

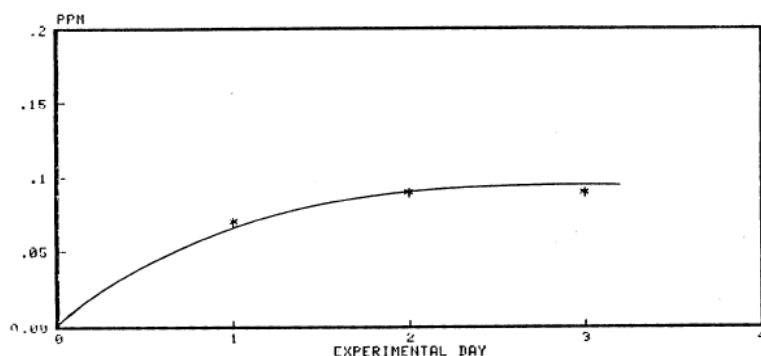
Table 7-123 TRRs determined in animal matrices

| Matrix           | TRR (mg eq/kg)             | Standard deviation ( $\pm$ mg eq/kg) |
|------------------|----------------------------|--------------------------------------|
| Whole milk       | 0.15 (0.09 <sup>†</sup> )  | 0.003 (0.001 <sup>†</sup> )          |
| Skimmed milk     | 0.11 (0.07 <sup>†</sup> )  | 0.001 (0.001 <sup>†</sup> )          |
| Cream            | 0.48 (0.34 <sup>†</sup> )  | 0.005 (0.012 <sup>†</sup> )          |
| Whole blood      | 0.44                       | 0.007                                |
| Blood plasma     | 0.51                       | 0.023                                |
| Blood cells      | 0.05                       | 0.001                                |
| Feces            | 50.0 (81.0 <sup>†</sup> )  | 2.9 (3.0 <sup>†</sup> )              |
| Urine            | 79.0 (199.0 <sup>†</sup> ) | 0.48 (8.5 <sup>†</sup> )             |
| Liver            | 4.14                       | 0.065                                |
| Kidney           | 3.84                       | 0.052                                |
| Mesenteric fat   | 0.17                       | 0.004                                |
| Subcutaneous fat | 0.15                       | 0.000                                |
| Back muscle      | 0.12                       | 0.001                                |
| Leg muscle       | 0.11                       | 0.002                                |

<sup>†</sup>The numbers in brackets refer to the TRR (mg eq/kg) in the matrix collected over the 24 hour period between the third and fourth dose

A figure is provided in the study to justify a plateau being reached in milk (copied into Figure 7-17); the results were not tabulated. The study states that the TRR reached a plateau within 2 days at 0.09 mg eq/kg however only 3 days of 24 hour-pooled milk data are available which is insufficient to draw a robust conclusion from.

Figure 7-17 TRR in whole milk from 24 hour pooled samples



### Extractability and characterisation

**Skimmed milk:** The distribution of radioactive residues between organic and aqueous phases and further extraction of the pellet resulting from centrifugation is shown in Table 7-124. Silica gel chromatography using hexane: ethyl acetate of the chloroform extract resulted in 25% TRR eluted from the column, 5% eluted with

methanol and 8% TRR bound to the column. The two largest peaks following silica gel chromatography were pooled and a two-dimensional TLC was performed. 17 metabolites were detected and the most abundant metabolite was present at <0.01 mg eq/kg and <1% TRR. No further characterisation work was performed on the aqueous phase.

Table 7-124 Distribution of radioactive residues between skimmed milk extracts

| Extract   | Distribution of radioactive residues |                       |
|---|--------------------------------------|-----------------------|
|   | %TRR                                 | mg eq/kg <sup>†</sup> |
| <b>TRR</b>  | <b>100</b>                           | <b>0.11</b>           |
| Organic phase (chloroform extract of TCA supernatant) | 44                                   | 0.048                 |
| Aqueous phase   | 42                                   | 0.046                 |
| Pellet  | 9                                    | 0.010                 |
| <i>Methanol extract of pellet</i>                     | <i>4</i>                             | <i>0.004</i>          |
| <i>Unextractable residue</i>                          | <i>5</i>                             | <i>0.006</i>          |

<sup>†</sup>The distribution of radioactive residues between extracts is only given in the report as %TRR values; the absolute mg eq/kg values have been calculated by HSE using these values and the TRR reported in Table 7-123.

**Liver:** The distribution of radioactive residues between organic and aqueous phases and further extraction of the pellet resulting from centrifugation is shown in Table 7-125. TLC of the organic phase resulted in 6 bands (maximum calculated by HSE approx. 8.0% TRR, 0.33 mg eq/kg; however report states maximum represents > 10% TRR and > 0.4 mg eq/kg). TLC of the organic extracts of the acid-hydrolysed aqueous phase resulted in 8 bands for the chloroform extract (maximum calculated by HSE approx. 2% TRR, 0.08 mg eq/kg) and 4 bands for the ethyl acetate extract (maximum calculated by HSE approx. 1.3% TRR, 0.05 mg eq/kg). TLC of the ethyl acetate phase following extraction of the pellet resulted in 3 bands (maximum approx. 3.4% TRR, 0.14 mg eq/kg).

Table 7-125 Distribution of radioactive residues between liver extracts

| Extract  | Distribution of radioactive residues |                       |
|--|--------------------------------------|-----------------------|
|  | %TRR                                 | mg eq/kg <sup>†</sup> |
| <b>TRR</b>   | <b>100</b>                           | <b>4.14</b>           |
| Organic phase (combined chloroform/ethyl acetate extract of TCA supernatant) | 24                                   | 0.99                  |
| Aqueous phase  | 23                                   | 0.95                  |
| <i>Acid hydrolysis of aqueous phase</i>                                      | <i>12</i>                            | <i>0.50</i>           |
| <i>Chloroform extract of the hydrolysate</i>                                 | <i>8</i>                             | <i>0.33</i>           |
| <i>Ethyl acetate extract of the hydrolysate</i>                              | <i>4</i>                             | <i>0.17</i>           |
| <i>Unextractable following acid hydrolysis of aqueous phase</i>              | <i>12</i>                            | <i>0.50</i>           |
| Pellet   | 40                                   | 1.66                  |
| <i>Methanol extract of pellet</i>  | <i>6</i>                             | <i>0.25</i>           |
| <i>Acetone extract of pellet</i>   | <i>10</i>                            | <i>0.41</i>           |
| <i>Unextractable residue</i>   | <i>24</i>                            | <i>0.99</i>           |

<sup>†</sup>The distribution of radioactive residues between extracts is only given in the report as %TRR values; the absolute mg eq/kg values have been calculated by HSE using these values and the TRR reported in Table 7-123.

### Conclusion

Whilst the study reports a plateau in the TRR was observed within 2 days at 0.09 mg eq/kg, with 75% of the radioactive residues in the skimmed milk fraction of the whole milk, only 3 days of milk data are available which is insufficient to draw a robust conclusion.

The TRR in liver and kidney (approx. 4.0 mg eq/kg) was higher than in muscle and fat (approx. 0.2 mg eq/kg).

TLC of the various fractions in both skim milk and liver indicate BAS 684 H is extensively metabolised to a variety of metabolites which are mostly less than 10% TRR with a possible exception of the organic extract of

liver with a number of metabolites larger than 10% TRR according to the study report. However, the study report does not state that any identification of metabolites was undertaken in either skim milk or liver.

The main deficiencies of the study are that it was not performed to OECD Guideline 503, nor to GLP. The purpose of study was for workers at the testing facility to gain confidence in handling goats and familiarising themselves with methods/techniques used to isolate metabolites of BAS 684 H from skimmed milk and liver. Only a single ring was labelled (phenyl-U-<sup>14</sup>C, however the modern studies in Section B.7.2.3 use both labels), the animal was only dosed for 4 days, and skim milk and liver were the only edible matrices extracted. Radiochemical purity was not determined at the time of administration to the goat but rather estimated based on interpolation between two storage intervals. Due to these deficiencies, the study cannot be relied upon. Given no identification of metabolites was performed, this study provides limited supporting information.

|                    |   |
|--------------------|---|
| <b>Report:</b>     | CA 6.2.3/4<br>Woodward M. et al., 1984 a<br>Use of goats for ruminant metabolism studies. Part 2 characterisation and identification of sd95481 metabolites in urine and faeces<br>CI-440-014 |
| <b>Guidelines:</b> | <none>  |
| <b>GLP:</b>        | no  |

This report describes the characterisation and identification of the metabolites in urine and faeces originating from the goat dosed with BAS 684 H in Report CA 6.2.3/3.

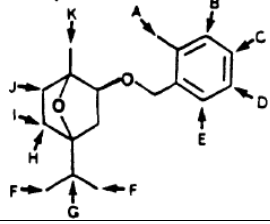
Given only urine and faeces were analysed, the report is considered as supporting information, however, given the metabolites identified in excreta may inform the livestock metabolic pathway, a brief summary of the methodology and results is given below.

The urine sample was adjusted to pH 3 using 6 N hydrochloric acid and extracted three times with chloroform. Further extraction of water-soluble conjugates was carried out using beta-glucuronidase enzymes and subsequently acid hydrolysis. Radioactivity was recovered by chloroform extraction separately after both enzyme and acid hydrolysis and analysed by two-dimensional TLC and LSC. The faecal sample was extracted by ether Soxhlet extraction and analysed directly by two-dimensional TLC and LSC.

Identification was performed primarily by TLC with at least 2 different solvent systems, and also autoradiography, radio-GLC, GLC and GC-MS. The combination of techniques used to achieve identification for each metabolite are given in Table 7-126. More than 25 metabolites were identified in excreta as laid out in Table 7-126. Identification was achieved by the methods stated in the table.



Table 7-126 Structures of major metabolites identified in goat excreta

| Metabolite           | Feces %TRR                  | Urine % TRR                 |                   |                 | Hydroxyl position substitution  | How identification/ characterisation was achieved                            |
|----------------------|-----------------------------|-----------------------------|-------------------|-----------------|---|--|
|                      |                             | Organic extractable         | Enzyme hydrolysis | Acid hydrolysis |   |  |
|                      |                             |                             |                   |                 |  |  |
| BAS 684 H (SD 95481) | 4.6                         | -                           | -                 | -               | -   | -  |
| M684H059 (SD 637)    | -                           | 0.6                         | -                 | 3.9             | -   | -  |
| M684H002 (SD 207856) | 29.4                        | 7.4                         | 7.0               | -               | A   | GLC retention time and EI MS consistent with reference standard              |
| M684H017 (SD 211648) | 1.0                         | 1.8                         | 2.6               | -               | B   | GLC retention time and EI MS consistent with reference standard              |
| M684H019 (SD 211368) | 0.8                         |                             |                   |                 | C   | GLC retention time and EI MS consistent with reference standard              |
| M684H018 (SD 211647) | 1.7                         |                             |                   |                 | D   | GLC retention time and EI MS consistent with reference standard              |
| M684H024 (SD 207430) | 19.8                        | 3.7                         | 4.1               | -               | F   | GLC retention time and EI MS consistent with reference standard              |
| SD 211732            |                             |                             |                   |                 | J   | GLC retention time and EI MS consistent with known soybean plant metabolite  |
| SD 213325            |                             | -                           | -                 | -               | F'  | EI MS of underivatised and TMS derivative consistent with proposed structure |
| M684H004 (SD 205588) | 6.8                         | -                           | -                 | -               | G   | GLC retention time and GC/MS consistent with reference standard              |
| M684H044 (SD 207855) | 13.7                        | 2.7                         | 7.4               | -               | A/G   | GC/EI-MS. TMS derivative GC/EI-MS  |
| SD 213323            | -                           | Detected but not quantified |                   |                 | B/G   | TMS derivative GC/EI-MS  |
| SD 213327            | Detected but not quantified | -                           | -                 | -               | C/G   | TMS derivative GC/EI-MS  |
| SD 211733            | Detected but not quantified | 3.0                         | -                 | -               | D/G   | TMS derivative GC/EI-MS  |

|                                       |      |                             |      |     |        |  |
|---------------------------------------|------|-----------------------------|------|-----|--------|--|
| SD 214014                             | 2.8  | 2.1                         | 4.1  | -   | A/F    | FAB-MS. TMS derivative GC/EI-MS  |
| SD 213324                             | -    | 0.5                         | 1.1  | -   | A/J    | TMS derivative GC/EI-MS  |
| SD 214013                             | -    | 0.8                         | 3.3  | -   | F/G    | CI-MS. TMS derivative GC/EI-MS   |
| SD 214012                             | 1.6  | -                           | 4.4  | -   | A/G/J  | TMS derivative GC/EI-MS  |
| SD 214011                             | -    | -                           | 3.7  | -   | A/F/G  | TMS derivative GC/EI-MS. Isobutane CI-MS   |
| SD 211892                             | 3.8  | -                           | -    | -   | A'     | GLC and EI MS of underivatized and TMS derivative consistent with known soybean plant metabolite |
| SD 214010                             | -    | Detected but not quantified |      |     | A/F'   | CI-MS. TMS derivative GC/EI-MS   |
| M684H013 (SD 207852)                  | -    | 2.6                         | -    | -   | A*/F   | TMS derivative GC/EI-MS consistent with reference standard                                       |
| M684H011 (SD 207574)                  | -    | 3.9                         | -    | -   | A*/G   | TMS derivative GC/EI-MS consistent with reference standard                                       |
| SD 214009                             | -    | Detected but not quantified |      |     | A*/J   | TMS derivative GC/EI-MS  |
| SD 214008                             | -    | Detected but not quantified |      |     | A*/G/J | TMS derivative GC/EI-MS  |
| Other minor and unidentified products | 14.0 | 10.3                        | 4.0  | 2.0 |        |  |
| Total                                 | 100  | 39.4                        | 41.8 | 5.9 |        |  |

\*indicates carboxylic acid group at position A

'indicates a hydroxyl group at the position indicated and a double bond in the isopropyl side chain

The isolation, characterisation and identification of approximately thirty BAS 684 H degradation products in the goat urinary and faecal excreta are reported. The majority of the isolated products are mono-, di- or trihydroxylated derivatives of BAS 684 H. Several hydroxylated products also contained a carboxylic acid moiety at the 2-benzyl position.

The deficiencies identified with the in-life phase given under CA 6.2.3/3 above also apply to this study. Additionally this study was not conducted to GLP or OECD Guideline 503: in addition to the deficiencies with the first part of the study given under CA 6.2.2/3, no details on storage stability are reported and metabolites were only characterised and identified in urinary and faecal excreta. Due to these deficiencies, the study cannot be relied upon but can be considered supporting information.

|                    |  |
|--------------------|--|
| <b>Report:</b>     | CA 6.2.3/5<br>Lee P. et al., 1989 a<br>Metabolic fate of cinmethylin in goat<br>CI-905-008 |
| <b>Guidelines:</b> | <none>   |
| <b>GLP:</b>        | no (not subject to GLP)  |

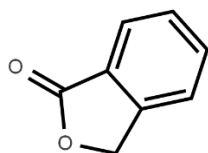
This document represents a peer reviewed literature article in the Journal of Agricultural and Food Chemistry published in 1989, which summarises the results of metabolism of BAS 684 H in goats from the studies above and additionally provides information on metabolites in liver. The information duplicated from study CA 6.2.3/3 has not been repeated, however, the additional information on metabolites in liver has been added below.

The article states that parent BAS 684 H was not recovered in tissues (only in faeces). In the excreta and liver, at least 25 metabolites were isolated and identified as organic-extractable and conjugated products, classified as mono-, di-, trihydroxylated, dehydrated, carboxylated, methoxylated and ether linkage cleavage products. The only quantitative results reported in the article are for excreta, which are mostly consistent with Table 7-126. There are some minor differences such as the F- and J- hydroxylated metabolites being determined as part of a sum including of B, C and D hydroxylated metabolites, which is not consistent with the study report.

In liver, 62% TRR precipitated following treatment with trichloroacetic acid. Extraction of the precipitate with hot acetone released an additional 30% TRR. No chromatographic characterisation of the acetone extract was achieved due to contamination with lipid and other biological components. The aqueous homogenate, after the removal of the proteinaceous materials, contained 38% TRR. Subsequent fractionation resulted in an organosoluble fraction containing 9% TRR, 3% TRR recovered after  $\beta$ -glucuronidase hydrolysis, 1% TRR after acid hydrolysis and 26% in the final aqueous phase.

TLC was the primary method for identification of liver residues. Parent BAS 684 H accounted for < 1% TRR., M684H059 (referred to in the report as *o*-(Hydroxy-methyl)benzoic acid lactone, structure shown in **Error! Reference source not found.**) was the major product detected in the organosoluble fraction. No information on how this metabolite was identified is given nor its quantitative level (in % TRR or mg eq/kg). However, given the organosoluble fraction contained 9% TRR, the level of M684H059 will be <9 % TRR given the radioactivity was distributed between >20 metabolites.

Figure 7-18 Structure of *o*-(Hydroxy-methyl)benzoic acid lactone (M684H059)



The major product after enzyme and acid hydrolysis was a monohydroxylated product (M684H004 (SD 205588)), which given that 3% TRR was present after enzyme hydrolysis, was present at < 3 % TRR. The article gives no information on the identity or quantitative level (in % TRR or mg eq/kg) of the other metabolites which are stated to have been identified in liver.

It is noted that the distribution of radioactivity between different liver fractions reported in the article is different to that reported in the study CA 6.2.3/3; additionally the identification of residues in liver was not reported in study CA 6.2.3/4 hence it is concluded that the results in the article must derive from other studies. Given this is not a study report conducted to GLP and OECD guidelines, it provides limited information, in particular, almost no quantitative information is given on the identification of metabolites in liver, other than parent BAS 684 H accounting for < 1% TRR. The article does not quantify the absolute (mg eq/kg) or relative (%TRR) amounts of metabolites in liver, and although reports over 20 metabolites were observed in liver, it does not provide the identity of these except M684H059. Therefore this article is considered to be of limited use, but can be considered supporting information.

A comparison of the metabolites identified in the old goat study with the metabolites identified in the new studies is made in Table 7-127. The metabolic pathway in the 1980s study is similar to the new goat study and there is a good correlation between the metabolite structures in the old and new studies, considering the new studies identified conjugated metabolites prior to further extraction whereas the 1980s studies only identified metabolites after deconjugation. As the new studies used LC-MS/MS for identification, the exact positions of hydroxylation were uncertain given regioisomers have the same m/z ratio and typical key fragments.

Table 7-127 Comparison of metabolites identified in old and new studies

| Metabolites identified in old goat study | Correlation in new studies (unconjugated) | Correlating metabolites identified in new goat study  |
|--|---|---|
| SD 207856                                | M684H002                                  | Direct: M684H002<br>Glucuronide M684H012  |
| SD 211648                                | M684H017                                  | One of the possible isomers of M684H034   |
| SD 211368                                | M684H019                                  | One of the possible isomers of M684H034   |
| SD 211647                                | M684H018                                  | One of the possible isomers of M684H034   |
| SD 207430                                | M684H024                                  | One of the possible isomers of M684H052   |
| SD 205588                                | M684H004                                  | One of the possible isomers of M684H052   |
| SD 211732                                | -----                                     | One of the possible isomers of M684H052   |
| SD 207855                                | M684H044                                  | One of the possible isomers of M684H022   |
| SD 213323                                | M684H039                                  | None  |
| SD 213327                                | M684H039                                  |   |
| SD 211733                                | M684H039                                  |   |
|  |   | M684H039 was observed in the new poultry study; in goat double hydroxylation only observed with hydroxylation at the methyl group of the phenyl ring; hydroxylation of other positions of the phenyl ring only observed with single hydroxylation in M684H034 |
| SD 214014                                | M684H039                                  | One of the possible isomers of M684H022   |
| SD 213324                                | M684H039                                  | One of the possible isomers of M684H022   |
| SD 214013                                | -----                                     | None  |
|  |   | Double hydroxylation in the isopropyl group not observed in new goat study, observed in poultry with M684H027   |
| SD 214012                                | -----                                     | One of the possible isomers of M684H057   |
| SD 214011                                | M684H053                                  | Direct: M684H056<br>One of the possible isomers of M684H057   |
| SD 211892                                | -----                                     | None  |
| SD 213325                                | -----                                     | None  |
| SD 214010                                | -----                                     | None  |
| SD 207852                                | M684H013                                  | None  |
|  |   | In new goat study, carboxyl group in connection with hydroxylation of isopropyl group only observed at tertiary carbon atom (M684H011)  |
| SD 207574                                | M684H011                                  | M684H011  |
| SD 214009                                | -----                                     | None  |

|           |          |  |
|-----------|----------|--|
|           |          | In new goat study, carboxyl group in connection with hydroxylation of isopropyl group only observed at tertiary carbon atom (M684H011) |
| SD 214008 | M684H027 | None   |
|           |          | Not observed in goat, but observed in new poultry study  |

#### B.7.2.4. Pigs

No study is needed for pig as the estimated maximum feed burden (1x level) is below 0.004 mg/kg bw/d (see Volume 1) and therefore none is provided in the present dossier. Additionally, the metabolic pathways of BAS 684 H in rodents (rats) and ruminants (goat) are sufficiently similar. Only three minor metabolites were detected in the new ruminant metabolism study that are not detected in the rat metabolism;

- M684H052 (detected up to 6.3 % TRR or 0.863 mg eq/kg in urine)
- M684H056 (detected up to 3.7 % TRR or 0.503 mg eq/kg in urine)
- M684H057 (detected up to 7.1 % TRR or 0.697 mg eq/kg in urine)

As these are minor metabolites in the ruminant metabolism study, and in general the same biotransformation steps occur in both rat and goat metabolism it can be concluded that a metabolism study in pigs is not required.

#### B.7.2.5. Fish

##### Applicant submission on fish metabolism:

With regards to the representative uses, the following matrices are considered fish feedstuffs according to SANCO/11187/2013: triticale (grain), wheat (extruded grain, bran, flour, germ, middlings, gluten, dried distiller's grain), soybean (treated seed, meal decorticated, protein), sunflower (seed, meal decorticated), barley (bran fractions, brewer's grain dried), cottonseed (meal), linseed (meal), mustard (meal), rape seed/canola (meal), sesame seed (meal), safflower (meal decorticated), vegetable oil.

The log Po/w for parent BAS 684 H is >3, i.e. 4.5, and thus a fish metabolism study was conducted. However, as residue studies on oilseeds and cereals show, residues of BAS 684 H are <LOQ in seeds and grain (see Section 7.3). Thus, it can be reasonably expected that fish are not exposed to BAS 684 H residues under realistic conditions.

Likewise, residues of metabolite M684H005 (including M684H006) were shown to be <LOQ in seeds and grain. Furthermore, the log Po/w for M684H005 and M684H006 is <3, thus no fish metabolism study is triggered for these compounds.

##### HSE comment on fish metabolism:

At present there is no agreed guidance on how to conduct fish metabolism studies in order to determine the residue definition for risk assessment and monitoring. Specifically, for fish metabolism and fish feeding studies, at the SCoPAFF (pesticide residues) meeting in November 2014, the COM stated the following in the minutes of the meeting:

*The Commission emphasised that for the time being there are no agreed test guidelines and that hence the pertinent data requirements can be waived. This was also clarified in general at the meeting of them Committee's section on Plant Protection Products – Legislation on 09/10 October 2014, and laid down in document SANCO/10181/2013 Rev 2.1. Such test guidelines must be published in the form of an update of the respective Commission Communications.*

Document SANCO/11187/2013 31 January 2013 rev. 3 is a working document on the nature of residues in fish. This was published on the EU Commission website to show how the status of the guidance (for fish metabolism studies) as in development. It has not been agreed that this document has been noted for use.

Consequently, the above data requirements do not need to be addressed at this time. However, the applicant has submitted a study on fish metabolism as part of the dossier and presented a detailed study summary within the submission. The study summary provided by the applicant is copied in full below (in italics). HSE has not amended or edited this summary, or evaluated the data presented due to the lack of available test guidelines. The

active substance BAS 684 H was observed to be metabolised by typical biotransformation reactions. Metabolite M684H026 resulted from cleavage of the phenyl group from the parent molecule and hydroxylation of the isopropyl moiety of the cyclohexane group. The metabolite M684H001 resulted from hydroxylation and oxidation of the parent compound BAS 684 H. These metabolites were also found in the goat and hen metabolism studies.

**Report:** CA 6.2.5/1  
 2018a  
*The Metabolism of [<sup>14</sup>C]-BAS 684 H in Rainbow Trout (*Oncorhynchus mykiss*)*  
 2018/1015281

**Guidelines:** 2004/10/EC of 11 February 2004, OECD 305, SANCO/11187/2013 (31 January 2013)

**GLP:** yes  
 (certified by Landesamt fuer Umwelt, Mainz, Germany)

## I. MATERIAL AND METHODS

### A. MATERIALS

#### 1. Test Material:

**Description:** BAS 684 H

**Lot/batch #:** 1147-2101 (phenyl-<sup>14</sup>C)  
 1159-1012 (benzyl-<sup>13</sup>C)  
 1146-2001 (cyclohexane-4-<sup>14</sup>C)  
 L87-84 (unlabeled)

**Purity:** Phenyl label: 98.0% (radiochemical purity)  
 16.5 MBq/mg (specific activity of a.s.)  
 Benzyl label: 99.6% (chemical purity)  
 Cyclohexane label (<sup>14</sup>C): 97.9% (radiochemical purity)  
 8.08 MBq/mg (specific activity of a.s.)  
 Unlabeled: 99.0% (chemical purity)

**CAS#:** 87818-31-3

#### 2. Test animals:

**Species:** Fish  
 Rainbow trout (*Oncorhynchus mykiss*)

**Variety:** Teleostei, Salmonidae

**Gender:** Unspecified

**Age:** Obtained at a size of 2-4 cm and raised to the experimental target weight

**Weight at dosing:** About 338-349 g (calculated based on the average fish weight measured on day -7 and the average feed uptake observed during days -7 to -1; no measurement at the start of experiment and during the exposure period was conducted to avoid stress)

**Number of animals:** 14 treated animals (7 per label)

**Acclimation period:** At least 14 days

**Diet:** Trout feed pellets, e.g. Milkivit® Type F-2P B40, Skretting, once daily

**Housing:** Experimental tank (2 m<sup>3</sup>) filled with approx. 1.4 m<sup>3</sup> pre-conditioned tap water

**Environmental conditions**

**Temperature:** 15±2 °C

**Air changes:** Flow-through rates between 60.0 and 85.2 L/h

**Photoperiod:** 16 h light / 8 h dark

### B. STUDY DESIGN AND METHODS

#### 1. Dosing regime

**Dosing route:** Oral

**Amount of dose:** Phenyl label: 14.86 mg/kg DW

**Food consumption:**

Cyclohexane label: 12.44 mg/kg DW  
 Phenyl label: 55.47 g/day (average)  
 Cyclohexane label: 59.81 g/day (average)

**Vehicle:**

Trout feed pellets

**Timing:**

Several times per day

**Duration:**

11 days

**2. Sample collection****Excreta collection:**

Daily

**Interval from last dose to sacrifice:**

6-12 h

**Tissues collected and analysed:**

Fillet without skin, skin, liver (entire organ), GI tract content and pyloric caeca, cleaned GI tract, carcass without GI tract and pyloric caeca (after dissection of liver and fillet)

**3. Test procedure**

The in-life phase of this study as well as TRR measurement was performed at [REDACTED]. The analytical phase of this study was performed at the [REDACTED].

For the phenyl label, the dose formulation consisted of [phenyl- $U$ - $^{14}C$ ], [benzyl- $^{13}C$ ] and unlabeled BAS 684 H in a 1:2:1 ratio. For the cyclohexane label, the dose formulation consisted of [cyclohexane-4- $^{14}C$ ] and unlabelled BAS 684 H in a 1:1 ratio. The specific activity was 3.00 MBq/mg (phenyl label) and 3.41 MBq/mg (cyclohexane label).

The dose formulation of each radiolabeled form was used to prepare 800 g fish feed each. The target test dose was 12 mg/kg diet (dry weight) based on results of non-GLP pre-tests. 4 batches of fish feed were spiked with the cyclohexane label and phenyl label, respectively, to prepare the full volume of feed required to run the test and necessary analysis. The different batches of spiked pellets were mixed carefully, to ensure an even distribution of the test item in the experimental diet. Until usage, the experimental diet was stored in a refrigerator (4-7°C). Stability of the test item on feed was analysed directly after preparation of feed, prior the first dosing, after one week and after the experimental phase. The feed was stored in the refrigerator (4°C). Analysis was performed by HPLC with online radio-detection after extraction of the feed with acetonitrile. The test item in the fish feed pellets was stable over the whole dosing period.

The effluent of the experimental tanks was passed through a filter column filled with activated charcoal and was finally collected in a separate tank to monitor the remaining total radioactivity. In addition to that, water in the tank was constantly recirculated through two filter columns filled with activated charcoal to avoid the accumulation of dissolved test item and metabolites in the water of the experimental tank.

**4. Description of analytical procedures**

**Dosing and sample collection:** Two radiolabelled forms of BAS 684 H (phenyl and cyclohexane label) were each administered to 14 fish for eleven consecutive days. Based on the results of a non-GLP pre-test, seven fish per label were dosed at a nominal level of 12 mg/kg BAS 684 H per dry weight diet. The daily ration was distributed in portions on the surface of the water. It was ensured that the ration was ingested immediately by the animals. Feces and any remaining uneaten feed (very unlikely) were siphoned daily prior to feeding and 90-150 minutes after feeding. The number of daily uneaten food pellets was used to derive the total weight of daily uneaten food pellets by multiplying the number of pellets with the average original weight (prior to immersion in water) per pellet.

The radioactive content of water in the experimental tanks was determined by LSC analysis of triplicate aliquots taken every day from the experimental tanks 90-150 min after feeding to monitor a potential exposure of fish via water caused by leaching of the test item from feces, urine or uneaten feed. In addition, triplicate water samples were taken from the effluent tank every day throughout the experiment to estimate the total radioactivity released with waste water.

At approximately 6-12 hours post final dose the fish were sacrificed and edible tissues (fillet, liver and skin), GI tract and pyloric caeca were removed post mortem.

At the end of the study, the activated charcoal was removed from the filter columns and mixed.

Determination of TRR: Fillet, liver and skin were homogenized and radioactive residues were determined in aliquots by combustion analysis followed by liquid scintillation counting (LSC).

The pooled feces samples were homogenized and analysed by combustion followed by LSC to determine the radiochemical content. The radioactivity in the water was low throughout the study. Therefore, the effect on tissue concentration caused by bioconcentration processes can be assessed to be negligible.

The radiochemical content of the activated charcoal was determined by combustion analysis of triplicate aliquots.

Pooling: Samples from all animals were pooled per matrix and radiolabel. Samples were stored on dry ice until further sample preparation and determination of the radiochemical content.

Homogenization/solvent extraction: Trout fillet, liver and skin samples were extracted three times with methanol. Additionally, trout liver and skin samples were subsequently extracted twice with water.

Prior to HPLC analysis and HPLC fractionation, samples were concentrated and subsequently diluted in an adequate solvent. When required the mixture was treated with a suitable amount of Triton X-100 and dissolved by ultrasonication. Prior to SPE purifications, samples were concentrated and subsequently diluted in adequate solvents. All concentration steps were performed at approx. 40°C and 160 rpm using a rotary evaporator.

Enantiomer specific analysis: To analyze if one enantiomer of BAS 684 H was preferably metabolized in fish, the parent compound was isolated from the extracts of trout fillet and analysed on a chiral HPLC column. For enantiomer specific analysis, a sub-sample of the methanol extract of trout fillet of workup sample 1 of the phenyl label was investigated. For sample preparation, the sample was concentrated and purified. The acetonitrile eluate after SPE purification was fractionated using HPLC and the peak representing the parent compound was investigated using an enantiomer-specific HPLC method.

Isolation and identification of metabolites: Sub-fractions of methanol extracts with a sufficient level of radioactive residues were analysed by HPLC. The peaks in the HPLC chromatograms used for quantification and confirmation were identified by comparison of their retention times and metabolic pattern with the reference items, that were generated in a study on the metabolism of  $^{14}\text{C}$ -BAS 684 H in rats using co-chromatography. The chemical structures of the reference items were elucidated by MS analyses in the same study.

## II. RESULTS AND DISCUSSION

### 1. Distribution of radioactive residues and total radioactive residues (TRR)

The overall recovery of radioactive residues is provided in Table 7-128. Approximately 94.5% and 92.9% of the administered dose were recovered in total for the phenyl and cyclohexane label, respectively. The main fraction was found in the charcoal accounting for approximately 66.5% (phenyl label) and 61.2% (cyclohexane label). The high amounts of radioactivity in the activated carbon filter system demonstrate the efficiency of the flow through filter system that constantly eliminated the excreted radioactive compounds. Thus, the only uptake pathway of radioactivity was via uptake of the fortified feed.

Radioactive residues recovered in water accounted for up to 17.1% (phenyl label) and 23.1% (cyclohexane label). In pyloric caeca 6.68% and 7.71% were recovered for the phenyl and cyclohexane label, respectively.

Radioactive residues associated with edible portions (tissues) only accounted for up to 0.21% (phenyl label) and 0.51% of the administered dose (cyclohexane label).

Table 7-128 Distribution of radioactive residues in tissue and excreta of fish after administration of BAS 684 H for 11 days



| Matrix               | [% dose]     |                   |
|----------------------|--------------|-------------------|
|                      | Phenyl label | Cyclohexane label |
| <i>Fillet</i>        | 0.21         | 0.51              |
| <i>Liver</i>         | 0.06         | 0.06              |
| <i>Skin</i>          | 0.04         | 0.08              |
| <i>Pyloric caeca</i> | 6.68         | 7.71              |
| <i>Faeces</i>        | 3.96         | 0.22              |
| <i>Charcoal</i>      | 66.47        | 61.20             |
| <i>Water</i>         | 17.06        | 23.12             |
| <b>Total</b>         | 94.47        | 92.92             |

In the present study, the TRR was calculated by summarizing the extractable radioactive residue (ERR) and the residual radioactive residue (RRR) after solvent extraction. This value was set to 100% and was used for further calculations. In general, the TRR measured was similar to the TRR calculated.

The results are summarized in Table 7-129. The main portions of radioactive residues were recovered in feces (0.30-4.13 mg/kg). In the relevant matrices, the highest TRR concentrations were calculated for liver (0.086-0.101 mg/kg). For the other matrices, the TRR was in a range from 0.016 mg/kg to 0.028 mg/kg (phenyl label) and from 0.040 mg/kg to 0.049 mg/kg (cyclohexane label).

For the phenyl label, 94.47% of the total administered radioactivity was recovered, the majority of which was present in the charcoal (66.47%) and water (17.06%). For the cyclohexane label, 92.92% of the dose was recovered, of which the majority was present in the charcoal (61.20%) and in the water (23.12%). Recovery of radioactivity in fillet samples was 0.21% of the applied dose (phenyl label) and 0.51% of the applied dose (cyclohexane label). In fillet skin 0.08% of the applied dose for cyclohexane label and 0.04% of the applied dose for phenyl label was recovered. The recovery rate for both the cyclohexane label and the phenyl label in liver was 0.06% of the applied dose. The highest TRR was measured in trout liver (phenyl label: 0.086 mg/kg and cyclohexane label: 0.101 mg/kg).

In trout fillet (phenyl label: 0.016 mg/kg and cyclohexane label: 0.040 mg/kg) and skin (phenyl label: 0.028 mg/kg and cyclohexane label: 0.049 mg/kg) the TRR was lower for both labels.

*Table 7-129 Total radioactive residues in samples from fish (trout) following treatment with BAS 684 H for 11 days*

| Matrix        | Sampling time | TRR measured<br>[mg/kg] | TRR calculated <sup>1</sup><br>[mg/kg] | TRR measured<br>[mg/kg] | TRR calculated <sup>1</sup><br>[mg/kg] |
|---------------|---------------|-------------------------|--|-------------------------|--|
|               |               | Phenyl label            |  | Cyclohexane label       |  |
| <i>Feces</i>  | Daily         | 4.13                    | Not determined                         | 0.30                    | Not determined                         |
| <i>Fillet</i> | Terminal      | 0.017                   | 0.016                                  | 0.037                   | 0.040                                  |
| <i>Liver</i>  | Terminal      | 0.102                   | 0.086                                  | 0.098                   | 0.101                                  |
| <i>Skin</i>   | Terminal      | 0.027                   | 0.028                                  | 0.047                   | 0.049                                  |

1 For trout fillet of both labels, the methanol extraction was repeated. Hence, the concentration of the TRR was calculated as the sum of the pooled methanol extracts and the corresponding residue of workup sample 2. The sum of ERR and RRR for workup sample 1 is 0.017 mg/kg or 107.4% TRR (phenyl label) and 0.041 mg/kg or 104.8% TRR (cyclohexane label) for trout fillet. Hence, the calculated TRR of fish fillet is slightly lower compared to the sum of ERR and RRR. The difference of both TRRs (0.001 mg/kg) is marginal and results from the two different methods applied (combustion vs. LSC measurement). For trout liver and skin, the TRR was calculated as the sum of the pooled methanol and water extracts and the corresponding residues.

## 2. Extractability of radioactive residues

Fillet, liver and skin were extracted and the results are summarized in Table 7-130.

From all tissues, high amounts of radioactive residues (phenyl label: 87.8-107.4% TRR and cyclohexane label: 92.9-104.8% TRR) were recovered in methanol. The portions of radioactive residues, extracted subsequently with water ranged from 1.7-5.5% TRR for the phenyl label and from 1.4-2.0% TRR for the cyclohexane label.

For fish fillet of both labels, very high amounts of radioactive residues were extracted with methanol (104.8-107.4% TRR) and therefore, no further extraction with water was required. Lower portions were extracted with methanol from liver (phenyl label: 90.0% TRR and cyclohexane label: 92.9% TRR) and skin (phenyl label: 87.8% TRR and cyclohexane label: 96.3% TRR) of both labels. Subsequent extraction with water released 1.7%

TRR (phenyl label) and 2.0 TRR (cyclohexane label) from trout liver and 5.5% TRR (phenyl label) and 1.4% TRR (cyclohexane label) from trout skin.

Altogether, high amounts of radioactive residues were extracted by solvent extraction. Amounts  $\geq 91.7\%$  TRR were extracted from trout fillet, liver and skin.

*Table 7-130 Extractability of radioactive residues from fish matrices following treatment with BAS 684 H for 11 days*

| Matrix            | Methanol extract <sup>1</sup> |         | Water extract  |         | ERR <sup>1,2</sup> |         | RRR <sup>1,3</sup> |         | TRR <sup>4</sup> |
|-------------------|-------------------------------|---------|----------------|---------|--------------------|---------|--------------------|---------|------------------|
|                   | [mg/kg]                       | [% TRR] | [mg/kg]        | [% TRR] | [mg/kg]            | [% TRR] | [mg/kg]            | [% TRR] |                  |
| Phenyl label      |                               |         |                |         |                    |         |                    |         |                  |
| Fillet            | 0.017                         | 107.4   | Not applicable |         | 0.017              | 107.4   | Not applicable     |         | 0.016            |
| Liver             | 0.078                         | 90.0    | 0.001          | 1.7     | 0.079              | 91.7    | 0.007              | 8.3     | 0.086            |
| Skin              | 0.024                         | 87.8    | 0.002          | 5.5     | 0.026              | 93.3    | 0.002              | 6.7     | 0.028            |
| Cyclohexane label |                               |         |                |         |                    |         |                    |         |                  |
| Fillet            | 0.041                         | 104.8   | Not applied    |         | 0.041              | 104.8   | Not applied        |         | 0.040            |
| Liver             | 0.094                         | 92.9    | 0.002          | 2.0     | 0.096              | 94.9    | 0.005              | 5.1     | 0.101            |
| Skin              | 0.047                         | 96.3    | 0.001          | 1.4     | 0.048              | 97.6    | 0.001              | 2.4     | 0.049            |

1 For trout fillet of both labels, the methanol extraction was repeated and the values of the extraction for workup sample 1 were used. For trout liver and skin, values of the pooled methanol extracts were used.

2 Extractable radioactive residues

3 Residual radioactive residues (after solvent extraction)

4 Sum of ERR + RRR

### 3. Identification, characterization and quantification of radioactive residues in fish matrices

A summary of identified and characterized radioactive residues is compiled in Table 7-131.

#### Identification, characterization and quantification of radioactive residues in fillet

BAS 684 H was identified as a major component in fish fillet and accounted for or 0.004 mg/kg or 27.7% TRR (phenyl label) and 0.007 mg/kg or 18.9% TRR (cyclohexane label).

The metabolite M684H026 is a cyclohexane-specific metabolite and was a major component in fish fillet (0.016 mg/kg or 39.9% TRR) of the cyclohexane label. Small amounts of metabolite M684H001 were identified in fish fillet (phenyl label: 0.001 mg/kg or 5.7% TRR and cyclohexane label: 0.001 mg/kg or 2.1% TRR).

#### Identification, characterization and quantification of radioactive residues in liver

In fish liver, the parent compound was strongly metabolized and accounted for only 0.005 mg/kg or 5.7% TRR (phenyl label) and 0.006 mg/kg or 6.2% TRR (cyclohexane label).

The metabolite M684H026 is a cyclohexane-specific metabolite and was a major component in fish liver (0.018 mg/kg or 17.8% TRR) of the cyclohexane label. The metabolite M684H001 was the predominant component in fish liver of the phenyl label (0.027 mg/kg or 31.6% TRR) and a major component in fish liver of the cyclohexane label (0.018 mg/kg or 18.0% TRR).

#### Identification, characterization and quantification of radioactive residues in skin

For fish skin of the phenyl label, BAS 684 H was the predominant compound and accounted for 0.010 mg/kg or 35.5% TRR. For fish skin of the cyclohexane label, BAS 684 H was further metabolized and accounted for only 0.002 mg/kg or 3.5% TRR.

The metabolite M684H026 is a cyclohexane-specific metabolite and was a major component in fish skin (0.012 mg/kg or 24.5% TRR) of the cyclohexane label. Small amounts of metabolite M684H001 were identified in fish skin (cyclohexane label: 0.002 mg/kg or 5.1% TRR).

Besides those main products of metabolic activity, up to 24 minor metabolites (up to 9.1% TRR) were detected in the extracts of trout matrices and classified as characterized by their extraction with water or methanol.

Taken together, 60.9-84.8% TRR were identified/ characterized for fish fillet, 81.6-89.0% TRR for fish liver and 64.2-88.9% TRR for fish skin. The lower amount of identification/ characterization in trout fillet (phenyl label) and trout skin (cyclohexane label) results from the overall low amount of radioactivity in the matrices (trout fillet: 0.016 mg/kg, trout skin: 0.049 mg/kg) as well as the loss of radioactivity in concentration, purification and fractionation steps. However, the extractability of all trout matrices was high (91.7-107.4% TRR) and all major components were identified.

*Table 7-131 Summary of identified and characterized radioactive residues in edible matrices from fish*

| <b>Designation</b>                 | <b>Fillet</b>  |         | <b>Liver</b> |         | <b>Skin</b> |         |
|------------------------------------|----------------|---------|--------------|---------|-------------|---------|
|                                    | [mg/kg]        | [% TRR] | [mg/kg]      | [% TRR] | [mg/kg]     | [% TRR] |
| <b>Phenyl label</b>                |                |         |              |         |             |         |
| BAS 684 H                          | 0.004          | 27.7    | 0.005        | 5.7     | 0.010       | 35.5    |
| M684H001                           | 0.001          | 5.7     | 0.027        | 31.6    | -           | -       |
| Total identified from ERR          | 0.005          | 33.4    | 0.032        | 37.3    | 0.010       | 35.5    |
| Total characterized from ERR       | 0.004          | 27.5    | 0.038        | 44.3    | 0.015       | 53.4    |
| Total identified and characterized | 0.010          | 60.9    | 0.070        | 81.6    | 0.025       | 88.9    |
| Final residue                      | Not applicable |         | 0.007        | 8.3     | 0.002       | 6.7     |
| Grand total                        | 0.010          | 60.9    | 0.078        | 89.9    | 0.027       | 95.6    |
| <b>Cyclohexane label</b>           |                |         |              |         |             |         |
| BAS 684 H                          | 0.007          | 18.9    | 0.006        | 6.2     | 0.002       | 3.5     |
| M684H001                           | 0.001          | 2.1     | 0.018        | 18.0    | 0.002       | 5.1     |
| M684H026                           | 0.016          | 39.9    | 0.018        | 17.8    | 0.012       | 24.5    |
| Total identified from ERR          | 0.024          | 60.9    | 0.042        | 42.0    | 0.016       | 33.1    |
| Total characterized from ERR       | 0.009          | 24.0    | 0.048        | 47.1    | 0.015       | 31.1    |
| Total identified and characterized | 0.034          | 84.8    | 0.090        | 89.0    | 0.031       | 64.2    |
| Final residue                      | Not detected   |         | 0.005        | 5.1     | 0.001       | 2.4     |
| Grand total                        | 0.034          | 84.8    | 0.095        | 94.1    | 0.033       | 66.6    |

#### 4. Enantiomer ratio

To analyze if one isomer of BAS 684 H was preferably metabolized in fish, enantiomer specific analysis of the parent compound, isolated from fillet (phenyl label), was performed representatively. The results are summarized in Table 7-132.

The ratio of the (-)-enantiomer : (+)-enantiomer of BAS 684 H in the application solution was 49:51 (representatively determined for the cyclohexane label). In an extract of fillet, containing a high portion of BAS 684 H, the relative ratio of the respective (-)-enantiomer : (+)-enantiomer of BAS 684 H was 14:86.

*Table 7-132 Determination of the enantiomer ratio of BAS 684 H*

| <b>Matrix</b>       | <b>(-)-enantiomer<br/>[%]</b> | <b>(+)-enantiomer<br/>[%]</b> |
|---------------------|-------------------------------|-------------------------------|
| <b>Phenyl label</b> |                               |                               |
| Fillet              | 14                            | 86                            |

#### 5. Metabolic pathway

The proposed metabolic pathway of BAS 684 H in fish is shown in

Figure 7-19.

The active substance BAS 684 H was observed to be metabolized via two major pathways in rainbow trout. Metabolite M684H026 resulted from the cleavage of the ether bridge and hydroxylation of the isopropyl moiety of the cyclohexane group. The metabolite M684H001 resulted from hydroxylation at the benzylic position on the parent compound BAS 684 H and oxidation.

#### 6. Storage stability

All samples were stored at -18°C or below prior to analysis or workup. All matrices were extracted within 239 days after sacrifice of the fish. By comparison of the HPLC profiles of early HPLC analyses of the concentrated methanol extracts with those of the corresponding late analyses, it was shown that the extracts were stable throughout the time of the study.

### III. CONCLUSION

BAS 684 H was administered orally to 14 rainbow trouts in two radiolabeled forms (phenyl and cyclohexane label) for eleven consecutive days (nominal dose of 12 mg/kg feed/day).

Approximately 94.5% and 92.2% of the total administered radioactivity was recovered in total for the phenyl and cyclohexane label, respectively. Thereby, the major portion of radioactive residues was determined in charcoal, water and pyloric caeca. Radioactive residues associated with edible portions (fillet) accounted for up to 0.21% (phenyl label) and 0.51% (cyclohexane label) of the administered dose.

The highest TRR was measured in trout liver (phenyl label: 0.086 mg/kg and cyclohexane label: 0.101 mg/kg). In trout fillet (phenyl label: 0.016 mg/kg and cyclohexane label: 0.040 mg/kg) and skin (phenyl label: 0.028 mg/kg and cyclohexane label: 0.049 mg/kg) the TRR was lower for both labels.

Extraction of the radioactive residues of BAS 684 H with methanol and water resulted in high extractabilities for all tissues of both labels (91.7-107.4% TRR). The predominant part of radioactivity was extracted with methanol in all matrices (87.8-107.4% TRR). For the phenyl label, 0.017 mg/kg or 107.4% TRR were extracted in this step from trout fillet, 0.078 mg/kg or 90.0% TRR from trout liver, 0.024 mg/kg or 87.8% TRR from trout skin. For the cyclohexane label, 0.041 mg/kg or 104.8% TRR were extracted in this step from trout fillet, 0.094 mg/kg or 92.9% TRR from trout liver, 0.047 mg/kg or 96.3% TRR from trout skin. For trout liver and trout skin of both labels, small amounts of radioactivity were subsequently extracted with water (up to 0.002 mg/kg or 5.5% TRR).

Sub-fractions of methanol extracts with a sufficient level of radioactive residues were analysed by HPLC. The peaks in the chromatograms were identified by comparison of their retention times and metabolic pattern with those of the reference items, using co-chromatography with HPLC. The chemical structures of reference items were elucidated by MS analyses in a related study on the metabolism of BAS 684 H in rats.

BAS 684 H was identified as a major component in fish fillet (0.004-0.007 mg/kg or 18.9-27.7% TRR). In fish liver, the parent compound was strongly metabolized (0.005-0.006 mg/kg or 5.7-6.2% TRR). For fish skin of the phenyl label, BAS 684 H was the predominant compound (0.010 mg/kg or 35.5% TRR). For fish skin of the cyclohexane label, BAS 684 H was further metabolized (only 0.002 mg/kg or 3.5% TRR).

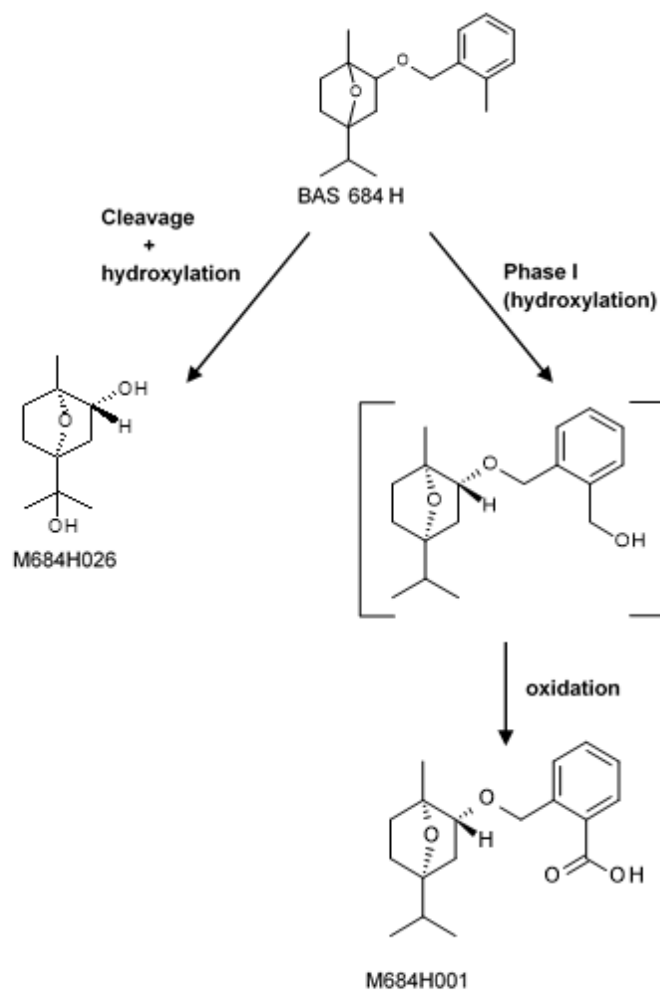
Metabolite M684H026 is a cyclohexane-specific metabolite and was a major component in fish fillet (0.016 mg/kg or 39.9% TRR), fish liver (0.018 mg/kg or 17.8% TRR) and fish skin (0.012 mg/kg or 24.5% TRR) of the cyclohexane label. Metabolite M684H001 was the predominant component in fish liver of the phenyl label (0.027 mg/kg or 31.6% TRR) and a major component in fish liver of the cyclohexane label (0.018 mg/kg or 18.0% TRR). Small amounts of this metabolite were also identified in fish fillet (0.001 mg/kg or 2.1-5.7% TRR) and fish skin (0.002 mg/kg or 5.1% TRR).

BAS 684 H was metabolized via the following reactions:

- Hydroxylation of the alkyl group at the benzyl ring and further oxidation
- Cleavage of the ether bridge and hydroxylation of the isopropyl moiety of the cyclohexane group

The active substance BAS 684 H was observed to be metabolized by typical biotransformation reactions. Metabolite M684H026 resulted from cleavage of the phenyl group from the parent molecule and hydroxylation of the isopropyl moiety of the cyclohexane group. The metabolite M684H001 resulted from hydroxylation and oxidation of the parent compound BAS 684 H.

*Figure 7-19 Proposed metabolic pathway of BAS 684 H in fish*



### B.7.2.6. Overall conclusion on metabolism in livestock

Metabolism in livestock was investigated using two radiolabels (BAS 684 H labelled in the phenyl ring and the cyclohexane ring). Investigations were done in laying hen and lactating goat, as well as in rat to support toxicology studies (see section 6) and in fish although the fish study has not been fully evaluated.

For goat and hen the residue was rapidly and extensively eliminated via excreta (approximately 90 % administered dose), and reached a plateau concentration in milk (approx. 2 – 7 days, noting a difference across the 4 goats dependant on the dose level of labelled BAS 684 H) and egg (7 – 9 days).

The major compounds found in products of animal origin were parent BAS 684 H, M684H001 and M684H012 (A-branch), M684H021, M684H022 and M684H039 (B-branch) and cleavage products M684H026 (D-branch), M684H009, M684H010 and M684H059 (E-branch). Metabolite M684H029 was exclusively found in goat urine and faeces, but not in any edible livestock matrix and is therefore is not considered relevant for consumer exposure. The metabolic routes in livestock are shown in Figure 7-21 and Figure 7-22 and the transformation reactions summarised in Figure 7-20.

In livestock, the metabolic pathway is largely based on:

- hydroxylation of the parent compound at various positions
- subsequent conjugation of these hydroxyl groups with glucuronide
- cleavage at the ether bridge

The new livestock metabolism data package is in agreement with the general metabolic pathway shown by the old goat metabolism studies of BAS 684 H performed in the 1980s (CA 6.2.3/3 – 6.2.3/5). Whilst the old studies are deficient and cannot be fully relied upon, they are considered to provide supporting information.

The parent BAS 684 H was applied as a racemic mixture of two enantiomers (a ratio of the (-) and (+) enantiomers of approximately 43:57 in the application solution). Chiral analysis of BAS 684 H revealed a ratio of the (-) and (+) enantiomers was approximately 62:38 in poultry (fat, cyclohexane label) and a ratio of the (-) and (+) enantiomers was approximately 53:47 in goat (liver, cyclohexane label).

Overall the *in-vitro* data for the metabolites M684H005 and M684H006 are considered appropriate to support the metabolism in poultry and lactating ruminants. Metabolites M684H002, M684H005 and M684H006 are hydroxylated or conjugated forms of parent BAS 684 H. The results of the *in-vitro* study are as expected, the metabolites M684H005 and M684H006 are cleaved to form M684H002. From a toxicological perspective, metabolite M684H002 is equivalent to parent BAS 684 H. Metabolite M684H002 is not found in the hen metabolism study but is found in the goat metabolism study of BAS 684 H in urine and the metabolic pathway shows it is a key intermediate for several other metabolites. Exposure to M684H002 would likely be comparable to exposure from BAS 684 H. Therefore, no additional data are required to support the lactating ruminant metabolism of the major plant metabolites M684H005 and M684H006.

Figure 7-20 BAS 684 H transformation reactions in animals

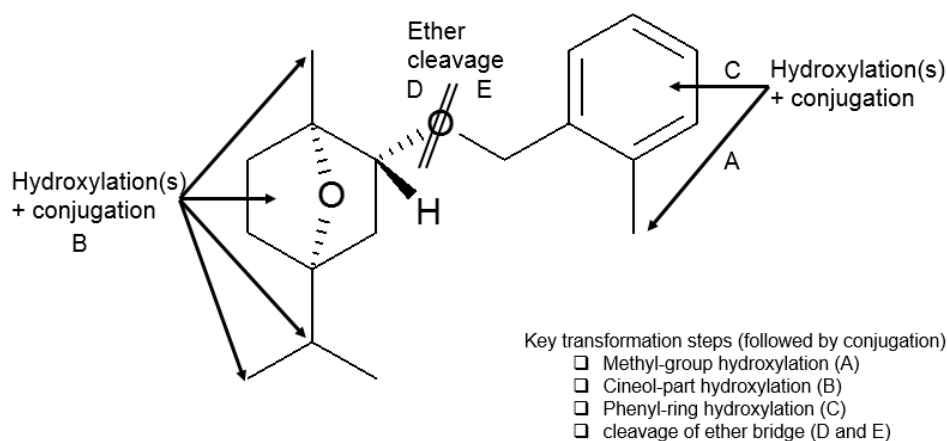


Figure 7-21 and Figure 7-22 show the key metabolism pathways determined for the animal commodities.

Metabolites occurring in edible livestock matrices in major amounts (> 10 % TRR) are listed in bold text in Table 7-133. This table groups the metabolites according to their chemical structure, together with their corresponding conjugates.

Table 7-133 Metabolites in edible livestock matrices

| <b>A-branch</b><br>(hydroxylation at the methyl group and further phase I and phase II metabolites) | <b>B-branch</b><br>(hydroxylation at the cineol-part and further phase I and phase II metabolites) | <b>C-branch</b><br>(hydroxylation at the phenyl-ring and further phase I and phase II metabolites) | <b>D-branch</b><br>(cleavage products cineol-part and further phase I and phase II metabolites) | <b>E-branch</b><br>(cleavage products phenyl part and further phase I and phase II metabolites) |
|---|--|--|---|---|
| <b>M684H001</b>   | <b>M684H021</b>  | M684H034   | <b>M684H026</b>   | <b>M684H009</b>   |
| M684H011  | <b>M684H022</b>  |  |   | <b>M684H010</b>   |
| <b>M684H012</b>   | M684H027   |  |   | M684H058  |
| M684H056  | <b>M684H039</b>  |  |   | <b>M684H059</b>   |
|   | M684H052   |  |   |   |
|   | M684H057   |  |   |   |

Metabolites with a content of >10% TRR are indicated in **bold** font



Figure 7-21 BAS 684 H: metabolic routes in livestock – A-, B- and C-branch (hydroxylated and conjugated metabolites)

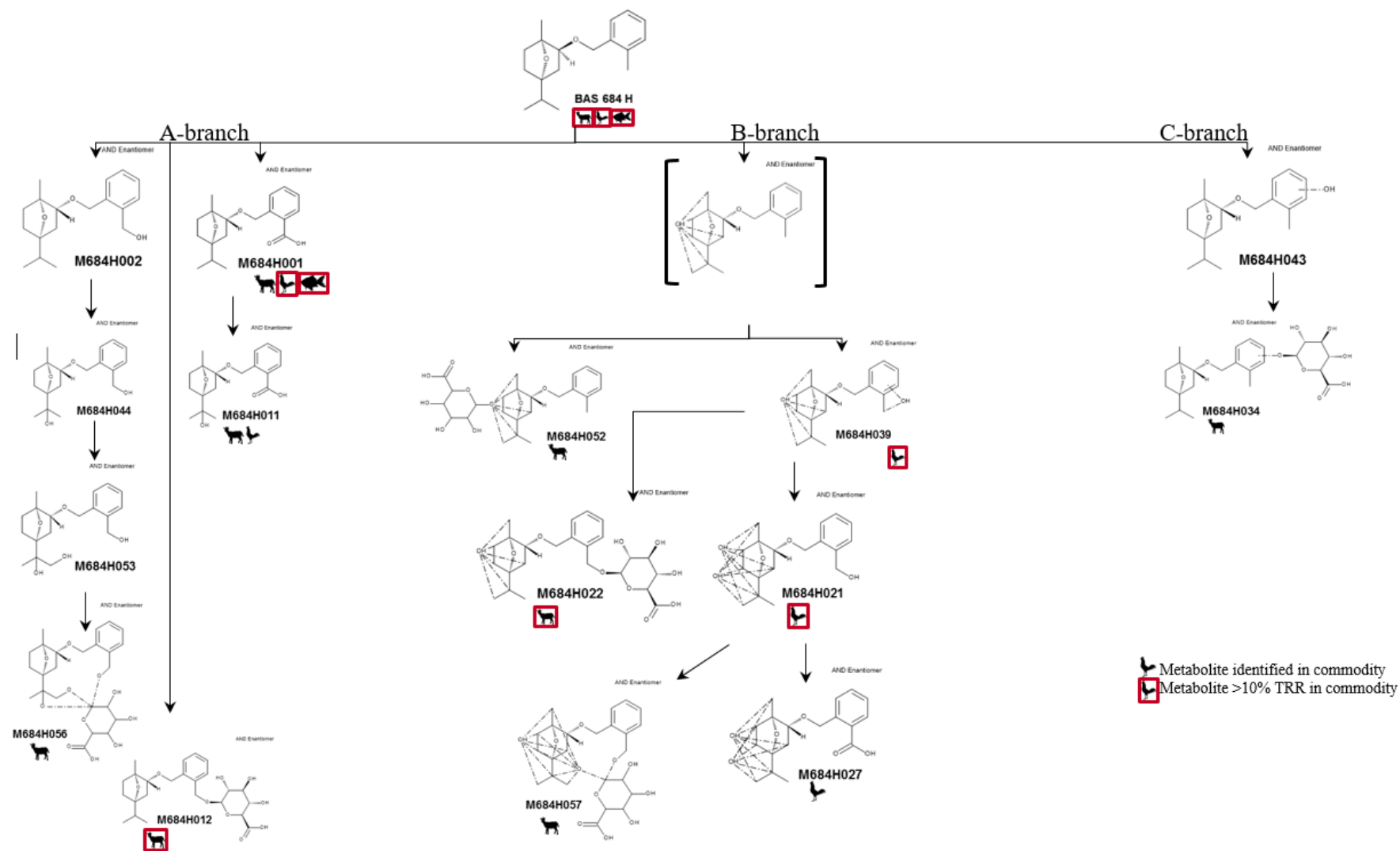
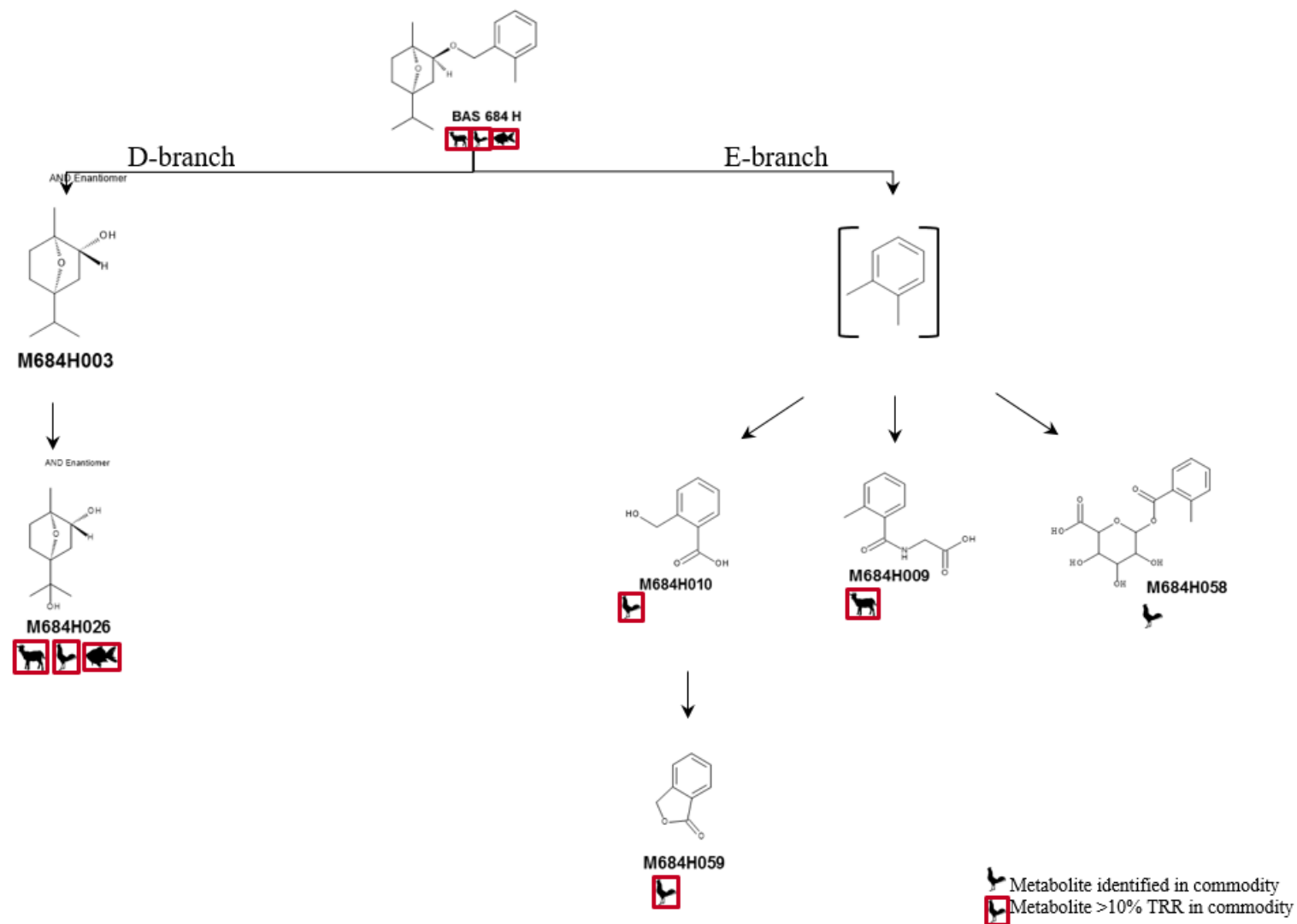


Figure 7-22 BAS 684 H: metabolic routes in livestock – D- and E-branch (cleavage products)



### B.7.3. MAGNITUDE OF RESIDUE TRIALS IN PLANTS

The present dossier supports the use of the formulation BAS 684 03 H on the crop wheat (extrapolated to barley) and oilseed rape. In this section, the supporting residue data on oilseed rape and wheat are summarised.

The basic criteria for acceptability are listed below:

#### Trials details

|  |                                       |
|--|---------------------------------------|
| Crop variety                             |                                       |
| Location, position and year of trial     | -acceptable spread of location/season |
| Formulations used                        | -formulation reported/as proposed     |
| Application/dilution rate                | -reported/as specified on label       |
| Maximum number of treatments             | -reported/applicable                  |
| Method of application                    | -reported/applicable                  |
| Growth stage of the crop at treatment or |                                       |
| Pre-harvest interval                     | -appropriate to proposed GAP          |
| Geo-climate information                  | - reported/applicable                 |
| Residue level (control and treated)      |                                       |

#### Analytical aspects

Method specified and submitted  
 Storage of samples prior to analysis (conditions and time period)  
 Limit of determination at an acceptable level  
 Acceptable recovery (means 70 - 110%).

In the residue trials presented below, two solo formulations containing 750 g/L of BAS 684 H were used: BAS 684 02 H and BAS 684 03 H. Both formulations are EC formulations therefore it is acceptable to accept residue trials data from either formulation. Full details on the formulations are provided in Volume 4.

#### **B.7.3.1. Wheat**

As cereals are a major crop, a minimum of eight trials are required for each geographical zone. The supporting trials are on wheat only, however the requested GAP is for winter cereals (wheat and barley). According to the “Guidelines on comparability, extrapolation, group tolerances and data requirements for setting MRLs”, SANCO 7525/VI/95 rev. 10.3 (June 2017) it is possible to extrapolate residue data between the various types of cereals; barley (0500010) and wheat (0500090), since the representative use involves application at an early stage, before the edible part of the crop is formed. Therefore the residues data provided support the requested cereal use of winter wheat and barley.

Four representative uses are being considered as shown in the GAP table. However, in this residue section only the worst-case representative use is being considered (cGAP). The critical use consists of one spray application to winter cereals (wheat or barley) at a rate of 500 g a.s./ha when the crop has reached the growth stage BBCH 29. No residues trials data have been submitted for the pre-emergence use (BBCH 00 – 08), however as this is considered a less critical use this is acceptable.

Table 7-134 Requested GAPs and critical GAP (in bold)

| Use-No.  | Member states/zones | Crop                                     | Application   |                                    |                        |                                  | PHI (days)  |
|----------|---------------------|--|---------------|------------------------------------|------------------------|----------------------------------|-------------|
|          |                     |  | Method / kind | Growth stage of crop               | Number of applications | Rate per application (g a.s./ha) |             |
| 1        | UK                  | Winter cereals (wheat and barley)        | Spray         | Pre-emergence (BBCH 00-08)         | 1                      | 500                              | n.a.        |
| <b>2</b> | <b>UK</b>           | <b>Winter cereals (wheat and barley)</b> | <b>Spray</b>  | <b>Post-emergence (BBCH 09-29)</b> | <b>1</b>               | <b>500</b>                       | <b>n.a.</b> |
| 3        | UK                  | Winter cereals (wheat and barley)        | Spray         | Pre-emergence (BBCH 00-08)         | 1                      | 250                              | n.a.        |
| 4        | UK                  | Winter cereals (wheat and barley)        | Spray         | Post-emergence (BBCH 09-29)        | 1                      | 250                              | n.a.        |

n.a – not applicable (PHI is covered by the time remaining between application and harvest).

A summary of the trials submitted to support the cGAP are given Table 7-135. This number of trials is considered adequate to address the requirements for the North EU (NEU). The dossier submitted also includes residue trials data from the South EU (SEU), these have been reported for completeness however the end-points from these have not been used further in the risk assessment.

Table 7-135 Number of residue trials per geographical region and vegetation period

| Crop                                     | Season | Number of trials |                |           |                |           | Reference          |
|--|--------|------------------|----------------|-----------|----------------|-----------|--------------------|
|  |        | N-EU             | Country        | S-EU      | Country        | Total     |                    |
| Wheat                                    | 2015   | 4                | DE, FR, NL, UK | 4         | ES, FR, GR, IT | 8         | 6.3.2/1            |
|  | 2016   | 4                | BE, DE, DK, FR | 4         | ES, FR, GR, IT | 8         | 6.3.2/2<br>6.3.2/3 |
|  | 2017   | 4                | AT, DE, NL, FR | 4         | ES, FR, GR, IT | 8         | 6.3.2/4            |
| <b>Total number of trials per region</b> |        | <b>12</b>        | <b>-</b>       | <b>12</b> | <b>-</b>       | <b>24</b> |                    |

**Report:** CA 6.3.2/1  
Ale E., 2017 a  
Residue study (Decline) with BAS 684 02 H applied to wheat in Northern and Southern Europe in 2015  
2016/1118116

**Guidelines:** Regulation 1107/2009 with Regulation 283/2013, Regulation 1107/2009 with Regulation 284/2013, OECD (2011) Guidance Document on Crop Field Trials (Series on Testing and Assessment No. 164 and Series On Pesticides No. 66), OECD 509 Crop Field Trial (2009), EEC 7525/VI/95 rev. 9 (March 2011), EEC 7029/VI/95 rev. 5 (July 22 1997)

**GLP:** yes

**Deviations:** A total of 4 deviations have been listed however these have been considered during evaluation to be minor and to not be of significance in terms of conduct or quality of the study.

During the 2015 growing season 8 field decline trials in wheat were conducted in Northern and Southern Europe to determine the residue level of BAS 684 H in or on raw agricultural commodities (RAC). BAS 684 02 H (EC) containing nominally 750 g/L BAS 684 H was applied once at a rate equivalent to 0.500 kg BAS 684 H /ha in a

spray volume of 200 L/ha. One untreated plot of each trial served as control. The application was performed at BBCH 29. Specimens of whole plants without roots were collected immediately after the application (BBCH 29) as well as 14-43 (BBCH 49) and 21-53 days thereafter (BBCH 65). Grain and straw were sampled at BBCH 89 (crop maturity), 61-102 days after the application. Samples were stored deep-frozen for a maximum of 314 (BAS 684 H) or 872 days (M684H005 and M684H006) until analysis. Residues of BAS 684 H have been shown to be stable for 24 months when stored at  $\leq -18^{\circ}\text{C}$  and therefore this storage period is acceptable. For the metabolite M684H005 (and hence M684H006) the overall storage stability conclusions are that the metabolite has only been shown to be stable to a maximum of 24 months. However, storage stability data were presented specifically for wheat grain and straw showing stability for 32 months. Therefore the trials within the study with storage periods of up to 872 days can be relied upon. Extracts are stored for up to 2 weeks. Storage stability of extracts for up to 7 days in is presented within the analytical method validation (Section B5). Within the study the stability of the analyte in final volume solutions was proven by procedural recovery samples which were stored for the same period of time between extraction and LC-MS/MS analysis therefore this is considered acceptable and no further data are required at this time.

The specimens were analysed for BAS 684 H with BASF method No L0337/01 quantifying the analyte with a limit of quantitation (LOQ) of 0.01 mg/kg. Method validation data (presented in Section B5) are available for the same matrices as tested in this residue trials study. The method is fully validated in accordance with SANCO 3029/99 rev. 4 with an LOQ of 0.01 mg/kg.

The specimens were analysed for M684H005 and M684H006 (as sum expressed as M684H005) with BASF method No L0337/02 quantifying the analytes with a limit of quantitation (LOQ) of 0.01 mg/kg. Method validation data (presented in Section B5) are available for the same matrices as tested in this residue trials study. The method is fully validated in accordance with SANCO 3029/99 rev. 4 with an LOQ of 0.01 mg/kg.

Recovery rates for BAS 684 H and the metabolites M684H005 and M684H006 were determined concurrently with the sample analysis in order to check the accuracy of the residue analysis. The recovery rates are reported in Table 7-136, the data are acceptable with all mean values within the range 70 – 110 %. The limit of quantification was 0.01 mg/kg for all sample materials.

Table 7-136 Procedural recoveries for BAS 684 H and the metabolites M684H005 and M684H006 in wheat

| Analyte   | Portion analysed          | n  | Fortification level (mg/kg) | Recovery (%)                                       |       |      |
|-----------|---------------------------|----|-----------------------------|--|-------|------|
|           |                           |    |                             | Individual recoveries                              | Mean  | RSD  |
| BAS 684 H | Whole plant without roots | 3  | 0.01                        | 109, 107, 104                                      | 106.7 | 2.4  |
|           |                           | 1  | 0.1                         | 90.0   | --    | --   |
|           |                           | 1  | 1.0                         | 95.0   | --    | --   |
|           |                           | 1  | 10                          | 93.8   | --    | --   |
|           |                           | 1  | 100                         | 98.5   | --    | --   |
|           |                           | 7  | overall                     |  | 99.6  | 7.3  |
|           | Grain                     | 5  | 0.01                        | 95.5, 94.5, 93.8, 90.3, 89.8                       | 92.8  | 2.8  |
|           |                           | 3  | 0.1                         | 93.5, 86.3, 83.3                                   | 87.7  | 6.0  |
|           |                           | 1  | 1.0                         | 94.75  | --    | --   |
|           |                           | 1  | 10                          | 82.0   | --    | --   |
|           |                           | 10 | overall                     |  | 90.4  | 5.5  |
|           | Straw                     | 5  | 0.01                        | 77.2, 76.6, 81.3, 77.2, 78.1                       | 78.1  | 2.4  |
|           |                           | 1  | 0.1                         | 90.0   | --    | --   |
|           |                           | 2  | 1.0                         | 96.6, 91.3   | 94.0  | --   |
|           |                           | 1  | 10                          | 94.1   | --    | --   |
|           |                           | 9  | overall                     |  | 84.7  | 10   |
| M684H005  | Whole plant without roots | 9  | 0.01                        | 85.0, 90.4, 113, 106, 91.6, 95.2, 84.8, 82.4, 93.6 | 93.6  | 10.8 |
|           |                           | 4  | 0.1                         | 107, 104, 103, 85.2                                | 99.8  | 9.9  |
|           |                           | 5  | 10                          | 88.2, 98.0, 90.0, 90.0, 91.4                       | 91.5  | 4.1  |

|          |                           |    |         |                                    |       |      |
|----------|---------------------------|----|---------|------------------------------------|-------|------|
|          |                           | 18 | overall |                                    | 94.4  | 9.4  |
|          | Grain                     | 3  | 0.01    | 102, 96.8, 105                     | 101.3 | 4.1  |
|          |                           | 3  | 0.1     | 92.2, 89.6, 90.0                   | 90.6  | 1.5  |
|          |                           | 6  | overall |                                    | 96.0  | 6.8  |
|          | Straw                     | 6  | 0.01    | 76.1, 79.0, 103, 89.0, 98.4, 98.8  | 90.7  | 12.4 |
|          |                           | 5  | 0.1     | 74.5, 73.5, 74.0, 86.4, 89.0       | 79.5  | 9.5  |
|          |                           | 3  | 1.0     | 80.9, 76.8, 109                    | 88.9  | 19.7 |
|          |                           | 14 | overall |                                    | 86.3  | 14.0 |
| M684H006 | Whole plant without roots | 5  | 0.01    | 102, 104, 102, 71.7, 95.0          | 94.9  | 14.1 |
|          |                           | 3  | 0.1     | 95.7, 95.5, 94.6                   | 95.3  | 0.6  |
|          |                           | 4  | 10      | 103, 104, 81.7, 83.5               | 93.1  | 13.0 |
|          |                           | 12 | overall |                                    | 94.3  | 11.0 |
|          | Grain                     | 3  | 0.01    | 99.2, 96.8, 82.6                   | 92.9  | 9.7  |
|          |                           | 3  | 0.1     | 90.1, 87.9, 87.0                   | 88.3  | 1.8  |
|          |                           | 6  | overall |                                    | 90.6  | 6    |
|          | Straw                     | 6  | 0.01    | 68.3, 82.1, 70.5, 72.2, 76.7, 79.1 | 74.8  | 7.1  |
|          |                           | 5  | 0.1     | 74.4, 86.9, 73.0, 75.9, 75.2       | 77.1  | 7.3  |
|          |                           | 3  | 1.0     | 72.7, 72.2, 100                    | 81.6  | 19.5 |
|          |                           | 14 | overall |                                    | 77.1  | 11   |

No residues of BAS 684 H or M684H005 (including M684H006) above the limit of quantitation (0.01 mg/kg) were found in any of the analysed untreated specimens.

The residues found in wheat samples from the individual trials are summarised in Table 7-137. Residues of BAS 684 H in treated whole plant without root specimens collected on the day of the application ranged from 13 to 42 mg/kg. At BBCH 49 residues declined to < 0.01 – 0.026 mg/kg and further to < 0.01 – 0.018 mg/kg at BBCH 65. Residues in mature grain and straw samples were below the LOQ of 0.01 mg/kg, except for one straw sample with 0.026 mg/kg.

Residues of M684H005 (including M684H006) in treated whole plant without root specimens collected on the day of the application ranged from 0.23 to 1.5 mg/kg. At BBCH 49 residues were < 0.01 – 1.2 mg/kg and declined to < 0.01 – 0.54 mg/kg at BBCH 65. Residues in mature grain samples were below the LOQ of 0.01 mg/kg; in straw, < 0.01 – 0.39 mg/kg were found.

Table 7-137 Residues of BAS 684 H, M684H005 and M684H006 in wheat (trials which support the critical GAP are underlined)

| Report No.<br>Location<br>(EU-region)<br>Trial No  | Commodity/<br>Variety      | Date of<br>1. Sowing or<br>planting<br>2. Flowering<br>3. Harvest | Method<br>of<br>treatment | Formulation                                     | Application rate per<br>treatment |               |            | No. of<br>treatments<br>and last<br>date | Growth<br>stage at<br>last<br>date | Portion analysed   | DALA <sup>1</sup> | Residues (mg/kg) |   |                    |
|--|----------------------------|---|---------------------------|---|-----------------------------------|---------------|------------|--|------------------------------------|--------------------|-------------------|------------------|---|--------------------|
|  |                            |   |                           |   | kg a.s./hL                        | Water<br>L/ha | kg a.s./ha |  |                                    |                    |                   | BAS 684 H        | Sum of<br>M684H005 and<br>M684H006,<br>expressed as<br>M684H005 | Total <sup>3</sup> |
| 741155<br>2016/1118116<br>67117<br>Limburgerhof<br>Germany (N)<br>L150075                  | GC 0654<br>Wheat<br>Kadrlj | 1. 19.03.2015<br>2. 08.06.-<br>24.06.2015<br>3. 20.07.2015        | Spray<br>application      | BAS 684 02<br>H<br>(EC)<br>750 g/L<br>BAS 684 H | 0.251                             | 200           | 0.503      | 1<br>23.04.2015                          | 29                                 | Plant <sup>2</sup> | 0                 | 42               | 1.1   | 43                 |
|  |                            |   |                           |   |                                   |               |            |  |                                    | Plant <sup>2</sup> | 43                | <0.01            | <0.01   | <0.016             |
|  |                            |   |                           |   |                                   |               |            |  |                                    | Plant <sup>2</sup> | 53                | <0.01            | <0.01   | <0.016             |
|  |                            |   |                           |   |                                   |               |            |  |                                    | Grain              | 88                | <0.01            | <0.01   | <0.016             |
|  |                            |   |                           |   |                                   |               |            |  |                                    | Straw              | 88                | <0.01            | <0.01   | <0.016             |
| 741155<br>2016/1118116<br>6599 AV Ven-<br>Zelderheide<br>The Netherlands<br>(N)<br>L150076 | GC 0654<br>Wheat<br>Tybalt | 1. 11.04.2015<br>2. 26.06.-<br>11.07.2015<br>3. 13.08.2015        | Spray<br>application      | BAS 684 02<br>H<br>(EC)<br>750 g/L<br>BAS 684 H | 0.251                             | 192           | 0.482      | 1<br>29.05.2015                          | 29                                 | Plant <sup>2</sup> | 0                 | 15               | 1.0   | 16                 |
|  |                            |   |                           |   |                                   |               |            |  |                                    | Plant <sup>2</sup> | 17                | 0.023            | 0.67  | 0.43               |
|  |                            |   |                           |   |                                   |               |            |  |                                    | Plant <sup>2</sup> | 34                | 0.018            | 0.31  | 0.21               |
|  |                            |   |                           |   |                                   |               |            |  |                                    | Grain              | 76                | <0.01            | <0.01   | <0.016             |
|  |                            |   |                           |   |                                   |               |            |  |                                    | Straw              | 76                | <0.01            | <0.01   | <0.016             |
| 741155<br>2016/1118116<br>51110<br>Auménancourt<br>France (N)<br>L150077                   | GC 0654<br>Wheat<br>Epos   | 1. 19.03.2015<br>2. 10.06.-<br>30.06.2015<br>3. 29.07.2015        | Spray<br>application      | BAS 684 02<br>H<br>(EC)<br>750 g/L<br>BAS 684 H | 0.251                             | 195           | 0.490      | 1<br>07.05.2015                          | 29                                 | Plant <sup>2</sup> | 0                 | 38               | 1.5   | 39                 |
|  |                            |   |                           |   |                                   |               |            |  |                                    | Plant <sup>2</sup> | 25                | <0.01            | 0.62  | 0.39               |
|  |                            |   |                           |   |                                   |               |            |  |                                    | Plant <sup>2</sup> | 35                | <0.01            | 0.092   | 0.066              |
|  |                            |   |                           |   |                                   |               |            |  |                                    | Grain              | 83                | <0.01            | <0.01   | <0.016             |
|  |                            |   |                           |   |                                   |               |            |  |                                    | Straw              | 83                | <0.01            | <0.01   | <0.016             |
| 741155<br>2016/1118116<br>CV35 0JH Kineton<br>United Kingdom<br>(N)<br>L150078             | GC 0654<br>Wheat<br>Tybalt | 1. 06.03.2015<br>2. 10.07.-<br>23.07.2015<br>3. 11.09.2015        | Spray<br>application      | BAS 684 02<br>H<br>(EC)<br>750 g/L<br>BAS 684 H | 0.251                             | 203           | 0.511      | 1<br>01.06.2015                          | 29                                 | Plant <sup>2</sup> | 0                 | 30               | 1.4   | 31                 |
|  |                            |   |                           |   |                                   |               |            |  |                                    | Plant <sup>2</sup> | 32                | 0.026            | 0.086   | 0.078              |
|  |                            |   |                           |   |                                   |               |            |  |                                    | Plant <sup>2</sup> | 45                | <0.01            | 0.041   | 0.035              |
|  |                            |   |                           |   |                                   |               |            |  |                                    | Grain              | 102               | <0.01            | <0.01   | <0.016             |
|  |                            |   |                           |   |                                   |               |            |  |                                    | Straw              | 102               | <0.01            | <0.01   | <0.016             |
| 741155<br>2016/1118116<br>47320 Lafitte-sur-<br>Lot<br>France (S)<br>L150079               | GC 0654<br>Wheat<br>Epos   | 1. 15.04.2015<br>2. 25.06.-<br>07.07.2015<br>3. 05.08.2015        | Spray<br>application      | BAS 684 02<br>H<br>(EC)<br>750 g/L<br>BAS 684 H | 0.251                             | 211           | 0.531      | 1<br>01.06.2015                          | 29                                 | Plant <sup>2</sup> | 0                 | 26               | 0.64  | 26                 |
|  |                            |   |                           |   |                                   |               |            |  |                                    | Plant <sup>2</sup> | 18                | 0.012            | 1.2   | 0.74               |
|  |                            |   |                           |   |                                   |               |            |  |                                    | Plant <sup>2</sup> | 28                | 0.016            | 0.49  | 0.31               |
|  |                            |   |                           |   |                                   |               |            |  |                                    | Grain              | 65                | <0.01            | <0.01   | <0.016             |
|  |                            |   |                           |   |                                   |               |            |  |                                    | Straw              | 65                | <0.01            | 0.39  | 0.25               |
| 741155   | GC 0654                    | 1. 01.02.2015   | Spray                     | BAS 684 02                                      | 0.250                             | 207           | 0.517      | 1  | 29                                 | Plant <sup>2</sup> | 0                 | 28               | 0.23  | 28                 |

| Report No.<br>Location<br>(EU-region)<br>Trial No                  | Commodity/<br>Variety         | Date of<br>1. Sowing or<br>planting<br>2. Flowering<br>3. Harvest | Method<br>of<br>treatment | Formulation                                     | Application rate per<br>treatment |               |            | No. of<br>treatments<br>and last<br>date | Growth<br>stage at<br>last<br>date | Portion analysed   | DALA <sup>1</sup> | Residues (mg/kg) |   |                    |
|--|-------------------------------|---|---------------------------|---|-----------------------------------|---------------|------------|--|------------------------------------|--------------------|-------------------|------------------|---|--------------------|
|  |                               |   |                           |   | kg a.s./hL                        | Water<br>L/ha | kg a.s./ha |  |                                    |                    |                   | BAS 684 H        | Sum of<br>M684H005 and<br>M684H006,<br>expressed as<br>M684H005 | Total <sup>3</sup> |
| 2016/1118116<br>61200 Chesotopos<br>Greece (S)<br>L150080          | Wheat<br>Maestrale            | 2. 05.05.-<br>15.05.2015<br>3. 16.06.2015                         | application               | H<br>(EC)<br>750 g/L<br>BAS 684 H               |                                   |               |            | 16.04.2015                               |                                    | Plant <sup>2</sup> | 14                | 0.017            | 0.50  | 0.32               |
|  |                               |   |                           |   |                                   |               |            |  |                                    | Plant <sup>2</sup> | 21                | <0.01            | 0.18  | 0.12               |
|  |                               |   |                           |   |                                   |               |            |  |                                    | Grain              | 61                | <0.01            | <0.01   | <0.016             |
|  |                               |   |                           |   |                                   |               |            |  |                                    | Straw              | 61                | 0.026            | 0.060   | 0.062              |
| 741155<br>2016/1118116<br>20060 Bellinzago<br>Italy (S)<br>L150081 | GC 0654<br>Wheat<br>Palesio   | 1. 13.01.2015<br>2. 03.05.-<br>13.05.2015<br>3. 26.06.2015        | Spray<br>application      | BAS 684 02<br>H<br>(EC)<br>750 g/L<br>BAS 684 H | 0.251                             | 204           | 0.513      | 1<br>02.04.2015                          | 29                                 | Plant <sup>2</sup> | 0                 | 13               | 0.40  | 13                 |
|  |                               |   |                           |   |                                   |               |            |  |                                    | Plant <sup>2</sup> | 26                | 0.014            | 0.47  | 0.30               |
|  |                               |   |                           |   |                                   |               |            |  |                                    | Plant <sup>2</sup> | 36                | <0.01            | 0.54  | 0.34               |
|  |                               |   |                           |   |                                   |               |            |  |                                    | Grain              | 85                | <0.01            | <0.01   | <0.016             |
|  |                               |   |                           |   |                                   |               |            |  |                                    | Straw              | 85                | <0.01            | 0.031   | 0.029              |
| 741155<br>2016/1118116<br>02110 La Gineta<br>Spain (S)<br>L150082  | GC 0654<br>Wheat<br>Mane Nick | 1. 22.01.2015<br>2. 15.05.-<br>25.05.2015<br>3. 02.07.2015        | Spray<br>application      | BAS 684 02<br>H<br>(EC)<br>750 g/L<br>BAS 684 H | 0.251                             | 193           | 0.485      | 1<br>10.04.2015                          | 29                                 | Plant <sup>2</sup> | 0                 | 25               | 0.88  | 26                 |
|  |                               |   |                           |   |                                   |               |            |  |                                    | Plant <sup>2</sup> | 26                | <0.01            | 0.31  | 0.20               |
|  |                               |   |                           |   |                                   |               |            |  |                                    | Plant <sup>2</sup> | 39                | <0.01            | 0.068   | 0.051              |
|  |                               |   |                           |   |                                   |               |            |  |                                    | Grain              | 83                | <0.01            | <0.01   | <0.016             |
|  |                               |   |                           |   |                                   |               |            |  |                                    | Straw              | 83                | <0.01            | 0.043   | 0.036              |

1 Days after last application

2 Whole plant without roots

3 Sum of BAS 684 H, M684H005 and M684H006, expressed as BAS 684 H. The conversion factor is 0.606, based on molecular weights (274.4 g/mol / 452.54 g/mol).



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|                    |  |
|--------------------|--|
| <b>Report:</b>     | CA 6.3.2/2<br>Mahlo C., Vagt I., 2017 a<br>Study on the residue behaviour of BAS 684 H in spring wheat after treatment with BAS 684 02 H under field conditions in Germany, Denmark, Northern France, Belgium, Southern France Greece, Italy and Spain, 2016<br>2017/1198202     |
| <b>Guidelines:</b> | EC 1107/2009 of the European Parliament and of the Council of 21 Oct 2009, EU Regulation 544/2011 (10 June 2011) implementing Regulation No 1107/2009, EEC 7029/VI/95 rev. 5 (July 22 1997), EEC 7525/VI/95 rev. 10.1, OECD 509 Crop Field Trial (2009)                          |
| <b>GLP:</b>        | yes  |
| <b>Deviations:</b> | A total of 4 deviations have been listed however these have been considered during evaluation to be minor and to not be of significance in terms of conduct or quality of the study.   |
| <b>Report:</b>     | CA 6.3.2/3<br>Mahlo C., 2018 a<br>Amendment 1: Study on the residue behaviour of BAS 684 H in spring wheat after treatment with BAS 684 02 H under field conditions in Germany, Denmark, Northern France, Belgium, Southern France Greece, Italy and Spain, 2016<br>2018/1030172 |
| <b>Guidelines:</b> | EC 1107/2009 of the European Parliament and of the Council of 21 Oct 2009, EU Regulation 544/2011 (10 June 2011) implementing Regulation No 1107/2009, EEC 7029/VI/95 rev. 5 (July 22 1997), EEC 7525/VI/95 rev. 10.1, OECD 509 Crop Field Trial (2009)                          |
| <b>GLP:</b>        | yes  |

During the 2016 growing season 8 field decline trials in wheat were conducted in Northern and Southern Europe to determine the residue level of BAS 684 H in or on raw agricultural commodities (RAC). BAS 684 02 H (EC) containing nominally 750 g/L BAS 684 H was applied once at a rate equivalent to 0.5 kg BAS 684 H /ha in a spray volume of 200 L/ha. One untreated plot of each trial served as control. The application was performed at BBCH 27-29. Specimens of whole plants without roots were collected immediately after the application (BBCH 27-29) as well as 18-37 (BBCH 49-59) and 28-48 days thereafter (BBCH 65). Grain and straw were sampled at BBCH 89 (crop maturity), 47-105 days after the application. Samples were stored deep-frozen for a maximum of 502 (BAS 684 H) or 482 days (M684H005 / M684H006) until analysis. These storage periods are adequately covered by the available storage stability data. Extracts are stored for up to 5 days, this is considered acceptable as the storage stability of extracts is presented within the analytical method validation for up to 7 days (Section B5).

The specimens were analysed for BAS 684 H with BASF method No L0337/01 quantifying the analyte with a limit of quantitation (LOQ) of 0.01 mg/kg. Method validation data (presented in Section B5) are available for the same matrices as tested in this residue trials study. The method is fully validated in accordance with SANCO 3029/99 rev. 4 with an LOQ of 0.01 mg/kg.

The specimens were analysed for M684H005 and M684H006 (as sum expressed as M684H005) with BASF method No L0337/02 quantifying the analytes with a limit of quantitation (LOQ) of 0.01 mg/kg. Method validation data (presented in Section B5) are available for the same matrices as tested in this residue trials study. The method is fully validated in accordance with SANCO 3029/99 rev. 4 with an LOQ of 0.01 mg/kg.

Recovery rates for BAS 684 H and the metabolites M684H005 and M684H006 were determined concurrently with the sample analysis in order to check the accuracy of the residue analysis. The recovery rates are reported in Table 7-138, the data are acceptable with all mean values within the range 70 – 110 % with the exception of BAS 684 H in straw at 0.01 mg/kg. A high procedural recovery would indicate a possible over-estimation of the residue levels, however as the residue levels in the trials data are < 0.01 mg/kg and the method has been fully validated with acceptable recovery values in Section B5 (Analytical Methods) no further consideration or data are required. The limit of quantification was 0.01 mg/kg for all sample materials.

Table 7-138 Procedural recoveries for BAS 684 H and the metabolites M684H005 and M684H006 in wheat

| Analyte   | Portion analysed          | n  | Fortification level (mg/kg) | Recovery (%)          |       |      |
|-----------|---------------------------|----|-----------------------------|-----------------------|-------|------|
|           |                           |    |                             | Individual recoveries | Mean  | RSD  |
| BAS 684 H | Whole plant without roots | 3  | 0.01                        | 96.5, 96.7, 100       | 97.7  | 2.0  |
|           |                           | 3  | 0.1                         | 99.0, 101, 96.0       | 98.7  | 2.6  |
|           |                           | 2  | 1.0                         | 95.2, 97.0            | 96.1  | --   |
|           |                           | 1  | 10                          | 101                   | --    | --   |
|           |                           | 1  | 50                          | 92.4                  | --    | --   |
|           |                           | 10 | overall                     |                       | 97.5  | 2.8  |
|           | Grain                     | 3  | 0.01                        | 96.5, 93.2, 96.5      | 95.4  | 2.0  |
|           |                           | 3  | 0.1                         | 91.9, 89.4, 98.5      | 93.3  | 5.0  |
|           |                           | 1  | 1.0                         | 100                   | --    | --   |
|           |                           | 7  | overall                     |                       | 95.2  | 4.0  |
|           | Straw                     | 3  | 0.01                        | 115, 121, 116         | 117.3 | 2.7  |
|           |                           | 3  | 0.1                         | 105, 100, 102         | 102.3 | 2.5  |
|           |                           | 1  | 1.0                         | 96.9                  | --    | --   |
|           |                           | 7  | overall                     |                       | 108   | 8.4  |
| M684H005  | Whole plant without roots | 3  | 0.01                        | 92.0, 97.0, 95.3      | 94.8  | 2.7  |
|           |                           | 3  | 0.1                         | 86.2, 87.3, 86.2      | 86.6  | 0.7  |
|           |                           | 3  | 10                          | 82.1, 86.1, 85.8      | 84.7  | 2.6  |
|           |                           | 9  | overall                     |                       | 88.7  | 5.6  |
|           | Grain                     | 3  | 0.01                        | 70.6, 74.1, 75.3      | 73.3  | 3.3  |
|           |                           | 3  | 0.1                         | 71.4, 67.5, 72.2      | 70.4  | 3.6  |
|           |                           | 1  | 1.0                         | 66.7                  | --    | --   |
|           |                           | 7  | overall                     |                       | 71.1  | 4.4  |
|           | Straw                     | 3  | 0.01                        | 73.3, 85.7, 82.0      | 80.3  | 7.9  |
|           |                           | 3  | 0.1                         | 69.1, 86.9, 78.5      | 78.2  | 11.4 |
|           |                           | 1  | 1.0                         | 73.8                  | --    | --   |
|           |                           | 7  | overall                     |                       | 78.5  | 8.6  |
| M684H006  | Whole plant without roots | 3  | 0.01                        | 106, 92.5, 86.8       | 95.1  | 10.4 |
|           |                           | 3  | 0.1                         | 80.7, 80.7, 77.6      | 79.7  | 2.2  |
|           |                           | 6  | overall                     |                       | 87.3  | 12.0 |
|           | Grain                     | 3  | 0.01                        | 71.5, 83.3, 75.4      | 76.7  | 7.8  |
|           |                           | 3  | 0.1                         | 74.5, 78.9, 73.6      | 75.7  | 3.7  |
|           |                           | 6  | overall                     |                       | 76.2  | 5.6  |
|           | Straw                     | 3  | 0.01                        | 83.0, 83.3, 83.6      | 83.3  | 0.4  |
|           |                           | 3  | 0.1                         | 78.1, 75.6, 82.7      | 78.8  | 4.6  |
|           |                           | 6  | overall                     |                       | 81.1  | 4.1  |

No residues of BAS 684 H or M684H005 (including M684H006) above the limit of quantitation (0.01 mg/kg) were found in any of the analysed untreated specimens, except for whole plant without roots immediately after the application in trial L160038 with 0.036 mg/kg of BAS 684 H. The applicant has provided the following information to attempt to explain this result and confirm it is unlikely to be due to drift from another trial plot:

*Drift from another plot could only mean drift from the treated plot in trial L160038. The application rate of the treated plot in this trial was within the range of  $\pm 10\%$  of the target application rate of 0.5 kg as/ha. Also, the residue levels observed in this trial for the treated plot are at the same level as in the all the other trials (BAS 684H residues in whole plant DALA0 range from 13-32 mg/kg, the residue in trial L160038 was 23 mg/kg), this speaks against a reduced application rate by drift issues. Also, if drift would be the issue, it should be observed most likely not only in a single trial out of 8 trials. The same type of application equipment as in trial L160038*

*(Boom sprayer Flat fan /Teejet 110 05) had been used also in trial L160036, where no residues in untreated samples occurred. We believe that the observed data supports the hypothesis of a possible slight contamination in the laboratory rather than a contamination in the field by drift since the sample only contains BAS 684 H, but no residues of the metabolite M684H005. With a contamination in the field from drift, it would be expected that residues of metabolite M684H005 would be observed in this contaminated untreated sample as well. Unfortunately, the analysis of this whole plant DALA 0 sample seems not to have been repeated, the report indicates only one result (no mean value), neither a repetition of the analysis with another replicate of the identical sample nor with the B sample was undertaken. Therefore, no final proof and explanation of the origin of this residue can be provided. However, compared to the high level of BAS 684 H in the corresponding treated sample of whole plant DALA 0 (23 mg/kg) a residue level of 0.036 mg/kg in the untreated sample should not falsify the result of the treated sample.*

Although this explanation does not give definitive reasoning for the result, the residues detected in the untreated plot are most likely due to contamination in the laboratory. No additional information is required and the results from this trial can be relied upon.

The residues found in wheat samples from the individual trials are summarised in Table 7-139. Residues of BAS 684 H in treated whole plant without root specimens collected on the day of the application ranged from 13 to 32 mg/kg. At BBCH 49-59 and BBCH 65 residues declined to < 0.01 mg/kg. Residues in mature grain and straw samples were also below the LOQ of 0.01 mg/kg.

Residues of M684H005 (including M684H006) in treated whole plant without root specimens collected on the day of the application ranged from 0.32 to 1.5 mg/kg. At BBCH 49-59 residues were < 0.01 – 1.1 mg/kg and declined to < 0.01 – 0.16 mg/kg at BBCH 65. Residues in mature grain samples were below the LOQ of 0.01 mg/kg; in straw, < 0.01 – 0.013 mg/kg were found.

Table 7-139 Residues of BAS 684 H, M684H005 and M684H006 in wheat (trials which support the critical GAP are underlined)

| Report No.<br>Location<br>(EU-region)<br>Trial No                      | Commodity/<br>Variety        | Date of<br>1. Sowing or<br>planting<br>2. Flowering<br>3. Harvest | Method of<br>treatment | Formulation                                     | Application rate per<br>treatment |               |               | No. of<br>treat-<br>ments<br>and last<br>date | Growth<br>stage at<br>last<br>date | Portion<br>analysed | DALA <sup>1</sup> | Residues (mg/kg) |   |                    |
|--|------------------------------|---|------------------------|---|-----------------------------------|---------------|---------------|---|------------------------------------|---------------------|-------------------|------------------|---|--------------------|
|  |                              |   |                        |   | kg<br>a.s./hL                     | Water<br>L/ha | kg<br>a.s./ha |   |                                    |                     |                   | BAS 684 H        | Sum of<br>M684H005 and<br>M684H006,<br>expressed as<br>M684H005 | Total <sup>3</sup> |
| 777105<br>2017/1198202<br>67117 Limburgerhof<br>Germany (N)<br>L160032 | GC 0654<br>Wheat<br>Kadrlj   | 1. 16.03.2016<br>2. 06.06.-14.06.2016<br>3. 27.07.2016            | Spray<br>application   | BAS 684 02<br>H<br>(EC)<br>750 g/L<br>BAS 684 H | 0.251                             | 200           | 0.502         | 1<br>04.05.2016                               | 27-29                              | Plant <sup>2</sup>  | 0                 | 28               | 1.5   | 29                 |
|  |                              |   |                        |   |                                   |               |               |   |                                    | Plant <sup>2</sup>  | 28                | <0.01            | 0.088   | 0.063              |
|  |                              |   |                        |   |                                   |               |               |   |                                    | Plant <sup>2</sup>  | 36                | <0.01            | 0.021   | 0.023              |
|  |                              |   |                        |   |                                   |               |               |   |                                    | Grain               | 84                | <0.01            | <0.01   | <0.016             |
|  |                              |   |                        |   |                                   |               |               |   |                                    | Straw               | 84                | <0.01            | <0.01   | <0.016             |
| 777105<br>2017/1198202<br>6580 Vamdrup<br>Denmark (N)<br>L160033       | GC 0654<br>Wheat<br>Lennox   | 1. 07.04.2016<br>2. 27.06.-01.07.2016<br>3. 26.08.2016            | Spray<br>application   | BAS 684 02<br>H<br>(EC)<br>750 g/L<br>BAS 684 H | 0.251                             | 202           | 0.507         | 1<br>01.06.2016                               | 29                                 | Plant <sup>2</sup>  | 0                 | 22               | 1.0   | 23                 |
|  |                              |   |                        |   |                                   |               |               |   |                                    | Plant <sup>2</sup>  | 19                | <0.01            | 0.81  | 0.50               |
|  |                              |   |                        |   |                                   |               |               |   |                                    | Plant <sup>2</sup>  | 28                | <0.01            | 0.11  | 0.077              |
|  |                              |   |                        |   |                                   |               |               |   |                                    | Grain               | 86                | <0.01            | <0.01   | <0.016             |
|  |                              |   |                        |   |                                   |               |               |   |                                    | Straw               | 86                | <0.01            | <0.01   | <0.016             |
| 777105<br>2017/1198202<br>60350 Jaulzy<br>France (N)<br>L160034        | GC 0654<br>Wheat<br>Granny   | 1. 15.03.2016<br>2. 20.06.-10.07.2016<br>3. 01.08.-15.08.2016     | Spray<br>application   | BAS 684 02<br>H<br>(EC)<br>750 g/L<br>BAS 684 H | 0.251                             | 207           | 0.519         | 1<br>27.05.2016                               | 29                                 | Plant <sup>2</sup>  | 0                 | 13               | 1.5   | 14                 |
|  |                              |   |                        |   |                                   |               |               |   |                                    | Plant <sup>2</sup>  | 18                | <0.01            | 1.1   | 0.68               |
|  |                              |   |                        |   |                                   |               |               |   |                                    | Plant <sup>2</sup>  | 39                | <0.01            | 0.16  | 0.11               |
|  |                              |   |                        |   |                                   |               |               |   |                                    | Grain               | 75                | <0.01            | <0.01   | <0.016             |
|  |                              |   |                        |   |                                   |               |               |   |                                    | Straw               | 75                | <0.01            | <0.01   | <0.016             |
| 777105<br>2017/1198202<br>6221 Saint-Amand<br>Belgium (N)<br>L160035   | GC 0654<br>Wheat<br>Triso    | 1. 18.03.2016<br>2. 15.06.-24.06.2016<br>3. 15.08.-31.08.2016     | Spray<br>application   | BAS 684 02<br>H<br>(EC)<br>750 g/L<br>BAS 684 H | 0.251                             | 204           | 0.513         | 1<br>09.05.2016                               | 29                                 | Plant <sup>2</sup>  | 0                 | 27               | 0.50  | 27                 |
|  |                              |   |                        |   |                                   |               |               |   |                                    | Plant <sup>2</sup>  | 30                | <0.01            | 0.056   | 0.044              |
|  |                              |   |                        |   |                                   |               |               |   |                                    | Plant <sup>2</sup>  | 39                | <0.01            | 0.013   | 0.018              |
|  |                              |   |                        |   |                                   |               |               |   |                                    | Grain               | 100               | <0.01            | <0.01   | <0.016             |
|  |                              |   |                        |   |                                   |               |               |   |                                    | Straw               | 100               | <0.01            | <0.01   | <0.016             |
| 777105<br>2017/1198202<br>47320 Bourran<br>France (S)<br>L160036       | GC 0654<br>Wheat<br>Specifik | 1. 20.09.2016<br>2. 12.07.-25.07.2016<br>3. 03.08.2016            | Spray<br>application   | BAS 684 02<br>H<br>(EC)<br>750 g/L<br>BAS 684 H | 0.252                             | 203           | 0.511         | 1<br>17.06.2016                               | 29                                 | Plant <sup>2</sup>  | 0                 | 32               | 0.36  | 32                 |
|  |                              |   |                        |   |                                   |               |               |   |                                    | Plant <sup>2</sup>  | 24                | <0.01            | <0.01   | <0.016             |
|  |                              |   |                        |   |                                   |               |               |   |                                    | Plant <sup>2</sup>  | 31                | <0.01            | <0.01   | <0.016             |
|  |                              |   |                        |   |                                   |               |               |   |                                    | Grain               | 47                | <0.01            | <0.01   | <0.016             |
|  |                              |   |                        |   |                                   |               |               |   |                                    | Straw               | 47                | <0.01            | <0.01   | <0.016             |
| 777105<br>2017/1198202<br>57020 Apollonia<br>Greece (S)<br>L160037     | GC 0654<br>Wheat<br>Africa   | 1. 10.11.2015<br>2. 15.04.-30.04.2016<br>3. 01.06.-15.06.2016     | Spray<br>application   | BAS 684 02<br>H<br>(EC)<br>750 g/L<br>BAS 684 H | 0.250                             | 202           | 0.506         | 1<br>02.03.2016                               | 29                                 | Plant <sup>2</sup>  | 0                 | 13               | 1.2   | 14                 |
|  |                              |   |                        |   |                                   |               |               |   |                                    | Plant <sup>2</sup>  | 37                | <0.01            | 0.24  | 0.16               |
|  |                              |   |                        |   |                                   |               |               |   |                                    | Plant <sup>2</sup>  | 48                | <0.01            | 0.042   | 0.035              |
|  |                              |   |                        |   |                                   |               |               |   |                                    | Grain               | 104               | <0.01            | <0.01   | <0.016             |
|  |                              |   |                        |   |                                   |               |               |   |                                    | Straw               | 104               | <0.01            | <0.01   | <0.016             |

| Report No.<br>Location<br>(EU-region)<br>Trial No               | Commodity/<br>Variety       | Date of<br>1. Sowing or<br>planting<br>2. Flowering<br>3. Harvest | Method of<br>treatment | Formulation                                     | Application rate per<br>treatment |               |               | No. of<br>treat-<br>ments<br>and last<br>date | Growth<br>stage at<br>last<br>date | Portion<br>analysed | DALA <sup>1</sup> | Residues (mg/kg) |   |                    |
|---|-----------------------------|---|------------------------|---|-----------------------------------|---------------|---------------|---|------------------------------------|---------------------|-------------------|------------------|---|--------------------|
|   |                             |   |                        |   | kg<br>a.s./hL                     | Water<br>L/ha | kg<br>a.s./ha |   |                                    |                     |                   | BAS 684 H        | Sum of<br>M684H005 and<br>M684H006,<br>expressed as<br>M684H005 | Total <sup>3</sup> |
| 777105<br>2017/1198202<br>71121 Foggia<br>Italy (S)<br>L160038  | GC 0654<br>Wheat<br>Kadrilj | 1. 23.01.2016<br>2. 25.04.-03.05.2016<br>3. 28.06.2016            | Spray<br>application   | BAS 684 02<br>H<br>(EC)<br>750 g/L<br>BAS 684 H | 0.251                             | 194           | 0.487         | 1<br>15.03.2016                               | 29                                 | Plant <sup>2</sup>  | 0                 | 23 <sup>4</sup>  | 0.55  | 23                 |
|   |                             |   |                        |   |                                   |               |               |   |                                    | Plant <sup>2</sup>  | 33                | <0.01            | 0.023   | 0.024              |
|   |                             |   |                        |   |                                   |               |               |   |                                    | Plant <sup>2</sup>  | 45                | <0.01            | <0.01   | <0.016             |
|   |                             |   |                        |   |                                   |               |               |   |                                    | Grain               | 105               | <0.01            | <0.01   | <0.016             |
|   |                             |   |                        |   |                                   |               |               |   |                                    | Straw               | 105               | <0.01            | <0.01   | <0.016             |
| 777105<br>2017/1198202<br>41410 Carmona<br>Spain (S)<br>L160039 | GC 0654<br>Wheat<br>Athoris | 1. 24.12.2015<br>2. 12.04.-04.05.2016<br>3. 08.06.2016            | Spray<br>application   | BAS 684 02<br>H<br>(EC)<br>750 g/L<br>BAS 684 H | 0.251                             | 207           | 0.519         | 1<br>08.03.2016                               | 29                                 | Plant <sup>2</sup>  | 0                 | 21               | 0.32  | 21                 |
|   |                             |   |                        |   |                                   |               |               |   |                                    | Plant <sup>2</sup>  | 30                | <0.01            | 0.13  | 0.089              |
|   |                             |   |                        |   |                                   |               |               |   |                                    | Plant <sup>2</sup>  | 37                | <0.01            | 0.090   | 0.065              |
|   |                             |   |                        |   |                                   |               |               |   |                                    | Grain               | 92                | <0.01            | <0.01   | <0.016             |
|   |                             |   |                        |   |                                   |               |               |   |                                    | Straw               | 92                | <0.01            | 0.013   | 0.018              |

1 Days after last application

2 Whole plant without roots

3 Sum of BAS 684 H, M684H005 and M684H006, expressed as BAS 684 H. The conversion factor is 0.606, based on molecular weights (274.4 g/mol / 452.54 g/mol).

4 Residue of 0.036 mg/kg found in untreated control sample

|                    |  |
|--------------------|--|
| <b>Report:</b>     | CA 6.3.2/4<br>Martin T., Ruiz E., 2018 a<br>Study on the residue behavior of BAS 684 H in wheat after the application of BAS 684 03 H under field conditions in Germany, Netherlands, Austria, France (North and South), Greece, Italy and Spain, 2017<br>2017/1202170 |
| <b>Guidelines:</b> | ENV/MC/CHEM(98)17, OECD-ENV/JM/MONO(2002)9 No 13 2002, Real Decreto 1369/2000, EC 1107/2009 of the European Parliament, EEC 79/117, EEC 91/414, EEC 7029/VI/95 rev. 5 (July 22 1997), EEC 7525/VI/95 rev. 10.3, OECD 509 Crop Field Trial (2009)                       |
| <b>GLP:</b>        | yes  |
| <b>Deviations:</b> | A total of 2 deviations have been listed however these have been considered during evaluation to be minor and to not be of significance in terms of conduct or quality of the study.   |

During the 2017 growing season 8 field decline trials in wheat were conducted in Northern and Southern Europe to determine the residue level of BAS 684 H in or on raw agricultural commodities (RAC). BAS 684 03 H (EC) containing nominally 750 g/L BAS 684 H was applied once at a rate equivalent to 0.5 kg BAS 684 H/ha in a spray volume of 200 L/ha. One untreated plot of each trial served as control. The application was performed at BBCH 29. Specimens of whole plants without roots were collected immediately after the application (BBCH 29, 0 DALA) as well as 15-70 (BBCH 49) and 22-86 days thereafter (BBCH 65-71). Grain and straw were sampled at BBCH 89 (crop maturity), 68-126 days after the application. Samples were stored deep-frozen at or below -18°C for a maximum of 252 (BAS 684 H) or 274 days (M684H005 / M684H006) until analysis. These storage periods are adequately covered by the available storage stability data. Extracts are stored for a maximum of 1 day, this is considered acceptable as the storage stability of extracts is presented within the analytical method validation for up to 7 days (Section B5).

The specimens were analysed for BAS 684 H with BASF method No L0337/01 quantifying the analyte with a limit of quantitation (LOQ) of 0.01 mg/kg. Method validation data (presented in Section B5) are available for the same matrices as tested in this residue trials study. The method is fully validated in accordance with SANCO 3029/99 rev. 4 with an LOQ of 0.01 mg/kg.

The specimens were analysed for M684H005 and M684H006 (as sum expressed as M684H005) with BASF method No L0337/02 quantifying the analytes with a limit of quantitation (LOQ) of 0.01 mg/kg. Method validation data (presented in Section B5) are available for the same matrices as tested in this residue trials study. The method is fully validated in accordance with SANCO 3029/99 rev. 4 with an LOQ of 0.01 mg/kg.

Recovery rates for BAS 684 H and the metabolites M684H005 and M684H006 were determined concurrently with the sample analysis in order to check the accuracy of the residue analysis. The recovery rates are reported in Table 7-140, the data are acceptable with all mean values within the range 70 – 110 %. The limit of quantification was 0.01 mg/kg for all sample materials.

Table 7-140 Procedural recoveries for BAS 684 H and the metabolites M684H005 and M684H006 in wheat

| Analyte   | Portion analysed          | n  | Fortification level (mg/kg) | Recovery (%)             |       |     |
|-----------|---------------------------|----|-----------------------------|--------------------------|-------|-----|
|           |                           |    |                             | Individual recoveries    | Mean  | RSD |
| BAS 684 H | Whole plant without roots | 5  | 0.01                        | 101, 102, 105, 96.6, 105 | 101.9 | 3.4 |
|           |                           | 3  | 0.1                         | 101, 105, 99.1           | 101.7 | 3.0 |
|           |                           | 1  | 1.0                         | 96.4                     | --    | --  |
|           |                           | 1  | 50                          | 91.6                     | --    | --  |
|           |                           | 10 | overall                     |                          | 100.0 | 4.4 |
|           | Grain                     | 3  | 0.01                        | 95.3, 95.1, 91.8         | 94.1  | 2.1 |
|           |                           | 3  | 0.1                         | 92.0, 93.1, 88.6         | 91.2  | 2.6 |
|           |                           | 6  | overall                     |                          | 92.6  | 2.7 |

|          |                           |    |         |                        |      |      |
|----------|---------------------------|----|---------|------------------------|------|------|
|          | Straw                     | 3  | 0.01    | 89.3, 79.9, 87.4       | 85.5 | 5.8  |
|          |                           | 3  | 0.1     | 84.0, 85.3, 85.3       | 84.9 | 0.9  |
|          |                           | 6  | overall |                        | 85.2 | 3.8  |
| M684H005 | Whole plant without roots | 4  | 0.01    | 85.7, 91.9, 88.4, 93.3 | 89.8 | 3.8  |
|          |                           | 4  | 0.1     | 80.2, 85.8, 85.9, 85.8 | 84.4 | 3.3  |
|          |                           | 3  | 10      | 82.4, 76.9, 87.2       | 82.2 | 6.3  |
|          |                           | 11 | overall |                        | 85.8 | 5.5  |
|          | Grain                     | 3  | 0.01    | 97.4, 83.7, 78.3       | 86.5 | 11.4 |
|          |                           | 3  | 0.1     | 86.2, 76.8, 79.2       | 80.7 | 6.0  |
|          |                           | 6  | overall |                        | 83.6 | 9.1  |
|          | Straw                     | 3  | 0.01    | 79.1, 73.9, 72.3       | 75.1 | 4.7  |
|          |                           | 3  | 0.1     | 78.2, 74.9, 77.1       | 76.7 | 2.2  |
|          |                           | 1  | 1.0     | 73.8                   | --   | --   |
|          |                           | 7  | overall |                        | 75.6 | 3.3  |
| M684H006 | Whole plant without roots | 3  | 0.01    | 93.5, 96.8, 101        | 97.1 | 3.9  |
|          |                           | 3  | 0.1     | 81.0, 83.0, 84.1       | 82.7 | 1.9  |
|          |                           | 1  | 10      | 79.9                   | --   | --   |
|          |                           | 7  | overall |                        | 88.5 | 9.7  |
|          | Grain                     | 3  | 0.01    | 76.0, 77.5, 78.7       | 77.4 | 1.7  |
|          |                           | 3  | 0.1     | 73.8, 68.6, 70.0       | 70.8 | 3.8  |
|          |                           | 6  | overall |                        | 74.1 | 5.5  |
|          | Straw                     | 3  | 0.01    | 79.2, 78.5, 77.7       | 78.5 | 1.0  |
|          |                           | 3  | 0.1     | 79.5, 81.5, 78.2       | 79.7 | 2.1  |
|          |                           | 1  | 1.0     | 82.9                   | --   | --   |
|          |                           | 7  | overall |                        | 79.6 | 2.4  |

No residues of BAS 684 H or M684H005 (including M684H006) above the limit of quantitation (0.01 mg/kg) were found in any of the analysed untreated specimens.

The residues found in wheat samples from the individual trials are summarised in Table 7-141. Residues of BAS 684 H in treated whole plant without root specimens collected on the day of the application ranged from 12 to 37 mg/kg. At BBCH 49 residues were < 0.01 – 0.017 mg/kg and declined to < 0.01 mg/kg at BBCH 65-71. Residues in mature grain and straw samples were also below the LOQ of 0.01 mg/kg.

Residues of M684H005 (including M684H006) in treated whole plant without root specimens collected on the day of the application ranged from 0.16 to 4.4 mg/kg. At BBCH 49 residues were < 0.01 – 1.4 mg/kg and declined to < 0.01 – 1.1 mg/kg at BBCH 65-71. Residues in mature grain samples were below the LOQ of 0.01 mg/kg; in straw, < 0.01 – 0.069 mg/kg were found.

Table 7-141 Residues of BAS 684 H, M684H005 and M684H006 in wheat (trials which support the critical GAP are underlined)

| Report No.<br>Location<br>(EU-region)<br>Trial No                                | Commodity/<br>Variety        | Date of<br>1. Sowing or<br>planting<br>2. Flowering<br>3. Harvest | Method of<br>treatment | Formulation                                     | Application rate per<br>treatment |               |               | No. of<br>treat-<br>ments<br>and last<br>date | Growth<br>stage at<br>last<br>date | Portion<br>analysed | DALA <sup>1</sup> | Residues (mg/kg) |   |                    |
|--|------------------------------|---|------------------------|---|-----------------------------------|---------------|---------------|---|------------------------------------|---------------------|-------------------|------------------|---|--------------------|
|  |                              |   |                        |   | kg<br>a.s./hL                     | Water<br>L/ha | kg<br>a.s./ha |   |                                    |                     |                   | BAS 684 H        | Sum of<br>M684H005 and<br>M684H006,<br>expressed as<br>M684H005 | Total <sup>3</sup> |
| 837496<br>2017/1202170<br>46342 Velen-Ramsdorf<br>Germany (N)<br>L170037         | GC 0654<br>Wheat<br>Tybalt   | 1. 28.03.2017<br>2. 10.06.-22.06.2017<br>3. 07.08.2017            | Spray<br>application   | BAS 684 03<br>H<br>(EC)<br>750 g/L<br>BAS 684 H | 0.250                             | 197.1         | 0.493         | 1<br>22.05.2017                               | 29                                 | Plant <sup>2</sup>  | 0                 | 37               | 2.1   | 38                 |
|  |                              |   |                        |   |                                   |               |               |   |                                    | Plant <sup>2</sup>  | 15                | <0.01            | 1.4   | 0.86               |
|  |                              |   |                        |   |                                   |               |               |   |                                    | Plant <sup>2</sup>  | 24                | <0.01            | 1.1   | 0.68               |
|  |                              |   |                        |   |                                   |               |               |   |                                    | Grain               | 77                | <0.01            | <0.01   | <0.016             |
|  |                              |   |                        |   |                                   |               |               |   |                                    | Straw               | 77                | <0.01            | 0.014   | 0.018              |
| 837496<br>2017/1202170<br>6599 Ven Zelderheide<br>The Netherlands (N)<br>L170038 | GC 0654<br>Wheat<br>Tybalt   | 1. 29.03.2017<br>2. 11.06.-19.06.2017<br>3. 07.08.2017            | Spray<br>application   | BAS 684 03<br>H<br>(EC)<br>750 g/L<br>BAS 684 H | 0.250                             | 207.8         | 0.520         | 1<br>23.05.2017                               | 29                                 | Plant <sup>2</sup>  | 0                 | 12               | 2.2   | 13                 |
|  |                              |   |                        |   |                                   |               |               |   |                                    | Plant <sup>2</sup>  | 15                | 0.012            | 0.57  | 0.36               |
|  |                              |   |                        |   |                                   |               |               |   |                                    | Plant <sup>2</sup>  | 22                | <0.01            | 0.38  | 0.24               |
|  |                              |   |                        |   |                                   |               |               |   |                                    | Grain               | 76                | <0.01            | <0.01   | <0.016             |
|  |                              |   |                        |   |                                   |               |               |   |                                    | Straw               | 76                | <0.01            | <0.01   | <0.016             |
| 837496<br>2017/1202170<br>4542 Nußbach<br>Austria (N)<br>L170039                 | GC 0654<br>Wheat<br>Liskamm  | 1. 10.04.2017<br>2. 18.06.-30.06.2017<br>3. 01.08.2017            | Spray<br>application   | BAS 684 03<br>H<br>(EC)<br>750 g/L<br>BAS 684 H | 0.250                             | 202.7         | 0.507         | 1<br>23.05.2017                               | 29                                 | Plant <sup>2</sup>  | 0                 | 12               | 4.4   | 15                 |
|  |                              |   |                        |   |                                   |               |               |   |                                    | Plant <sup>2</sup>  | 17                | <0.01            | 1.1   | 0.68               |
|  |                              |   |                        |   |                                   |               |               |   |                                    | Plant <sup>2</sup>  | 31                | <0.01            | 0.33  | 0.21               |
|  |                              |   |                        |   |                                   |               |               |   |                                    | Grain               | 68                | <0.01            | <0.01   | <0.016             |
|  |                              |   |                        |   |                                   |               |               |   |                                    | Straw               | 68                | <0.01            | 0.023   | 0.024              |
| 837496<br>2017/1202170<br>37340 Ambillou<br>France (N)<br>L170040                | GC 0654<br>Wheat<br>Sculptur | 1. 30.03.2017<br>2. 08.06.-12.06.2017<br>3. 20.07.2017            | Spray<br>application   | BAS 684 03<br>H<br>(EC)<br>750 g/L<br>BAS 684 H | 0.251                             | 193.3         | 0.486         | 1<br>28.04.2017                               | 29                                 | Plant <sup>2</sup>  | 0                 | 34               | 0.16  | 34                 |
|  |                              |   |                        |   |                                   |               |               |   |                                    | Plant <sup>2</sup>  | 26                | 0.017            | 0.16  | 0.11               |
|  |                              |   |                        |   |                                   |               |               |   |                                    | Plant <sup>2</sup>  | 49                | <0.01            | 0.14  | 0.095              |
|  |                              |   |                        |   |                                   |               |               |   |                                    | Grain               | 83                | <0.01            | <0.01   | <0.016             |
|  |                              |   |                        |   |                                   |               |               |   |                                    | Straw               | 83                | <0.01            | <0.01   | <0.016             |
| 837496<br>2017/1202170<br>32600 Endoufielle<br>France (S)<br>L170041             | GC 0654<br>Wheat<br>Valbona  | 1. 03.03.2017<br>2. 14.05.-23.05.2017<br>3. 03.08.2017            | Spray<br>application   | BAS 684 03<br>H<br>(EC)<br>750 g/L<br>BAS 684 H | 0.250                             | 195.0         | 0.488         | 1<br>19.04.2017                               | 29                                 | Plant <sup>2</sup>  | 0                 | 16               | 0.65  | 16                 |
|  |                              |   |                        |   |                                   |               |               |   |                                    | Plant <sup>2</sup>  | 21                | <0.01            | 0.83  | 0.51               |
|  |                              |   |                        |   |                                   |               |               |   |                                    | Plant <sup>2</sup>  | 29                | <0.01            | 0.44  | 0.28               |
|  |                              |   |                        |   |                                   |               |               |   |                                    | Grain               | 99                | <0.01            | <0.01   | <0.016             |
|  |                              |   |                        |   |                                   |               |               |   |                                    | Straw               | 99                | <0.01            | 0.019   | 0.022              |
| 837496<br>2017/1202170<br>59300 Platanos<br>Greece (S)<br>L170042                | GC 0654<br>Wheat<br>Africa   | 1. 20.02.2017<br>2. 01.07.-15.07.2017<br>3. 15.08.-20.08.2017     | Spray<br>application   | BAS 684 03<br>H<br>(EC)<br>750 g/L<br>BAS 684 H | 0.250                             | 201.0         | 0.503         | 1<br>12.04.2017                               | 29                                 | Plant <sup>2</sup>  | 0                 | 30               | 1.3   | 31                 |
|  |                              |   |                        |   |                                   |               |               |   |                                    | Plant <sup>2</sup>  | 70                | <0.01            | <0.01   | <0.016             |
|  |                              |   |                        |   |                                   |               |               |   |                                    | Plant <sup>2</sup>  | 86                | <0.01            | <0.01   | <0.016             |
|  |                              |   |                        |   |                                   |               |               |   |                                    | Grain               | 126               | <0.01            | <0.01   | <0.016             |
|  |                              |   |                        |   |                                   |               |               |   |                                    | Straw               | 126               | <0.01            | <0.01   | <0.016             |



| Report No.<br>Location<br>(EU-region)<br>Trial No               | Commodity/<br>Variety      | Date of<br>1. Sowing or<br>planting<br>2. Flowering<br>3. Harvest | Method of<br>treatment | Formulation                                     | Application rate per<br>treatment |               |               | No. of<br>treat-<br>ments<br>and last<br>date | Growth<br>stage at<br>last<br>date | Portion<br>analysed | DALA <sup>1</sup> | Residues (mg/kg) |   |                    |
|---|----------------------------|---|------------------------|---|-----------------------------------|---------------|---------------|---|------------------------------------|---------------------|-------------------|------------------|---|--------------------|
|   |                            |   |                        |   | kg<br>a.s./hL                     | Water<br>L/ha | kg<br>a.s./ha |   |                                    |                     |                   | BAS 684 H        | Sum of<br>M684H005 and<br>M684H006,<br>expressed as<br>M684H005 | Total <sup>3</sup> |
| 837496<br>2017/1202170<br>44048 Argenta<br>Italy (S)<br>L170043 | GC 0654<br>Wheat<br>Cesare | 1. 06.01.2017<br>2. 03.05.-13.05.2017<br>3. 23.06.2017            | Spray<br>application   | BAS 684 03<br>H<br>(EC)<br>750 g/L<br>BAS 684 H | 0.250                             | 215.0         | 0.538         | 1<br>07.04.2017                               | 29                                 | Plant <sup>2</sup>  | 0                 | 18               | 0.18  | 18                 |
|   |                            |   |                        |   |                                   |               |               |   |                                    | Plant <sup>2</sup>  | 21                | <0.01            | 0.67  | 0.42               |
|   |                            |   |                        |   |                                   |               |               |   |                                    | Plant <sup>2</sup>  | 31                | <0.01            | 0.38  | 0.24               |
|   |                            |   |                        |   |                                   |               |               |   |                                    | Grain               | 76                | <0.01            | <0.01   | <0.016             |
|   |                            |   |                        |   |                                   |               |               |   |                                    | Straw               | 76                | <0.01            | 0.069   | 0.052              |
| 837496<br>2017/1202170<br>41710 Utrera<br>Spain (S)<br>L170044  | GC 0654<br>Wheat<br>Galera | 1. 26.01.2017<br>2. 20.04.-01.05.2017<br>3. 01.06.2017            | Spray<br>application   | BAS 684 03<br>H<br>(EC)<br>750 g/L<br>BAS 684 H | 0.250                             | 195.0         | 0.488         | 1<br>15.03.2017                               | 29                                 | Plant <sup>2</sup>  | 0                 | 17               | 0.25  | 17                 |
|   |                            |   |                        |   |                                   |               |               |   |                                    | Plant <sup>2</sup>  | 21                | <0.01            | 0.73  | 0.45               |
|   |                            |   |                        |   |                                   |               |               |   |                                    | Plant <sup>2</sup>  | 42                | <0.01            | 0.062   | 0.048              |
|   |                            |   |                        |   |                                   |               |               |   |                                    | Grain               | 77                | <0.01            | <0.01   | <0.016             |
|   |                            |   |                        |   |                                   |               |               |   |                                    | Straw               | 77                | <0.01            | 0.023   | 0.024              |

1 Days after last application

2 Whole plant without roots

3 Sum of BAS 684 H, M684H005 and M684H006, expressed as BAS 684 H. The conversion factor is 0.606, based on molecular weights (274.4 g/mol / 452.54 g/mol).

Table 7-142 Summary of residue trials on wheat

| Crop        | Region/<br>Indoor | <b>RD-Mo – BAS 684 H</b><br>Residue levels (mg/kg)<br>observed in the<br>supervised residue<br>trials relevant to the<br>supported GAPs | <b><u>RD-RA - Sum of BAS<br/>684 H, M684H005 and<br/>M684H006, expressed<br/>as BAS 684 H</u></b><br>Residue levels (mg/kg)<br>observed in the<br>supervised residue<br>trials relevant to the<br>supported GAPs | HR<br>(RD-RA)<br>(mg/kg) | STMR<br>(RD-RA)<br>(mg/kg) |
|-------------|-------------------|---|--|--------------------------|----------------------------|
| Wheat grain | NEU<br>Outdoor    | 12 x < 0.01*  | 12 x < 0.016*  | 0.016*                   | 0.016*                     |
| Wheat grain | SEU<br>Outdoor    | 12 x < 0.01*  | 12 x < 0.016*  | -                        | -                          |
| Wheat straw | NEU<br>Outdoor    | 12 x < 0.01*  | 10 x < 0.016*, 0.018,<br>0.024   | 0.024                    | 0.016*                     |
| Wheat straw | SEU<br>Outdoor    | 11 x < 0.01*, 0.026   | 4 x < 0.016*, 0.018,<br>0.022, 0.024, 0.029,<br>0.036, 0.052, 0.062,<br>0.25   | -                        | -                          |

Note data from the SEU are not being used in the risk assessment therefore HR and STMR values have not been determined.

### B.7.3.2. Oilseed rape

A proposed use in winter oilseed rape is included in the EU Annex III dossier. To facilitate a future application for an extension of use into oilseed rape an evaluation of the oilseed rape residues data has been completed.

As oilseed rape is a major crop, a minimum of eight trials are required for each geographical zone.

Two uses are being considered as shown in the GAP table below. However, in this residue section only the worst-case use is being considered (cGAP). The critical use consists of one spray application to winter oilseed rape at a rate of 250 g a.s./ha when the crop has reached the growth stage BBCH 18. No residues trials data have been submitted for the pre-emergence use (BBCH 00 – 08), however as this is considered a less critical use this is acceptable.

Table 7-143 Requested GAPs and critical GAP (in bold)

| Use-<br>No. | Member<br>states/<br>zones | Crop                           | Application      |  |                           |  | PHI<br>(days) |
|-------------|----------------------------|--------------------------------|------------------|--|---------------------------|--|---------------|
|             |                            |                                | Method /<br>kind | Growth stage of<br>crop                | Number of<br>applications | Rate per<br>application<br>(g a.s./ha) |               |
| 1           | UK                         | Winter oilseed<br>rape         | Spray            | Pre-emergence<br>(BBCH 00-08)          | 1                         | 250                                    | n.a.          |
| 2           | UK                         | <b>Winter oilseed<br/>rape</b> | <b>Spray</b>     | <b>Post-emergence<br/>(BBCH 09-18)</b> | <b>1</b>                  | <b>250</b>                             | <b>n.a.</b>   |

A summary of the trials submitted to support the cGAP are given

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Table 7-144. This number of trials is considered adequate to address the requirements for the North EU (NEU). The dossier submitted also includes residue trials data from the South EU (SEU), these have been reported for completeness however the end-points from these have not been used further in the risk assessment.

Table 7-144 Number of residue trials per geographical region and vegetation period

| Crop                              | Season | Number of trials |                |      |                |       | Reference          |
|-----------------------------------|--------|------------------|----------------|------|----------------|-------|--------------------|
|                                   |        | N-EU             | Country        | S-EU | Country        | Total |                    |
| Oilseed rape                      | 2016   | 4                | DE, FR, NL, UK | 4    | ES, GR, 2xIT   | 8     | 6.3.1/1<br>6.3.1/2 |
|                                   | 2017   | 4                | DE, FR, HU, NL | 4    | ES, FR, GR, IT | 8     | 6.3.1/3            |
| Total number of trials per region |        | 8                | -              | 8    | -              | 16    |                    |

**Report:** CA 6.3.1/1  
Klimmek S., Bruhn F., 2017 a  
Study on the residue behaviour of BAS 684 H in oilseed rape after one application with BAS 684 02 H under field conditions in Germany, Northern France, The Netherlands, United Kingdom, Greece, Italy and Spain, 2016  
2017/1219191

**Guidelines:** EU1999: 1607/VI/97, EEC 7029/VI/95 rev. 5, EU Regulation 1107/2009 with Regulation 283/2013, EU Regulation 1107/2009 with Regulation 284/2013, EU Regulation 1107/2009 with Regulation 544/2011 (former Annex II), EU Regulation 1107/2009 with Regulation 545/2011 (former Annex III), OECD 509 Crop Field Trial (2009), SANCO/3029/99 rev. 4, EEC 7525/VI/95 rev. 10.1

**GLP:** yes

**Deviations:** A total of 5 deviations have been listed however these have been considered during evaluation to be minor and to not be of significance in terms of conduct or quality of the study.

**Report:** CA 6.3.1/2  
Klimmek S., Bruhn F., 2018 a  
Amendment No.1, study on the residue behaviour of BAS 684 H in oilseed rape after one application with BAS 684 02 H under field conditions in Germany, Northern France, The Netherlands, United Kingdom, Greece, Italy and Spain, 2016  
2018/1028316

**Guidelines:** EU1999: 1607/VI/97, EEC 7029/VI/95 rev. 5, EU Regulation 1107/2009 with Regulation 283/2013, EU Regulation 1107/2009 with Regulation 284/2013, EU Regulation 1107/2009 with Regulation 544/2011 (former Annex II), EU Regulation 1107/2009 with Regulation 545/2011 (former Annex III), OECD 509 Crop Field Trial (2009), SANCO/3029/99 rev. 4, EEC 7525/VI/95 rev. 10.1

**GLP:** yes

During the 2016 growing season 8 field decline trials in oilseed rape were conducted in Northern and Southern Europe to determine the residue level of BAS 684 H in or on raw agricultural commodities (RAC). BAS 684 02 H (EC) containing nominally 750 g/L BAS 684 H was applied once at a rate equivalent to 0.250 kg BAS 684 H /ha in a spray volume of 200 L/ha. One untreated plot of each trial served as control. The application was performed at BBCH 18-21. Specimens of whole plants without roots were collected immediately after the application (BBCH 18-21) as well as 8-29 (BBCH 51) and 22-69 days thereafter (BBCH 65). Seed and rest of plant without roots were sampled at BBCH 89 (crop maturity), 65-136 days after the application. Samples were stored deep-frozen for a maximum of 497 (BAS 684 H) or 512 days (M684H005 and M684H006) until analysis. These storage periods are adequately covered by the available storage stability data. Extracts are stored for up to 8 days, this is considered acceptable as the storage stability of extracts is presented within the analytical method validation for up to 7 days (Section B5) and in addition the procedural recoveries are acceptable.

The specimens were analysed for BAS 684 H with BASF method No L0337/01 quantifying the analyte with a limit of quantitation (LOQ) of 0.01 mg/kg. Method validation data (presented in Section B5) are available for

the same matrices as tested in this residue trials study. The method is fully validated in accordance with SANCO 3029/99 rev. 4 with an LOQ of 0.01 mg/kg.

The specimens were analysed for M684H005 and M684H006 (as sum expressed as M684H005) with BASF method No L0337/02 quantifying the analytes with a limit of quantitation (LOQ) of 0.01 mg/kg. It is noted that the method validation data are presented for wheat whole plant, wheat straw, wheat grain, sunflower seeds, citrus fruit, bean dried seed and lettuce heads. These are not the same matrices as analysed in the magnitude of residues trials study, no data have been provided for rape seeds, or oilseed rape plant. However, as commodities have been tested from the same matrix groups, no matrix effects have been noted, procedural recoveries are acceptable at LOQ and 10 x LOQ and the study report states no residues of the analyte at or above the limit of quantitation were detected which proves that no interferences of the specimen material with the analytical procedure occurred, the data provided are considered acceptable. The method is fully validated in accordance with SANCO 3029/99 rev. 4 with an LOQ of 0.01 mg/kg.

Recovery rates for BAS 684 H and the metabolites M684H005 and M684H006 were determined concurrently with the sample analysis in order to check the accuracy of the residue analysis. The recovery rates are reported in Table 7-145, the data are acceptable with all mean values within the range 70 – 110 %. The limit of quantification was 0.01 mg/kg for all sample materials.

Table 7-145 Procedural recoveries for BAS 684 H and the metabolites M684H005 and M684H006 in oilseed rape

| Analyte   | Portion analysed            | n  | Fortification level (mg/kg) | Recovery (%)                      |       |      |
|-----------|-----------------------------|----|-----------------------------|-----------------------------------|-------|------|
|           |                             |    |                             | Individual recoveries             | Mean  | RSD  |
| BAS 684 H | Whole plant without roots   | 5  | 0.01                        | 81.4, 89.3, 80.3, 83.5, 86.0      | 84.1  | 4.3  |
|           |                             | 3  | 0.1                         | 86.0, 86.3, 83.8                  | 85.4  | 1.6  |
|           |                             | 2  | 15                          | 74.7, 81.8                        | 78.3  | --   |
|           |                             | 10 | overall                     |                                   | 83.3  | 4.9  |
|           | Rest of plant without roots | 3  | 0.01                        | 71.9, 76.3, 85.6                  | 77.9  | 9.0  |
|           |                             | 3  | 0.1                         | 70.8, 75.0, 73.1                  | 73.0  | 2.9  |
|           |                             | 6  | overall                     |                                   | 75.4  | 7.1  |
|           | Seed                        | 3  | 0.01                        | 82.1, 89.0, 76.0                  | 82.4  | 7.9  |
|           |                             | 3  | 0.1                         | 76.0, 82.0, 77.3                  | 78.4  | 4.0  |
|           |                             | 6  | overall                     |                                   | 80.4  | 6.3  |
| M684H005  | Whole plant without roots   | 4  | 0.01                        | 100, 105, 93.0, 104               | 100.5 | 5.4  |
|           |                             | 3  | 0.1                         | 105, 104, 98.0                    | 102.3 | 3.7  |
|           |                             | 2  | 10                          | 81.4, 80.8                        | 81.1  | --   |
|           |                             | 9  | overall                     |                                   | 96.8  | 10.0 |
|           | Rest of plant without roots | 3  | 0.01                        | 85.5, 85.5, 87.0                  | 86.0  | 1.0  |
|           |                             | 3  | 0.1                         | 75.0, 79.8, 82.8                  | 79.2  | 5.0  |
|           |                             | 6  | overall                     |                                   | 82.6  | 5.5  |
|           | Seed                        | 3  | 0.01                        | 88.6, 86.4, 92.8                  | 89.3  | 3.6  |
|           |                             | 3  | 0.1                         | 90.4, 92.4, 87.2                  | 90.0  | 2.9  |
|           |                             | 6  | overall                     |                                   | 89.6  | 3.0  |
| M684H006  | Whole plant without roots   | 3  | 0.01                        | 91.2, 93.2, 100                   | 94.8  | 4.9  |
|           |                             | 6  | 0.1                         | 100, 89.2, 92.8, 79.9, 79.9, 82.6 | 87.4  | 9.2  |
|           |                             | 2  | 10                          | 86.8, 86.8                        | 86.8  | --   |
|           |                             | 11 | overall                     |                                   | 89.3  | 7.9  |
|           | Rest of plant               | 3  | 0.01                        | 90.0, 99.9, 96.0                  | 95.3  | 5.2  |
|           |                             | 3  | 0.1                         | 89.9, 96.6, 93.2                  | 93.2  | 3.6  |

|  |               |   |         |                  |      |     |
|--|---------------|---|---------|------------------|------|-----|
|  | without roots | 6 | overall |                  | 94.3 | 4.2 |
|  | Seed          | 3 | 0.01    | 84.8, 87.5, 94.6 | 89.0 | 5.7 |
|  |               | 3 | 0.1     | 84.8, 75.5, 79.0 | 79.8 | 5.9 |
|  |               | 6 | overall |                  | 84.4 | 7.9 |

No residues of BAS 684 H or M684H005 (including M684H006) above the limit of quantitation (0.01 mg/kg) were found in any of the analysed untreated specimens.

The residues found in oilseed rape samples from the individual trials are summarised in Table 7-146. Residues of BAS 684 H in treated whole plant without root specimens collected on the day of the application ranged from 5.4 to 11 mg/kg. At BBCH 51 residues declined to < 0.01 – 0.062 mg/kg and further to < 0.01 mg/kg at BBCH 65. Residues in mature seed and rest of plant without roots samples were below the LOQ of 0.01 mg/kg.

Residues of M684H005 (including M684H006) in treated whole plant without root specimens collected on the day of the application ranged from 0.012 to 0.20 mg/kg. At BBCH 51 residues were 0.25 – 1.5 mg/kg and declined to < 0.01 – 0.32 mg/kg at BBCH 65. Residues in mature seed and rest of plant without roots samples were below the LOQ of 0.01 mg/kg.

Table 7-146 Residues of BAS 684 H, M684H005 and M684H006 in oilseed rape (trials which support the critical GAP are underlined)

| Report No.<br>Location<br>(EU-region)<br>Trial No                                      | Commodity/<br>Variety                   | Date of<br>1. Sowing or<br>planting<br>2. Flowering<br>3. Harvest | Method of<br>treatment | Formulation                                     | Application rate per<br>treatment |               |               | No. of<br>treat-<br>ments<br>and last<br>date | Growth<br>stage at<br>last<br>date | Portion<br>analysed   | DALA <sup>1</sup>           | Residues (mg/kg)                        |   |   |
|--|---|---|------------------------|---|-----------------------------------|---------------|---------------|---|------------------------------------|---|-----------------------------|---|---|---|
|  |   |   |                        |   | kg<br>a.s./hL                     | Water<br>L/ha | kg<br>a.s./ha |   |                                    |   |                             | BAS 684 H                               | Sum of<br>M684H005 and<br>M684H006,<br>expressed as<br>M684H005 | Total <sup>4</sup>                                  |
| 741157<br>2017/1219191<br>67117 Limburgerhof<br>Germany (N)<br>L160024                 | SO 0495<br>Oilseed<br>rape<br>Heros     | 1. 21.03.2016<br>2. 02.06.-13.06.2016<br>3. 22.07.2016            | Spray<br>application   | BAS 684 02<br>H<br>(EC)<br>750 g/L<br>BAS 684 H | 0.124                             | 203.8         | 0.252         | 1<br>18.05.2016                               | 18                                 | Plant <sup>2</sup><br>Plant <sup>2</sup><br>Rest <sup>3</sup><br>Seed                       | 0<br>22<br>65<br>65         | 8.9<br><0.01<br><0.01<br><0.01          | 0.16<br>0.32<br><0.01<br><0.01                                  | 9.0<br>0.20<br><0.016<br><u>&lt;0.016</u>           |
| 741157<br>2017/1219191<br>45300 Audeville<br>France (N)<br>L160025                     | SO 0495<br>Oilseed<br>rape<br>Mosaik    | 1. 06.04.2016<br>2. 20.06.-15.07.2016<br>3. 08.09.2016            | Spray<br>application   | BAS 684 02<br>H<br>(EC)<br>750 g/L<br>BAS 684 H | 0.125                             | 209           | 0.261         | 1<br>24.05.2016                               | 21                                 | Plant <sup>2</sup><br>Plant <sup>2</sup><br>Plant <sup>2</sup><br>Rest <sup>3</sup><br>Seed | 0<br>8<br>34<br>107<br>107  | 11<br>0.011<br><0.01<br><0.01<br><0.01  | 0.049<br>1.3<br><0.01<br><0.01<br><0.01                         | 11<br>0.80<br><0.016<br><0.016<br><u>&lt;0.016</u>  |
| 741157<br>2017/1219191<br>6662 PK Elst<br>The Netherlands (N)<br>L160026               | SO 0495<br>Oilseed<br>rape<br>Royal Pro | 1. 11.09.2015<br>2. not reported<br>3. 18.07.2016                 | Spray<br>application   | BAS 684 02<br>H<br>(EC)<br>750 g/L<br>BAS 684 H | 0.125                             | 203           | 0.253         | 1<br>24.03.2016                               | 18                                 | Plant <sup>2</sup><br>Plant <sup>2</sup><br>Plant <sup>2</sup><br>Rest <sup>3</sup><br>Seed | 0<br>11<br>36<br>116<br>116 | 9.6<br>0.023<br><0.01<br><0.01<br><0.01 | 0.017<br>1.1<br>0.11<br><0.01<br><0.01                          | 9.6<br>0.69<br>0.077<br><0.016<br><u>&lt;0.016</u>  |
| 741157<br>2017/1219191<br>DE695AT Church<br>Broughton<br>United Kingdom (N)<br>L160027 | SO 0495<br>Oilseed<br>rape<br>Picto     | 1. 02.09.2015<br>2. 01.05.-16.06.2016<br>3. 25.07.2016            | Spray<br>application   | BAS 684 02<br>H<br>(EC)<br>750 g/L<br>BAS 684 H | 0.124                             | 220           | 0.272         | 1<br>11.03.2016                               | 18                                 | Plant <sup>2</sup><br>Plant <sup>2</sup><br>Plant <sup>2</sup><br>Rest <sup>3</sup><br>Seed | 0<br>25<br>69<br>136<br>136 | 10<br>0.022<br><0.01<br><0.01<br><0.01  | 0.013<br>1.0<br><0.01<br><0.01<br><0.01                         | 10<br>0.63<br><0.016<br><0.016<br><u>&lt;0.016</u>  |
| 741157<br>2017/1219191<br>40052 Baricella<br>Italy (S)<br>L160028                      | SO 0495<br>Oilseed<br>rape<br>Pulsar    | 1. 30.09.2015<br>2. 02.04.-25.05.2016<br>3. 16.06.2016            | Spray<br>application   | BAS 684 02<br>H<br>(EC)<br>750 g/L<br>BAS 684 H | 0.125                             | 210           | 0.263         | 1<br>24.02.2016                               | 18                                 | Plant <sup>2</sup><br>Plant <sup>2</sup><br>Plant <sup>2</sup><br>Rest <sup>3</sup><br>Seed | 0<br>26<br>47<br>113<br>113 | 5.4<br><0.01<br><0.01<br><0.01<br><0.01 | 0.012<br>0.25<br><0.01<br><0.01<br><0.01                        | 5.4<br>0.16<br><0.016<br><0.016<br><u>&lt;0.016</u> |
| 741157<br>2017/1219191<br>57018 Melissachori<br>Greece (S)<br>L160029                  | SO 0495<br>Oilseed<br>rape<br>Karun     | 1. 12.12.2015<br>2. 30.03.-20.04.2016<br>3. 23.06.2016            | Spray<br>application   | BAS 684 02<br>H<br>(EC)<br>750 g/L<br>BAS 684 H | 0.125                             | 218           | 0.272         | 1<br>22.02.2016                               | 18                                 | Plant <sup>2</sup><br>Plant <sup>2</sup><br>Plant <sup>2</sup><br>Rest <sup>3</sup><br>Seed | 0<br>16<br>44<br>122<br>122 | 11<br>0.020<br><0.01<br><0.01<br><0.01  | 0.037<br>1.5<br>0.081<br><0.01<br><0.01                         | 11<br>0.93<br>0.059<br><0.016<br><u>&lt;0.016</u>   |

| Report No.<br>Location<br>(EU-region)<br>Trial No                            | Commodity/<br>Variety                   | Date of<br>1. Sowing or<br>planting<br>2. Flowering<br>3. Harvest | Method of<br>treatment | Formulation                                     | Application rate per<br>treatment |               |               | No. of<br>treat-<br>ments<br>and last<br>date | Growth<br>stage at<br>last<br>date | Portion<br>analysed | DALA <sup>1</sup> | Residues (mg/kg) |   |                    |
|--|---|---|------------------------|---|-----------------------------------|---------------|---------------|---|------------------------------------|---------------------|-------------------|------------------|---|--------------------|
|  |   |   |                        |   | kg<br>a.s./hL                     | Water<br>L/ha | kg<br>a.s./ha |   |                                    |                     |                   | BAS 684 H        | Sum of<br>M684H005 and<br>M684H006,<br>expressed as<br>M684H005 | Total <sup>4</sup> |
| 741157<br>2017/1219191<br>40050 Castello<br>D'Argile<br>Italy (S)<br>L160030 | SO 0495<br>Oilseed<br>rape<br>Excalibur | 1. 10.10.2015<br>2. 04.04.-02.05.2016<br>3. 23.06.2016            | Spray<br>application   | BAS 684 02<br>H<br>(EC)<br>750 g/L<br>BAS 684 H | 0.125                             | 207           | 0.258         | 1<br>24.02.2016                               | 18                                 | Plant <sup>2</sup>  | 0                 | 7.6              | 0.20  | 7.7                |
|  |   |   |                        |   |                                   |               |               |   |                                    | Plant <sup>2</sup>  | 29                | <0.01            | 0.41  | 0.26               |
|  |   |   |                        |   |                                   |               |               |   |                                    | Plant <sup>2</sup>  | 52                | <0.01            | <0.01   | <0.016             |
|  |   |   |                        |   |                                   |               |               |   |                                    | Rest <sup>3</sup>   | 121               | <0.01            | <0.01   | <0.016             |
|  |   |   |                        |   |                                   |               |               |   |                                    | Seed                | 121               | <0.01            | <0.01   | <0.016             |
| 741157<br>2017/1219191<br>22193 Arascues<br>Spain (S)<br>L160031             | SO 0495<br>Oilseed<br>rape<br>Hydromel  | 1. 01.10.2015<br>2. not reported<br>3. 28.06.2016                 | Spray<br>application   | BAS 684 02<br>H<br>(EC)<br>750 g/L<br>BAS 684 H | 0.123                             | 199           | 0.244         | 1<br>10.03.2016                               | 18                                 | Plant <sup>2</sup>  | 0                 | 6.0              | 0.10  | 6.1                |
|  |   |   |                        |   |                                   |               |               |   |                                    | Plant <sup>2</sup>  | 12                | 0.062            | 1.1   | 0.73               |
|  |   |   |                        |   |                                   |               |               |   |                                    | Plant <sup>2</sup>  | 32                | <0.01            | 0.067   | 0.051              |
|  |   |   |                        |   |                                   |               |               |   |                                    | Rest <sup>3</sup>   | 110               | <0.01            | <0.01   | <0.016             |
|  |   |   |                        |   |                                   |               |               |   |                                    | Seed                | 110               | <0.01            | <0.01   | <0.016             |

1 Days after last application

2 Whole plant without roots

3 Rest of plant without roots

4 Sum of BAS 684 H, M684H005 and M684H006, expressed as BAS 684 H. The conversion factor is 0.606, based on molecular weights (274.4 g/mol / 452.54 g/mol).



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|                    |   |
|--------------------|---|
| <b>Report:</b>     | CA 6.3.1/3<br>Klimmek S., Bruhn F., 2018 b<br>Study on the residue behaviour of BAS 684 H in oilseed rape after one application with BAS 684 03 H under field conditions in Germany, Northern France<br>2017/1219684  |
| <b>Guidelines:</b> | EU1999: 1607/VI/97, EEC 7029/VI/95 rev. 5, EEC 91/414, EU Regulation 544/2011 (10 June 2011) implementing Regulation No 1107/2009, Regulations (EU) 545/2011 implementing Regulation (EC) 1107/2009, European Commission Regulation No 283/2013, European Commission Regulation No 284/2013, OECD 509 Crop Field Trial (2009), SANCO/3029/99 rev. 4, EEC 7525/VI/95 rev. 10.1 |
| <b>GLP:</b>        | yes   |
| <b>Deviations:</b> | A total of 4 deviations have been listed however these have been considered during evaluation to be minor and to not be of significance in terms of conduct or quality of the study.  |

During the 2017 growing season 8 field decline trials in oilseed rape were conducted in Northern and Southern Europe to determine the residue level of BAS 684 H in or on raw agricultural commodities (RAC). BAS 684 03 H (EC) containing nominally 750 g/L BAS 684 H was applied once at a rate equivalent to 0.250 kg BAS 684 H/ha in a spray volume of 200 L/ha. One untreated plot of each trial served as control. The application was performed at BBCH 18-19. Specimens of whole plants without roots were collected immediately after the application (BBCH 18-19) as well as 9-20 (BBCH 51-53) and 35-50 days thereafter (BBCH 65). Seed and rest of plant without roots were sampled at BBCH 89 (crop maturity), 95-144 days after the application. Samples were stored deep-frozen at or below -18 °C for a maximum of 316 (BAS 684 H) or 293 days (M684H005 and M684H006) until analysis. These storage periods are adequately covered by the available storage stability data. Extracts are stored for up to 22 days, however this time period is only for some of the whole plant samples, the extracts from seeds were stored for a maximum of 4 days. Within the study the stability of the analyte in final volume solutions was proven by procedural recovery samples which were stored for the same period of time between extraction and LC-MS/MS analysis therefore this is considered acceptable and no further data are required at this time.

The specimens were analysed for BAS 684 H with BASF method No L0337/01 quantifying the analyte with a limit of quantitation (LOQ) of 0.01 mg/kg. Method validation data (presented in Section B5) are available for the same matrices as tested in this residue trials study. The method is fully validated in accordance with SANCO 3029/99 rev. 4 with an LOQ of 0.01 mg/kg.

The specimens were analysed for M684H005 and M684H006 (as sum expressed as M684H005) with BASF method No L0337/02 quantifying the analytes with a limit of quantitation (LOQ) of 0.01 mg/kg. It is noted that the method validation data are presented for wheat whole plant, wheat straw, wheat grain, sunflower seeds, citrus fruit, bean dried seed and lettuce heads. These are not the same matrices as analysed in the magnitude of residues trials study, no data have been provided for rape seeds, or oilseed rape plant. However, as commodities have been tested from the same matrix groups, no matrix effects have been noted, procedural recoveries are acceptable at LOQ and 10 x LOQ and the study report states no residues of the analyte at or above the limit of quantitation were detected which proves that no interferences of the specimen material with the analytical procedure occurred, the data provided are considered acceptable. The method is fully validated in accordance with SANCO 3029/99 rev. 4 with an LOQ of 0.01 mg/kg.

Recovery rates for BAS 684 H and the metabolites M684H005 and M684H006 were determined concurrently with the sample analysis in order to check the accuracy of the residue analysis. The recovery rates are reported in

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Table 7-147, the data are acceptable with all mean values within the range 70 – 110 %. It is noted that the %RSD for M684H005 in the rest of plant without roots is > 20 %. This value is not of significant concern as the procedural recovery data are only a small dataset and the full validation of the analytical method presented in section B5 show acceptable precision data for a larger set of data. No additional consideration or data are required. The limit of quantification was 0.01 mg/kg for all sample materials.

Table 7-147 Procedural recoveries for BAS 684 H and the metabolites M684H005 and M684H006 in oilseed rape

| Analyte   | Portion analysed            | n | Fortification level (mg/kg) | Recovery (%)           |       |      |
|-----------|-----------------------------|---|-----------------------------|------------------------|-------|------|
|           |                             |   |                             | Individual recoveries  | Mean  | RSD  |
| BAS 684 H | Whole plant without roots   | 3 | 0.01                        | 76.3, 91.1, 86.1       | 84.5  | 8.9  |
|           |                             | 3 | 0.1                         | 68.9, 86.6, 82.8       | 79.4  | 11.7 |
|           |                             | 1 | 16                          | 78.9                   | --    | --   |
|           |                             | 7 | overall                     |                        | 81.5  | 9.1  |
|           | Rest of plant without roots | 3 | 0.01                        | 80.0, 84.6, 77.4       | 80.7  | 4.5  |
|           |                             | 3 | 0.1                         | 75.8, 84.8, 75.9       | 78.8  | 6.6  |
|           |                             | 6 | overall                     |                        | 79.8  | 5.2  |
|           | Seed                        | 3 | 0.01                        | 74.8, 71.2, 71.8       | 72.6  | 2.7  |
|           |                             | 3 | 0.1                         | 72.4, 77.3, 87.6       | 79.1  | 9.8  |
|           |                             | 6 | overall                     |                        | 75.9  | 8.2  |
| M684H005  | Whole plant without roots   | 3 | 0.01                        | 95.3, 106*, 92.3       | 97.9  | 7.4  |
|           |                             | 3 | 0.1                         | 101, 90.3*, 83.2       | 91.5  | 9.8  |
|           |                             | 1 | 2.0                         | 88.8                   | --    | --   |
|           |                             | 7 | overall                     |                        | 93.8  | 8.1  |
|           | Rest of plant without roots | 3 | 0.01                        | 109, 94.2, 91.3        | 98.2  | 9.7  |
|           |                             | 3 | 0.1                         | 108, 72.7, 75.2        | 85.3  | 23.1 |
|           |                             | 6 | overall                     |                        | 91.7  | 17.0 |
|           | Seed                        | 3 | 0.01                        | 108, 101, 90.6         | 99.9  | 8.8  |
|           |                             | 3 | 0.1                         | 96.8, 73.1, 85.1       | 85.0  | 13.9 |
|           |                             | 6 | overall                     |                        | 92.4  | 13.0 |
| M684H006  | Whole plant without roots   | 4 | 0.01                        | 87.9, 96.5, 94.0, 81.3 | 89.9  | 7.6  |
|           |                             | 3 | 0.1                         | 89.3, 93.5, 88.9       | 90.6  | 2.8  |
|           |                             | 1 | 2.0                         | 93.4                   | --    | --   |
|           |                             | 8 | overall                     |                        | 90.6  | 5.3  |
|           | Rest of plant without roots | 3 | 0.01                        | 98.2, 93.6, 96.5       | 96.1  | 2.4  |
|           |                             | 3 | 0.1                         | 88.8, 92.4, 93.6       | 91.6  | 2.7  |
|           |                             | 6 | overall                     |                        | 93.9  | 3.5  |
|           | Seed                        | 3 | 0.01                        | 127, 94.5, 96.7        | 106.1 | 17.1 |
|           |                             | 3 | 0.1                         | 80.0, 85.3, 105        | 90.1  | 14.6 |
|           |                             | 6 | overall                     |                        | 98.1  | 17.0 |

\* Mean of two determinations due to dilution of the treated samples (0.01 mg/kg : 113 %, 98.0 % ; 0.1 mg/kg : 94.5 %, 86.0 %)

No residues of BAS 684 H or M684H005 (including M684H006) above the limit of quantitation (0.01 mg/kg) were found in any of the analysed untreated specimens.

The residues found in oilseed rape samples from the individual trials are summarised in Table 7-148. Residues of BAS 684 H in treated whole plant without root specimens collected on the day of the application ranged from 5.7 to 12 mg/kg. At BBCH 51-53 residues declined to < 0.01 – 0.065 mg/kg and further to < 0.01 mg/kg at BBCH 65. Residues in mature seed and rest of plant without roots samples were below the LOQ of 0.01 mg/kg.

Residues of M684H005 (including M684H006) in treated whole plant without root specimens collected on the day of the application ranged from 0.010 to 0.33 mg/kg. At BBCH 51-53 residues were 0.23 – 1.4 mg/kg and declined to < 0.01 – 0.17 mg/kg at BBCH 65. Residues in mature seed and rest of plant without roots samples were below the LOQ of 0.01 mg/kg.

Table 7-148 Residues of BAS 684 H, M684H005 and M684H006 in oilseed rape (trials which support the critical GAP are underlined)

| Report No.<br>Location<br>(EU-region)<br>Trial No                             | Commodity/<br>Variety                         | Date of<br>1. Sowing or<br>planting<br>2. Flowering<br>3. Harvest | Method of<br>treatment | Formulation                                     | Application rate per<br>treatment |               |               | No. of<br>treat-<br>ments<br>and last<br>date | Growth<br>stage at<br>last<br>date | Portion<br>analysed   | DALA <sup>1</sup>           | Residues (mg/kg)                        |   |   |
|---|---|---|------------------------|---|-----------------------------------|---------------|---------------|---|------------------------------------|---|-----------------------------|---|---|---|
|   |   |   |                        |   | kg<br>a.s./hL                     | Water<br>L/ha | kg<br>a.s./ha |   |                                    |   |                             | BAS 684 H                               | Sum of<br>M684H005 and<br>M684H006,<br>expressed as<br>M684H005 | Total <sup>4</sup>                        |
| 741158<br>2017/1219684<br>27449 Mulsum<br>Germany (N)<br>L170029              | SO 0495<br>Oilseed<br>rape<br>DK Imperial     | 1. 31.08.2016<br>2. 18.04.-10.05.2017<br>3. 05.08.2017            | Spray<br>application   | BAS 684 03<br>H<br>(EC)<br>750 g/L<br>BAS 684 H | 0.126                             | 215           | 0.270         | 1<br>14.03.2017                               | 18                                 | Plant <sup>2</sup><br>Plant <sup>2</sup><br>Plant <sup>2</sup><br>Rest <sup>3</sup><br>Seed | 0<br>16<br>50<br>144<br>144 | 6.8<br>0.013<br><0.01<br><0.01<br><0.01 | 0.063<br>0.60<br>0.065<br><0.01<br><0.01                        | 6.8<br>0.38<br>0.049<br><0.016<br><0.016  |
| 741158<br>2017/1219684<br>6675 AD Valburg<br>The Netherlands (N)<br>L170030   | SO 0495<br>Oilseed<br>rape<br>Pt 211          | 1. 09.09.2016<br>2. not reported<br>3. 19.07.2017                 | Spray<br>application   | BAS 684 03<br>H<br>(EC)<br>750 g/L<br>BAS 684 H | 0.127                             | 227           | 0.288         | 1<br>10.03.2017                               | 18                                 | Plant <sup>2</sup><br>Plant <sup>2</sup><br>Plant <sup>2</sup><br>Rest <sup>3</sup><br>Seed | 0<br>14<br>49<br>131<br>131 | 12<br>0.065<br><0.01<br><0.01<br><0.01  | 0.075<br>1.2<br>0.043<br><0.01<br><0.01                         | 12<br>0.79<br>0.036<br><0.016<br><0.016   |
| 741158<br>2017/1219684<br>91150 Mespuits<br>France (N)<br>L170031             | SO 0495<br>Oilseed<br>rape<br>DK<br>Exception | 1. 10.09.2016<br>2. 05.01.-18.05.2017<br>3. 04.07.2017            | Spray<br>application   | BAS 684 03<br>H<br>(EC)<br>750 g/L<br>BAS 684 H | 0.125                             | 184           | 0.230         | 1<br>03.03.2017                               | 19                                 | Plant <sup>2</sup><br>Plant <sup>2</sup><br>Plant <sup>2</sup><br>Rest <sup>3</sup><br>Seed | 0<br>14<br>40<br>123<br>123 | 9.0<br>0.018<br><0.01<br><0.01<br><0.01 | 0.010<br>0.57<br>0.015<br><0.01<br><0.01                        | 9.0<br>0.36<br>0.019<br><0.016<br><0.016  |
| 741158<br>2017/1219684<br>2476 Pázmánd<br>Hungary (N)<br>L170032              | SO 0495<br>Oilseed<br>rape<br>DK Exquisite    | 1. 24.10.2016<br>2. 05.04.-25.04.2017<br>3. 29.06.2017            | Spray<br>application   | BAS 684 03<br>H<br>(EC)<br>750 g/L<br>BAS 684 H | 0.127                             | 213           | 0.271         | 1<br>17.03.2017                               | 18                                 | Plant <sup>2</sup><br>Plant <sup>2</sup><br>Plant <sup>2</sup><br>Rest <sup>3</sup><br>Seed | 0<br>20<br>35<br>109<br>109 | 9.2<br><0.01<br><0.01<br><0.01<br><0.01 | 0.14<br>0.50<br><0.01<br><0.01<br><0.01                         | 9.3<br>0.31<br><0.016<br><0.016<br><0.016 |
| 741158<br>2017/1219684<br>82290 Barry-<br>d'islemade<br>France (S)<br>L170033 | SO 0495<br>Oilseed<br>rape<br>Trezzor         | 1. 24.10.2016<br>2. 05.04.-25.04.2017<br>3. 29.06.2017            | Spray<br>application   | BAS 684 03<br>H<br>(EC)<br>750 g/L<br>BAS 684 H | 0.125                             | 203           | 0.253         | 1<br>03.03.2017                               | 18                                 | Plant <sup>2</sup><br>Plant <sup>2</sup><br>Plant <sup>2</sup><br>Rest <sup>3</sup><br>Seed | 0<br>18<br>42<br>118<br>118 | 9.8<br><0.01<br><0.01<br><0.01<br><0.01 | 0.018<br>0.42<br>0.020<br><0.01<br><0.01                        | 9.8<br>0.26<br>0.022<br><0.016<br><0.016  |
| 741158<br>2017/1219684<br>57018 Melissachori<br>Greece (S)<br>L170034         | SO 0495<br>Oilseed<br>rape<br>SY Cassidy      | 1. 26.09.2016<br>2. 18.04.-19.05.2017<br>3. 23.06.2017            | Spray<br>application   | BAS 684 03<br>H<br>(EC)<br>750 g/L<br>BAS 684 H | 0.125                             | 214           | 0.267         | 1<br>20.03.2017                               | 18                                 | Plant <sup>2</sup><br>Plant <sup>2</sup><br>Plant <sup>2</sup><br>Rest <sup>3</sup><br>Seed | 0<br>9<br>37<br>95<br>95    | 8.1<br>0.025<br><0.01<br><0.01<br><0.01 | 0.17<br>1.4<br>0.17<br><0.01<br><0.01                           | 8.2<br>0.87<br>0.11<br><0.016<br><0.016   |

| Report No.<br>Location<br>(EU-region)<br>Trial No                                | Commodity/<br>Variety                  | Date of<br>1. Sowing or<br>planting<br>2. Flowering<br>3. Harvest | Method of<br>treatment | Formulation                                     | Application rate per<br>treatment |               |               | No. of<br>treat-<br>ments<br>and last<br>date | Growth<br>stage at<br>last<br>date | Portion<br>analysed | DALA <sup>1</sup> | Residues (mg/kg) |   |                    |
|--|--|---|------------------------|---|-----------------------------------|---------------|---------------|---|------------------------------------|---------------------|-------------------|------------------|---|--------------------|
|  |  |   |                        |   | kg<br>a.s./hL                     | Water<br>L/ha | kg<br>a.s./ha |   |                                    |                     |                   | BAS 684 H        | Sum of<br>M684H005 and<br>M684H006,<br>expressed as<br>M684H005 | Total <sup>4</sup> |
| 741158<br>2017/1219684<br>40059 Fossatone di<br>Medicina<br>Italy (S)<br>L170035 | SO 0495<br>Oilseed<br>rape<br>Pulsar   | 1. 25.10.2016<br>2. 07.04.-10.05.2017<br>3. 12.06.2017            | Spray<br>application   | BAS 684 03<br>H<br>(EC)<br>750 g/L<br>BAS 684 H | 0.126                             | 217           | 0.273         | 1<br>02.03.2017                               | 18                                 | Plant <sup>2</sup>  | 0                 | 9.9              | 0.33  | 10                 |
|  |  |   |                        |   |                                   |               |               |   |                                    | Plant <sup>2</sup>  | 19                | <0.01            | 0.23  | 0.15               |
|  |  |   |                        |   |                                   |               |               |   |                                    | Plant <sup>2</sup>  | 36                | <0.01            | 0.025   | 0.025              |
|  |  |   |                        |   |                                   |               |               |   |                                    | Rest <sup>3</sup>   | 102               | <0.01            | <0.01   | <0.016             |
|  |  |   |                        |   |                                   |               |               |   |                                    | Seed                | 102               | <0.01            | <0.01   | <u>&lt;0.016</u>   |
| 741158<br>2017/1219684<br>22193 Arascues<br>Spain (S)<br>L170036                 | SO 0495<br>Oilseed<br>rape<br>Hydromel | 1. 10.09.2016<br>2. 20.03.-02.05.2017<br>3. 19.06.2017            | Spray<br>application   | BAS 684 03<br>H<br>(EC)<br>750 g/L<br>BAS 684 H | 0.125                             | 213           | 0.266         | 1<br>02.03.2017                               | 18                                 | Plant <sup>2</sup>  | 0                 | 5.7              | 0.18  | 5.8                |
|  |  |   |                        |   |                                   |               |               |   |                                    | Plant <sup>2</sup>  | 12                | <0.01            | 0.81  | 0.50               |
|  |  |   |                        |   |                                   |               |               |   |                                    | Plant <sup>2</sup>  | 35                | <0.01            | 0.056   | 0.044              |
|  |  |   |                        |   |                                   |               |               |   |                                    | Rest <sup>3</sup>   | 109               | <0.01            | <0.01   | <0.016             |
|  |  |   |                        |   |                                   |               |               |   |                                    | Seed                | 109               | <0.01            | <0.01   | <0.016             |

1 Days after last application

2 Whole plant without roots

3 Rest of plant without roots

4 Sum of BAS 684 H, M684H005 and M684H006, expressed as BAS 684 H. The conversion factor is 0.606, based on molecular weights (274.4 g/mol / 452.54 g/mol).

Table 7-149 Summary of residue trials on oilseed rape

| Crop                    | Region/<br>Indoor | RD-Mo – BAS 684 H<br>Residue levels (mg/kg)<br>observed in the<br>supervised residue<br>trials relevant to the<br>supported GAPs | RD-RA - Sum of BAS<br>684 H, M684H005 and<br>M684H006, expressed<br>as BAS 684 H<br>Residue levels (mg/kg)<br>observed in the<br>supervised residue<br>trials relevant to the<br>supported GAPs | HR<br>(RD-RA)<br>(mg/kg) | STMR<br>(RD-RA)<br>(mg/kg) |
|-------------------------|-------------------|--|---|--------------------------|----------------------------|
| Oilseed<br>rape<br>seed | NEU<br>Outdoor    | 8 x < 0.01*  | 8 x < 0.016*  | 0.016*                   | 0.016*                     |
| Oilseed<br>rape<br>seed | SEU<br>Outdoor    | 8 x < 0.01*  | 8 x < 0.016*  | -                        | -                          |

Note data from the SEU are not being used in the risk assessment therefore HR and STMR values have not been determined.

#### B.7.4. FEEDING STUDIES

No feeding study is required for ruminants, poultry and pigs and for fish.

The requirements for feeding studies are set out according to Commission Regulation (EU) No 283/2013 with data requirements for active substances, in accordance with Regulation (EC) No 1107/2009 of the European Parliament and of the Council concerning the placing of plant protection products on the market as well as and in OECD guidelines.

Feeding studies are required:

- (1) if metabolism studies indicate that significant residues (above 0.01 mg/kg for each analyte) may occur in any edible animal tissue, considering the residue levels in potential feeding stuff obtained at the 1x dose rate.
- (2) However, feeding studies shall not be required where intake is below 0.004 mg/kg bw/d, except in cases where the residue, namely the active substance, its metabolites or breakdown products, as defined in the residue definition for risk assessment, tends to accumulate.

In the context of this document, feed burden calculations were performed (see Volume 1) using the EU Animal Model 2017 considering only the representative (wheat and barley) and future (oilseed rape) uses and the residues according to the risk assessment residue definition (sum of BAS 684 H, metabolites M684H005 and M684H006, expressed as BAS 684 H).

The resulting maximum dietary burden for all the various livestock species are 0.001 mg/kg bw/d (0.02 mg/kg feed DM).

Thus, for poultry, pigs and cattle, the intakes are not exceeding the trigger value of 0.004 mg/kg bw/d.

Comparing the feed burdens with the metabolism studies on hens and goats overdosing factors of 900 – 940 N for poultry and 300 – 390 N for ruminants have been derived. When the over-dosing factors are applied to the TRR measured in animal feedstuffs in the metabolism studies it shows the residues in all edible animal tissues are expected to be < 0.01 mg/kg at the maximum reasonable worst-case feedburden, see

Table 7-150.

Table 7-150 Extrapolation of residues in ruminant and poultry matrices relevant for consumer exposure

| Matrix      | TRR measured<br>(mg eq/kg) |                   | Extrapolated residues for 1N feed burden<br>(mg eq/kg) |                                |
|-------------|----------------------------|-------------------|--|--------------------------------|
|             | Phenyl label               | Cyclohexane label | Phenyl label   | Cyclohexane label              |
| <b>Goat</b> |                            |                   | <b>(overdosing factor 300)</b>                         | <b>(overdosing factor 390)</b> |
| Milk        | 0.011                      | 0.013             | 0.00004  | 0.00003                        |
| Liver       | 0.690                      | 0.673             | 0.00230  | 0.00173                        |
| Kidney      | 0.372                      | 0.474             | 0.00124  | 0.00122                        |
| Muscle      | 0.012                      | 0.022             | 0.00004  | 0.00006                        |
| Fat         | 0.008                      | 0.019             | 0.00003  | 0.00005                        |
| <b>Hen</b>  |                            |                   | <b>(overdosing factor 900)</b>                         | <b>(overdosing factor 940)</b> |
| Egg white   | 0.063                      | 0.112             | 0.00007  | 0.00012                        |
| Egg yolk    | 0.051                      | 0.071             | 0.00006  | 0.00008                        |
| Muscle      | 0.036                      | 0.079             | 0.00004  | 0.00008                        |
| Fat         | 0.058                      | 0.071             | 0.00006  | 0.00008                        |
| Liver       | 0.183                      | 0.202             | 0.00020  | 0.00021                        |

Therefore, no feeding studies in poultry, ruminants or pigs are necessary.

#### B.7.4.1. Poultry

No study required, see above for further details.

#### B.7.4.2. Ruminants

No study required, see above for further details.

#### B.7.4.3. Pigs

No study required, see above for further details.

#### B.7.4.4. Fish

A fish feeding study is not required. Currently no test method or guidance document is available. Therefore, waiving of this particular data requirement is considered acceptable.

### B.7.5. EFFECTS OF PROCESSING

#### B.7.5.1. Nature of the residue

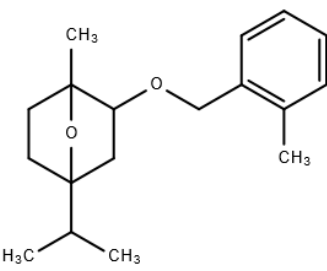
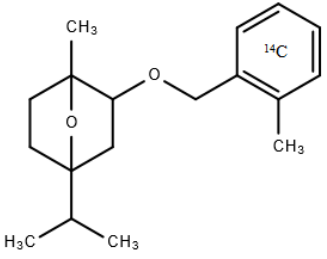
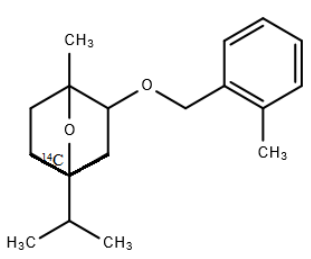
The effect of processing on the nature of the residues of BAS 684 H was investigated using the active substance radiolabelled in the phenyl-U-<sup>14</sup>C and cyclohexane-4-<sup>14</sup>C positions. These labelling positions are considered appropriate to provide sufficient information.

The molecular structures and position of the <sup>13</sup>C/<sup>14</sup>C label in the test items used in the studies are shown in



Table 7-151.

Table 7-151: Structural details of test items

|                     |  |  |
|---------------------|--|--|
| Chemical structure  |   |  |
| Common name         | Cinmethylin  |  |
| IUPAC names         | (1RS, 2R, 4SR)-1,4-epoxy-p-menth-2-yl 2-methylbenzyl-ether (ISO)   |  |
| Radiolabel position | <br>[Phenyl-U- <sup>14</sup> C]-BAS 684 H | <br>[Cyclohexane-4- <sup>14</sup> C]-BAS 684 H |

**Report:**

CA 6.5.1/1  
Wijntjes C. et al., 2016 a  
<sup>14</sup>C BAS 684 H: Simulated processing - Hydrolysis at 90 C, 100 C and 120 C  
2015/1198477

**Guidelines:**

OECD 507 - Nature of the residues in processed commodities - High temperature hydrolysis, EC 1107/2009 of the European Parliament and of the Council of 21 Oct 2009, European Commission Regulation No 283/2013 Part A Section 6

**GLP:**

yes

*Materials and methods**Materials*1. C-label BAS 684 H (CAS No. 87818-31-3)

**Description:** Phenyl-U-<sup>14</sup>C (spec. activity of a.s. 17.1 MBq/mg)

**Lot/Batch #:** 1147-2001

**Radiochemical Purity:** 98.9% on CoA (97.2 % redetermined before use)

2. C-label BAS 684 H (CAS No. 87818-31-3)

**Description:** Cyclohexane-4-<sup>14</sup>C (spec. activity of a.s. 7.75 MBq/mg)

**Lot/Batch #:** 1146-1001

**Radiochemical Purity:** 99.4% on CoA (98.4 % redetermined before use)

*Methods*

A standard hydrolysis study was performed with <sup>14</sup>C-BAS 684 H, labelled either in the phenyl or cyclohexane ring. The conditions in

Table 7-152 were investigated.

Table 7-152: Representative processing conditions studied

| Temperature (°C) | pH | Time (min) | Simulated processing procedure |
|------------------|----|------------|--------------------------------|
| 90               | 4  | 20         | Pasteurisation                 |
| 100              | 5  | 60         | Baking, boiling, brewing       |
| 120              | 6  | 20         | Sterilisation                  |

Aqueous acetate buffer solutions containing the radiolabelled test item at an initial concentration of 0.094 mg/L (phenyl-label) and 0.859 mg/L (cyclohexane-label; prepared by mixing  $^{14}\text{C}$ : $^{12}\text{C}$  in the ratio 1:1 with unlabelled BAS 684 H (96.3 % purity)) were incubated in closed high pressure stainless steel vessels placed in a water bath (pH 4 and 5) or autoclave (pH 6) respectively. As the initial concentration for the phenyl label was lower than 1 mg/L, this affects the triggers for characterisation and identification; and these have been adjusted in the conclusions section below.

At time 0 and after 20 or 60 minutes of incubation, 3 samples per pH value were taken. The temperatures maintained constant throughout incubation time. No significant variation of pH values was observed in the buffer solutions.

Total radioactivity was determined by LSC and the composition of the radioactive residue was analysed by HPLC and TLC co-chromatography with reference item BAS 684 H only.

### Results

The total radioactivity recovery (based on the applied radioactivity) was 95.0-97.6% TAR, 94.6-98.7% TAR and 96.8-103.5% TAR for the pH 4, pH 5, and pH 6 hydrolysis samples, respectively.

The main component detected in the pH 4, pH 5 and pH 6 test vessels was identified as parent BAS 684 H (93.0-101.9% TAR).

Three impurities/degradation products were present in the test solutions prior to hydrolysis which either increased slightly, remained at similar levels or were not detected following hydrolysis. Impurity 1 (phenyl label only) accounted for 1.2 – 1.7 % AR prior to hydrolysis and 1.2 – 2.2 % AR following hydrolysis. Impurity 2 (cyclohexane label only) accounted for 1.2 – 1.4 % AR prior to hydrolysis and 1.0 – 1.8 % AR following hydrolysis. Impurity 3 (cyclohexane label only) accounted for 0.7 – 0.8 % AR prior to hydrolysis and was not detected following hydrolysis. The detailed results are given in

Table 7-153.

Table 7-153: Results of standard hydrolysis study of BAS 684 H

| Process represented                      | Test conditions           | Initial conc.<br>[ <sup>14</sup> C]<br>BAS 684 H<br>(mg/L) | Analyte                     | Recovery<br>(% TAR) |            |                  |              |
|--|---------------------------|--|-----------------------------|---------------------|------------|------------------|--------------|
|  |                           |  |                             | Before hydrolysis   |            | After hydrolysis |              |
|  |                           |  |                             | Mean                | Range      | Mean             | Range        |
| Phenyl-U- <sup>14</sup> C-BAS 684 H      |                           |  |                             |                     |            |                  |              |
| Pasteurisation                           | pH 4,<br>90°C,<br>20 min  | 0.094  | BAS 684 H                   | 98.8                | 98.4-99.3  | 95.8             | 93.8-97.2    |
|  |                           |  | Impurity 1<br>(ca 31.8 min) | 1.2                 | 1.2-1.2    | 1.8              | 1.4-2.2      |
|  |                           |  | Total characterised         | 100                 | -          | 97.6             | -            |
| Baking/<br>brewing/<br>boiling           | pH 5,<br>100°C,<br>60 min | 0.095  | BAS 684 H                   | 98.8                | 98.0-100.0 | 93.0             | 88.5-96.4    |
|  |                           |  | Impurity 1<br>(ca 31.8 min) | 1.2                 | 1.2-1.2    | 1.7              | 1.2-2.2      |
|  |                           |  | Total characterised         | 100                 | -          | 94.6             | -            |
| Sterilisation                            | pH 6,<br>120°C, 20 min    | 0.093  | BAS 684 H                   | 98.3                | 96.5-99.3  | 101.9            | 100.8-103.0  |
|  |                           |  | Impurity 1<br>(ca 31.8 min) | 1.7                 | 1.7-1.7    | 1.6              | 1.5-1.9      |
|  |                           |  | Total characterised         | 100                 | -          | 103.5            | -            |
| Cyclohexane-4- <sup>14</sup> C-BAS 684 H |                           |  |                             |                     |            |                  |              |
| Pasteurisation                           | pH 4,<br>90°C,<br>20 min  | 0.864  | BAS 684 H                   | 98.0                | 97.0-98.7  | 93.4             | 90.3-95.9    |
|  |                           |  | Impurity 2<br>(ca 20.8 min) | 1.2                 | 1.2-1.2    | 1.6              | 1.5-1.8      |
|  |                           |  | Impurity 3<br>(ca 61.4 min) | 0.8                 | 0.8-0.8    | Not detected     | Not detected |
|  |                           |  | Total characterised         | 100                 | -          | 95.0             | -            |
| Baking/<br>brewing/<br>boiling           | pH 5,<br>100°C,<br>60 min | 0.850  | BAS 684 H                   | 98.1                | 96.4-99.5  | 97.3             | 95.0-100.5   |
|  |                           |  | Impurity 2<br>(ca 20.8 min) | 1.2                 | 1.2-1.2    | 1.5              | 1.0-1.8      |
|  |                           |  | Impurity 3<br>(ca 61.4 min) | 0.7                 | 0.7-0.8    | Not detected     | Not detected |
|  |                           |  | Total characterised         | 100                 | -          | 98.7             | -            |
| Sterilisation                            | pH 6,<br>120°C,<br>20 min | 0.863  | BAS 684 H                   | 98.0                | 96.6-98.7  | 95.4             | 94.6-96.7    |
|  |                           |  | Impurity 2<br>(ca 20.8 min) | 1.4                 | 1.4-1.4    | 1.4              | 1.2-1.6      |
|  |                           |  | Impurity 3<br>(ca 61.4 min) | 0.7                 | 0.7-0.7    | Not detected     | Not detected |
|  |                           |  | Total characterised         | 100.1               | -          | 96.8             | -            |

No attempt to further characterise/identify the impurities present was made.

Impurity 1 (phenyl label) was present at a maximum of 1.8 % TAR, 0.002 mg/L. The maximum increase in Impurity 1 was 1.0 % TAR, this is <10% TRR and <0.001 mg/L (considering the dosing is approximately one order of magnitude below the required level), Therefore it is acceptable no further characterisation/identification was performed.

Impurity 2 (cyclohexane label) is present at 0.014 mg/L which slightly exceeds the trigger for characterisation, however given the maximum increase in Impurity 2 was 0.6 % TAR, this is <10% TRR and <0.01 mg/L, it is acceptable no further characterisation/identification was performed.

Impurity 3 (cyclohexane label) was present at a maximum of 0.8 % TAR, 0.007 mg/L. No increase in Impurity 3 was observed. Therefore it is acceptable no further characterisation/identification was performed.

#### *Conclusion*

Three minor impurities/degradation products did not significantly increase under all three hydrolytic conditions.

Under conditions representative of pasteurisation (pH 4, 90 °C, 20 min), baking, boiling, brewing (pH 5, 100 °C, 60 min) and sterilisation (pH 6, 120 °C, 20 min), BAS 684 H is stable.

### **B.7.5.2. Distribution of the residue in peel and pulp**

Not relevant for the representative uses on cereals nor the proposed future uses on oilseeds.

### **B.7.5.3. Magnitude of residues in processed commodities**

Studies on the magnitude of residues in processed commodities are not required as residues were < 0.1 mg/kg in all the RACs analysed in the NEU trials on wheat and oilseed rape, parent BAS 684 H is stable upon processing and no degradation products of toxicological concern are formed upon processing.

### **B.7.6. RESIDUES IN SUCCEEDING OR ROTATIONAL CROPS**

Representative uses on cereals (wheat and barley) and the proposed future use on oilseed rape can be grown in rotation and field soil degradation studies indicate the DT<sub>90</sub> value for BAS 684 H is a maximum of 207.6 days (Volume 3 CA B.8.1) therefore a consideration of residues in rotational crops is required, but there is no potential for accumulation over multiple years of use. There are no major soil metabolites for BAS 684 H and therefore no potential for accumulation of soil metabolites over multiple years of use.

#### **B.7.6.1. Metabolism in rotational crops**

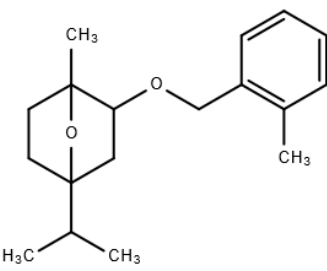
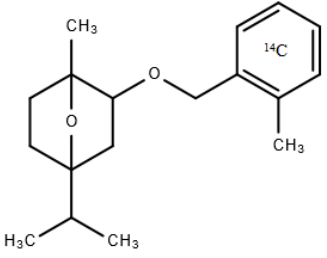
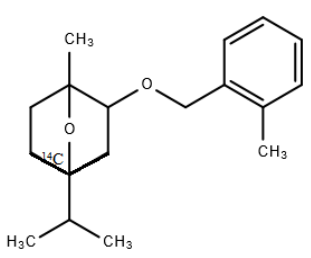
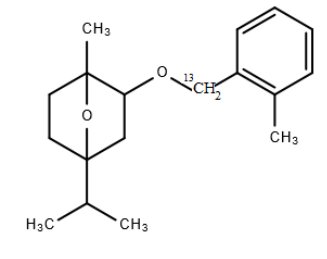
The metabolism and distribution of BAS 684 H in rotational crops (spinach (leafy vegetable group), radish (root and tuber group) and wheat (small grain group)) was investigated using the active substance radiolabelled in the phenyl-U-<sup>14</sup>C and cyclohexane-4-<sup>14</sup>C positions. These labelling positions are considered appropriate to provide sufficient information.

The molecular structures and position of the <sup>13</sup>C/<sup>14</sup>C label in the test items used in the studies are shown in

Table 7-154.



Table 7-154: Structural details of test items

|                                |  |  |
|--------------------------------|--|--|
| Chemical structure             |   |  |
| Common name                    | Cinmethylin  |  |
| IUPAC names                    | (1RS, 2R, 4SR)-1,4-epoxy-p-menth-2yl 2-methylbenzyl-ether (ISO)  |  |
| Radiolabel position            | <br>[Phenyl-U- <sup>14</sup> C]-BAS 684 H | <br>[Cyclohexane-4- <sup>14</sup> C]-BAS 684 H |
| <sup>13</sup> C label position | <br>[Benzyl- <sup>13</sup> C]-BAS 684 H |  |

mg eq/kg

**Report:**

CA 6.6.1/1

Wenzel N. et al., 2018 a

Confined rotational crop study with [<sup>14</sup>C]-BAS 684 H

2016/1321090

**Guidelines:**

EPA 860.1850: Confined Accumulation in Rotational Crops, EPA 860.1000, PMRA Residue Chemistry Guidelines Section 97.13 Confined Accumulation in Rotational Crops (Canada), Lundehn III: 7524/VI/95 Rev. 2 Appendix C (EU) Testing of plant protection products in rotational crops (draft), OECD 502 Metabolism in Rotational Crops (January 2007)

**GLP:**

yes

**Materials and methods***Materials*1. C-label BAS 684 H (CAS No. 87818-31-3)**Description:** Phenyl-U-<sup>14</sup>C (spec. activity of a.s. 17.1 MBq/mg)**Lot/Batch #:** 1147-2001**Radiochemical Purity:** 98.9%**Chemical Purity:** 97.0%

2. C-label BAS 684 H (CAS No. 87818-31-3)

**Description:** Cyclohexane-4-<sup>14</sup>C (spec. activity of a.s. 7.75 MBq/mg)

**Lot/Batch #:** 1146-1001

**Radiochemical Purity:** 99.4%

**Chemical Purity:** 99.3%

3. C-label BAS 684 H (CAS No. 87818-31-3)

**Description:** Benzyl-<sup>13</sup>C-BAS 684 H

**Lot/Batch #:** 1159-1012

**Chemical Purity:** 99.6%

4. BAS 684 H (CAS No. 87818-31-3)

**Description:** Unlabelled BAS 684 H

**Lot/Batch #:** COD-001950

**Chemical Purity:** 96.3%

MethodsTest system

The metabolism and distribution of BAS 684 H in rotational crops was investigated using BAS 684 H radiolabelled in the cyclohexane ring (cyclohexane-label) or in the phenyl ring (phenyl-label) on the following crops:

- Spinach (variety *Corvette F1*) (leafy vegetable group)
- Radish (variety *April Cross*) (root and tuber group)
- Wheat (variety *Thasos*) (small grain group)

The study was carried out in 2015-2018 indoors at the Agricultural Research Centre of BASF SE in Limburgerhof, Germany. Plant back intervals of 30, 120 and 365 days were studied. For each radiolabel, 12 boxes (0.4 m x 0.6 m) were filled with soil (USA: sandy loam; DIN 4220: loamy sand [SI3]) corresponding to a total testing area of 2.88 m<sup>2</sup>.

Applications

For each label, a single spray application of BAS 684 H, as an EC formulation, at a target rate of 500 g a.s./ha (phenyl label actual rate: 503.96 g a.s./ha; cyclohexane label actual rate: 496.69 g a.s./ha) was made to bare soil in a spray volume of 300 L/ha. The application rate for each label (g a.s./ha) corresponds to 1.0 N compared to the representative critical GAPs on barley and wheat (1 x 500 g a.s./ha) and 2.0 N compared to the proposed future critical GAP on oilseed rape (1 x 250 g a.s./ha). The phenyl-labelled test item was applied as two successive spray applications with a total rate of 503.96 g a.s./ha.

**Preparation 1 (phenyl label):** For the preparation of the application formulation of the phenyl label, phenyl-U-<sup>14</sup>C- (dissolved in toluene), benzyl-<sup>13</sup>C- and unlabelled BAS 684 H were mixed to obtain a ratio of approximately 1:1:1 (<sup>13</sup>C was used to produce a specific MS isotopic pattern).

**Preparation 2 (cyclohexane label):** For the preparation of the application formulation of the cyclohexane label, cyclohexane-4-<sup>14</sup>C- (dissolved in toluene) and unlabelled BAS 684 H were mixed in an approximate ratio of 1:1.

The solvents were evaporated and stored in a freezer. On the day of application, the mixtures were taken up in water and blank formulation assisted by ultrasonication. For the phenyl label, methanol was added due to incomplete homogenisation. The purity of the application solution was confirmed using HPLC and the isotopic pattern as well as the identity was determined and verified by HPLC-MS analysis.

Planting

After 30 days of ageing and simulated ploughing of the top 20 cm of soil, the crops were planted or sowed (representing the 30 day PBI). After harvest of the mature crops, the top 20 cm of the soil was mixed before planting of crops again (representing the 120 day PBI). Likewise, after harvest of these crops, the soil was mixed and crops planted (representing the 360 day PBI). The maintenance of the growing crops was performed using normal agricultural practice.

Table 7-155 outlines the crops sown at each plant back interval. The initial soil ageing for 30 days was carried out in a vegetation hall. The cultivation of the crops was conducted in a climatic chamber, glass house and vegetation hall. Boxes which were not replanted and left as bare soil for the second rotation 120 DAT continued in a glass house; and for the third rotation 365 DAT in a vegetation hall.

Table 7-155: Crops sown at each plant back interval

| Box no. | 30 DAT    | 120 DAT   | 365 DAT   |
|---------|-----------|-----------|-----------|
| 1       | Radish    | Wheat     | Spinach   |
| 2       | Spinach   | Wheat     | Radish    |
| 3       | Radish    | Wheat     | Wheat     |
| 4       | Wheat     | Bare soil | Wheat     |
| 5       | Radish    | Spinach   | Wheat     |
| 6       | Spinach   | Radish    | Wheat     |
| 7       | Wheat     | Bare soil | Spinach   |
| 8       | Wheat     | Bare soil | Radish    |
| 9       | Wheat     | Bare soil | Bare soil |
| 10      | Spinach   | Radish    | Bare soil |
| 11      | Bare soil | Spinach   | Bare soil |
| 12      | Bare soil | Wheat     | Bare soil |

### Sampling

The spinach plant population was thinned at growth stage BBCH 15–19, by cutting the leaves above the roots (designated as immature spinach). Mature spinach was harvested similarly at growth stage BBCH 49. The roots of both immature and mature spinach remained in the soil.

Radish plants including roots were harvested at growth stage BBCH 49 and separated into radish leaves and radish roots.

The wheat plant population was thinned at growth stage BBCH 37–39. Two equivalent portions of the removed plants were chopped (designated as wheat forage) or allowed to dry at room temperature for seven days (designated as wheat hay). Mature wheat plants were harvested at growth stage BBCH 89. Ears of wheat and straw were cut off. The straw was chopped and the ears were separated into grain and chaff using a thresher. Chaff and chopped straw were combined (designated as wheat straw).

After application, soil was collected from petri dishes placed at the edge of the boxes and combined to form the “soil after mixing” sample at 0 DAT. At each plant back interval, soil samples were taken from all boxes after ploughing and combined to form the “soil after mixing” sample at 30 DAT, 120 DAT and 365 DAT. For the cyclohexane label at 30 DAT and 120 DAT, at harvest of the crops from every box with wheat plants, small soil samples were taken from the surface and combined to form the “soil (wheat)” sample.

Samples were stored in a freezer at -18°C or below.

### Analysis

**TRR:** The TRR by combustion was determined by combustion analysis. Subsamples of the crop matrices were homogenised with dry ice. Aliquots of homogenised solid plant and soil samples were combusted by means of a sample oxidiser. The resultant  $^{14}\text{CO}_2$  was absorbed, mixed with scintillation fluid and the amount of radioactive residues was determined by LSC.

**Solvent extraction:** The homogenised crop samples were extracted with 3 x methanol and 2 x water using a homogeniser. Subsamples of homogenised soil samples were extracted with 3 x methanol using a mechanical shaker. A further subsample of the soil sample collected at 120 DAT (phenyl label) was extracted with 3 x acetonitrile:water (1:1) and 2 x acetonitrile. After each extraction step, the solid material was separated from the extract by centrifugation followed by filtration with a filter paper. The filtrates of the respective solvent extracts were combined.

The residues after solvent extractions were dried at room temperature and combusted for the determination of the TRR.

Further extraction: The residual radioactive residues after solvent extraction, which contained sufficient amounts of radioactive residues, were subjected to sequential solubilising procedures. The following attempts were made to release additional radioactive residues from selected matrices:

- 2x 1% ammonia
- Macerozyme and cellulose
- Tyrosinase
- $\alpha$ -amylase,  $\beta$ -amylase and amyloglucosidase
- Artificial gastric juice containing pepsin
- Artificial intestinal fluid containing pancreatin

In general, all enzyme incubations were carried out at 37°C. The solubilisates were separated from the remaining solid residues after each step by centrifugation. The radioactive residues in the supernatants were determined by the LSC and the remaining amount of radioactive residues in the final residue was determined by combustion.

Partitioning/SPE-cleanup: For plant samples, aliquots of the methanol extracts were partitioned against ethyl acetate and the ethyl acetate and water phases were collected separately. The ethyl acetate phases containing relevant levels of radioactive residues were either purified by solid phase extraction (SPE) and concentrated or subjected directly to HPLC analysis. Aliquots of the water extract and water phases containing sufficient amounts of radioactive residues were analysed by HPLC either directly or after concentration.

For soil, the methanol extract was directly subjected to HPLC analysis.

Characterisation and identification: Structure elucidation was based on HPLC-MS analysis of isolated fractions of methanol extracts of wheat straw 120 DAT (phenyl label) and of soil 30 DAT (cyclohexane label). Peak assignment for all extracts was based on co-chromatography with the reference item BAS 684 H and comparison of retention times with those of samples analysed by HPLC-MS and reference items: BAS 684 H, M684H014, M684H059, Reg. Nos 545654 and 4108046 (structures in Table 7-1).

There are no major soil metabolites (Volume 3 CA B.8.1). The major plant metabolites M684H005 and M684H006 have not been used as reference standards however there are no significant peaks ( $>0.001$  mg eq/kg) in the chromatograms at the retention times of these metabolites from the primary crop metabolism studies in which the same HPLC system was used.

Attempts were made to identify a component that has been denoted as “peak at 76 min” based on its retention time in HPLC chromatograms used for quantification. Therefore, 1.7 kg of homogenised wheat straw (phenyl label, 120 DAT) was sequentially extracted with methanol and water. Subsamples were consecutively purified by SPE, HPLC fractionation and flash chromatography. Isolated fractions thereof, were analysed by HPLC and/or HPLC-MS and against reference standards BAS 684 H, M684H014, M684H059, Reg. Nos 545654 and 4108046. Further subsamples of the isolated fractions containing the peak of interest were subjected to a derivatisation using pyridine and acetic anhydride or to a cleavage experiment applying  $\beta$ -glucosidase or  $\alpha$ -amylase /  $\beta$ -amylase / amyloglucosidase incubation.

## Results and discussion

### Total radioactive residue (TRR)

The results are presented in Table 7-156 and Table 7-157. There are no significant differences between the TRR measured and TRR calculated for each label and matrix.

For spinach, highest TRRs were detected for the plant back interval 30 DAT ranging from 0.008 mg eq/kg to 0.013 mg eq/kg. The TRR declined to  $\leq 0.007$  mg eq/kg at 120 DAT for both labels and  $\leq 0.005$  mg eq/kg at 365 DAT for both labels.

For radish, TRRs  $>0.010$  mg eq/kg were only determined for radish leaves at 30 DAT (0.013 mg eq/kg, phenyl label) and 365 DAT (0.011 mg eq/kg, phenyl label). In radish roots, the TRR was  $<0.01$  mg eq/kg at all plant back intervals for both radiolabels.

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For wheat, highest TRRs were determined for wheat hay (0.132 mg eq/kg, both labels) and wheat straw (0.088 mg eq/kg phenyl label; 0.061 mg eq/kg cyclohexane label) at 30 DAT. For the phenyl label, the TRR decreased for both matrices accounting for 0.057 mg eq/kg (wheat hay) and 0.058 mg eq/kg (wheat straw) at 120 DAT and 0.060 mg eq/kg (wheat hay) and 0.024 mg eq/kg (wheat straw) at 365 DAT. For the cyclohexane label, the TRR decreased for both matrices accounting for 0.018 mg eq/kg (wheat hay) and 0.020 mg eq/kg (wheat straw) at 120 DAT and was < 0.010 mg eq/kg for both matrices at 365 DAT. TRRs in wheat grain were up to 0.016 mg eq/kg at 30 DAT (cyclohexane label), declining to below 0.01 mg eq/kg at 365 DAT.

For soil, the initial TRR accounted for 2.246 mg eq/kg (phenyl label) and 3.249 mg eq/kg (cyclohexane label) straight after application. During ageing, the TRR decreased to 0.024 mg eq/kg for the phenyl label and 0.020 mg eq/kg for the cyclohexane label at 365 DAT.

Table 7-156: TRR and extractable residues in rotational crop samples (phenyl label)

| Matrix                                    | Days<br>after<br>sowing | TRR<br>measured | TRR <sup>1</sup><br>calculated | Distribution of radioactive residues |             |                     |             |                  |             |                  |             |
|---|-------------------------|-----------------|--------------------------------|--------------------------------------|-------------|---------------------|-------------|------------------|-------------|------------------|-------------|
|   |                         | [mg<br>eq/kg]   | [mg<br>eq/kg]                  | Methanol<br>extract                  |             | Water<br>extract    |             | ERR <sup>2</sup> |             | RRR <sup>3</sup> |             |
|   |                         |                 |                                | [mg<br>eq/kg]                        | [% TR<br>R] | [mg<br>eq/kg]       | [% T<br>RR] | [mg<br>eq/kg]    | [% TR<br>R] | [mg<br>eq/kg]    | [% TR<br>R] |
| Phenyl label                              |                         |                 |                                |                                      |             |                     |             |                  |             |                  |             |
| Initial: 0 DAT                            |                         |                 |                                |                                      |             |                     |             |                  |             |                  |             |
| Soil after mixing                         | --                      | 2.246           | not extracted                  |                                      |             |                     |             |                  |             |                  |             |
| Plant back interval: 30 DAT <sup>4</sup>  |                         |                 |                                |                                      |             |                     |             |                  |             |                  |             |
| Soil after mixing                         | --                      | 0.092           | --                             | 0.031                                | 33.4        | not applied         |             | 0.031            | 33.4        | not analysed     |             |
| Immature spinach                          | 26                      | 0.012           | 0.012                          | 0.005                                | 45.1        | 0.001               | 4.9         | 0.006            | 50.1        | 0.006            | 49.9        |
| Mature spinach                            | 39                      | 0.012           | 0.011                          | 0.006                                | 52.0        | 0.001               | 5.1         | 0.007            | 57.1        | 0.005            | 42.9        |
| Radish leaves                             | 56                      | 0.014           | 0.013                          | 0.006                                | 46.9        | 0.001               | 8.7         | 0.007            | 55.6        | 0.006            | 44.4        |
| Radish roots                              | 56                      | 0.006           | not extracted                  |                                      |             |                     |             |                  |             |                  |             |
| Wheat forage                              | 48                      | 0.029           | 0.028                          | 0.009                                | 32.3        | 0.001               | 4.8         | 0.010            | 37.1        | 0.018            | 62.9        |
| Wheat hay                                 | 48                      | 0.145           | 0.132                          | 0.033                                | 25.2        | 0.012               | 8.8         | 0.045            | 34.0        | 0.087            | 66.0        |
| Wheat straw                               | 104                     | 0.091           | 0.088                          | 0.020                                | 23.0        | 0.010               | 11.2        | 0.030            | 34.2        | 0.058            | 65.8        |
| Wheat grain                               | 104                     | 0.013           | 0.014                          | 0.001                                | 4.6         | 0.001               | 6.3         | 0.001            | 10.9        | 0.012            | 89.1        |
| Plant back interval: 120 DAT <sup>4</sup> |                         |                 |                                |                                      |             |                     |             |                  |             |                  |             |
| Soil after mixing                         | --                      | 0.036           | --                             | 0.003 <sup>5</sup>                   | 8.2         | <0.001 <sup>6</sup> | 0.6         | 0.003            | 8.8         | not analysed     |             |
|   |                         |                 | --                             | 0.002                                | 4.4         | not applied         |             | 0.002            | 4.4         | not analysed     |             |
|   |                         |                 | --                             | 0.002                                | 4.4         | not applied         |             | 0.002            | 4.4         | not analysed     |             |
| Immature spinach                          | 33                      | 0.007           | not extracted                  |                                      |             |                     |             |                  |             |                  |             |
| Mature spinach                            | 48                      | 0.006           | not extracted                  |                                      |             |                     |             |                  |             |                  |             |
| Radish leaves                             | 69                      | 0.005           | not extracted                  |                                      |             |                     |             |                  |             |                  |             |
| Radish roots                              | 69                      | 0.001           | not extracted                  |                                      |             |                     |             |                  |             |                  |             |
| Wheat forage                              | 55                      | 0.006           | not extracted                  |                                      |             |                     |             |                  |             |                  |             |
| Wheat hay                                 | 55                      | 0.059           | 0.057                          | 0.034                                | 58.6        | 0.007               | 12.2        | 0.041            | 70.8        | 0.017            | 29.2        |
| Wheat straw                               | 169                     | 0.062           | 0.058                          | 0.039                                | 66.6        | 0.006               | 9.5         | 0.044            | 76.1        | 0.014            | 23.9        |
|   |                         | 0.050           | not extracted                  |                                      |             |                     |             |                  |             |                  |             |
|   |                         | 0.062           | not extracted                  |                                      |             |                     |             |                  |             |                  |             |
| Wheat grain                               | 169                     | 0.009           | not extracted                  |                                      |             |                     |             |                  |             |                  |             |
| Plant back interval: 365 DAT <sup>4</sup> |                         |                 |                                |                                      |             |                     |             |                  |             |                  |             |
| Soil after mixing                         | --                      | 0.024           | --                             | 0.001                                | 4.2         | not applied         |             | 0.001            | 4.2         | not analysed     |             |
| Immature spinach                          | 28                      | 0.005           | not extracted                  |                                      |             |                     |             |                  |             |                  |             |
| Mature spinach                            | 41                      | 0.005           | not extracted                  |                                      |             |                     |             |                  |             |                  |             |
| Radish leaves                             | 62                      | 0.011           | 0.010                          | 0.007                                | 67.2        | 0.001               | 9.4         | 0.008            | 76.6        | 0.002            | 23.4        |
| Radish roots                              | 62                      | 0.002           | not extracted                  |                                      |             |                     |             |                  |             |                  |             |
| Wheat forage                              | 54                      | 0.011           | 0.011                          | 0.007                                | 61.4        | 0.001               | 5.6         | 0.008            | 67.0        | 0.004            | 33.0        |
| Wheat hay                                 | 54                      | 0.062           | 0.060                          | 0.027                                | 45.5        | 0.010               | 16.1        | 0.037            | 61.7        | 0.023            | 38.3        |
| Wheat straw                               | 112                     | 0.028           | 0.024                          | 0.009                                | 37.1        | 0.003               | 13.7        | 0.012            | 50.8        | 0.012            | 49.2        |
| Wheat grain                               | 112                     | 0.004           | not extracted                  |                                      |             |                     |             |                  |             |                  |             |

1 Total radioactive residue; was calculated as the sum of ERR + RRR

2 Extractable radioactive residue calculated as the sum of the combined methanol extract and the combined water extract

3 Residual radioactive residue

4 Days after treatment

5 Extraction was conducted three times with acetonitrile / water instead of methanol

6 Extraction was conducted two times with acetonitrile instead of water

Table 7-157: TRR and extractable residues in rotational crop samples (cyclohexane label)

| Matrix                                    | Days after sowing | TRR measured | TRR <sup>1</sup> calculated | Distribution of radioactive residues |         |               |         |                  |         |                  |         |
|---|-------------------|--------------|-----------------------------|--------------------------------------|---------|---------------|---------|------------------|---------|------------------|---------|
|   |                   |              |                             | Methanol extract                     |         | Water extract |         | ERR <sup>2</sup> |         | RRR <sup>3</sup> |         |
|   |                   | [mg eq/kg]   | [mg eq/kg]                  | [mg eq/kg]                           | [% TRR] | [mg eq/kg]    | [% TRR] | [mg eq/kg]       | [% TRR] | [mg eq/kg]       | [% TRR] |
| Cyclohexane label                         |                   |              |                             |                                      |         |               |         |                  |         |                  |         |
| Initial: 0 DAT                            |                   |              |                             |                                      |         |               |         |                  |         |                  |         |
| Soil after mixing                         | --                | 3.249        | not extracted               |                                      |         |               |         |                  |         |                  |         |
| Plant back interval: 30 DAT <sup>4</sup>  |                   |              |                             |                                      |         |               |         |                  |         |                  |         |
| Soil after mixing                         | --                | 0.089        | --                          | 0.028                                | 30.9    | not applied   |         | 0.028            | 30.9    | not analysed     |         |
|   |                   |              | --                          | 0.030                                | 33.2    | not applied   |         | 0.030            | 33.2    | not analysed     |         |
| Soil (wheat)                              | --                | 0.056        | --                          | 0.003                                | 5.0     | not applied   |         | 0.003            | 5.0     | not analysed     |         |
| Immature spinach                          | 26                | 0.013        | 0.013                       | 0.005                                | 38.5    | 0.001         | 6.5     | 0.006            | 45.1    | 0.007            | 54.9    |
| Mature spinach                            | 39                | 0.008        | not extracted               |                                      |         |               |         |                  |         |                  |         |
| Radish leaves                             | 56                | 0.010        | 0.009                       | 0.004                                | 47.7    | 0.001         | 8.4     | 0.005            | 56.1    | 0.004            | 43.9    |
| Radish roots                              | 56                | 0.004        | not extracted               |                                      |         |               |         |                  |         |                  |         |
| Wheat forage                              | 48                | 0.026        | 0.025                       | 0.007                                | 28.9    | 0.001         | 4.3     | 0.008            | 33.2    | 0.017            | 66.8    |
| Wheat hay                                 | 48                | 0.141        | 0.132                       | 0.033                                | 25.3    | 0.009         | 6.9     | 0.043            | 32.2    | 0.090            | 67.8    |
| Wheat straw                               | 104               | 0.073        | 0.061                       | 0.014                                | 23.8    | 0.005         | 8.2     | 0.019            | 32.0    | 0.041            | 68.0    |
| Wheat grain                               | 104               | 0.016        | 0.016                       | 0.001                                | 6.5     | 0.001         | 7.8     | 0.002            | 14.4    | 0.014            | 85.6    |
| Plant back interval: 120 DAT <sup>4</sup> |                   |              |                             |                                      |         |               |         |                  |         |                  |         |
| Soil after mixing                         | --                | 0.027        | --                          | 0.002                                | 7.4     | not applied   |         | 0.002            | 7.4     | not analysed     |         |
| Soil (wheat)                              | --                | 0.022        | --                          | 0.001                                | 4.5     | not applied   |         | 0.001            | 4.5     | not analysed     |         |
| Immature spinach                          | 33                | 0.001        | not extracted               |                                      |         |               |         |                  |         |                  |         |
| Mature spinach                            | 48                | 0.002        | not extracted               |                                      |         |               |         |                  |         |                  |         |
| Radish leaves                             | 69                | 0.002        | not extracted               |                                      |         |               |         |                  |         |                  |         |
| Radish roots                              | 69                | 0.001        | not extracted               |                                      |         |               |         |                  |         |                  |         |
| Wheat forage                              | 55                | 0.001        | not extracted               |                                      |         |               |         |                  |         |                  |         |
| Wheat hay                                 | 55                | 0.020        | 0.018                       | 0.003                                | 16.1    | 0.002         | 13.0    | 0.005            | 29.1    | 0.013            | 70.9    |
| Wheat straw                               | 169               | 0.020        | 0.020                       | 0.007                                | 34.8    | 0.003         | 13.6    | 0.010            | 48.3    | 0.010            | 51.7    |
| Wheat grain                               | 169               | 0.012        | 0.013                       | 0.002                                | 16.9    | 0.002         | 16.9    | 0.005            | 33.8    | 0.009            | 66.2    |
| Plant back interval: 365 DAT <sup>4</sup> |                   |              |                             |                                      |         |               |         |                  |         |                  |         |
| Soil after mixing                         | --                | 0.020        | not extracted               |                                      |         |               |         |                  |         |                  |         |
| Immature spinach                          | 28                | 0.001        | not extracted               |                                      |         |               |         |                  |         |                  |         |
| Mature spinach                            | 41                | 0.002        | not extracted               |                                      |         |               |         |                  |         |                  |         |
| Radish leaves                             | 62                | 0.003        | not extracted               |                                      |         |               |         |                  |         |                  |         |
| Radish roots                              | 62                | 0.001        | not extracted               |                                      |         |               |         |                  |         |                  |         |
| Wheat forage                              | 54                | 0.003        | not extracted               |                                      |         |               |         |                  |         |                  |         |
| Wheat hay                                 | 54                | 0.009        | not extracted               |                                      |         |               |         |                  |         |                  |         |
| Wheat straw                               | 112               | 0.006        | not extracted               |                                      |         |               |         |                  |         |                  |         |
| Wheat grain                               | 112               | 0.002        | not extracted               |                                      |         |               |         |                  |         |                  |         |

1 Total radioactive residue; was calculated as the sum of ERR + RRR

2 Extractable radioactive residue calculated as the sum of the combined methanol extract and the combined water extract

3 Residual radioactive residue

4 Days after treatment

*Extraction with solvent*

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For both labels, the extractability of radioactive residues with methanol and water ranged from 29.1 – 76.6% TRR (0.005 – 0.045 mg eq/kg) for immature and mature spinach, radish leaves and roots, wheat forage, hay and straw. For wheat grain, the extractability with methanol/water was lower with 10.9 – 14.4% TRR (0.001 – 0.002 mg eq/kg) at 30 DAT. At 120 DAT, the extractability of wheat grain increased to 33.8% TRR (0.005 mg eq/kg).

The main part of the radioactive residues was generally extracted with methanol, and lower portions were subsequently extracted with water. In wheat grain, considerably lower portions were extractable with methanol compared to the other matrices. The extractability of radioactive residues was similar for the two labels.

For both labels, the residual radioactive residues after solvent extraction (RRR) showed values in the range of 23.4 – 70.9% TRR (0.002 – 0.090 mg eq/kg) in immature lettuce, white radish leaf and spring wheat forage, hay and straw. The RRR in wheat grain amounted to 66.2 – 89.1% TRR (0.009 – 0.014 mg eq/kg).

For soil, the extractability was low ranging from 30.9 – 33.2% TRR (0.003 – 0.031 mg eq/kg) at 30 DAT and decreased to 4.2 % TRR (0.001 mg eq/kg) at 365 DAT. Extraction using acetonitrile instead of methanol had a negligible influence on the outcome.

#### *Further extraction (solubilisation)*

The residues after solvent extraction of all matrices were further characterised using sequential solubilisation procedure applying ammonia treatment and enzymatic cleavage steps. Selected samples were also characterised by consecutive treatment with simulated gastric fluid (pepsin) and simulated intestinal fluid (pancreatin). The results are summarised in **Error! Reference source not found.**



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Table 7-158 – Table 7-160 **Error! Reference source not found..**

In general, the amounts of released radioactive residues were similar in both labels and the main portions of radioactive residues were released in the macerozyme solubilisation steps. For wheat grain, the main portions were released with ammonia ( $\leq 19.4\%$  TRR, 0.003 mg eq/kg), macerozyme ( $\leq 17.5\%$  TRR, 0.003 mg eq/kg) and amylase ( $\leq 29.2\%$  TRR, 0.005 mg eq/kg). The final unextractable residues ( $\leq 0.044$  mg eq/kg (maximum: wheat hay 30 d PBI) or  $\leq 44.8\%$  TRR (maximum: wheat straw 30 d PBI)) are not considered bioavailable, since they are not released upon incubation with artificial gastric juice and artificial intestinal fluid.

Table 7-158: Residues released by further extraction (solubilisation) at 30 day PBI

| Fraction /<br>solubilisate   | Matrix                      |                             |                             |                             |                             |                             |                             |
|--|-----------------------------|-----------------------------|-----------------------------|-----------------------------|-----------------------------|-----------------------------|-----------------------------|
|  | Immature<br>spinach         | Mature<br>spinach           | Radish<br>leaves            | Wheat<br>forage             | Wheat<br>hay                | Wheat<br>straw              | Wheat<br>grain              |
|  | [mg eq/kg]<br>[% TRR]       | [mg eq/kg]<br>[% TRR]       | [mg<br>eq/kg]<br>[% TRR]    | [mg<br>eq/kg]<br>[% TRR]    | [mg<br>eq/kg]<br>[% TRR]    | [mg<br>eq/kg]<br>[% TRR]    | [mg<br>eq/kg]<br>[% TRR]    |
| <b>Phenyl label</b>  |                             |                             |                             |                             |                             |                             |                             |
| <i>RRR after solvent extraction</i>  | <i>0.006</i><br><i>49.9</i> | <i>0.005</i><br><i>42.9</i> | <i>0.006</i><br><i>44.4</i> | <i>0.018</i><br><i>62.9</i> | <i>0.087</i><br><i>66.0</i> | <i>0.058</i><br><i>65.8</i> | <i>0.012</i><br><i>89.1</i> |
| Ammonia solubilisate   | < 0.001<br>2.2              | 0.001<br>7.5                | < 0.001<br>2.1              | 0.001<br>4.3                | 0.006<br>4.7                | 0.005<br>5.2                | 0.003<br>19.4               |
| Macerozyme /<br>cellulase solubilisate   | 0.004<br>31.0               | 0.002<br>18.4               | 0.003<br>22.3               | 0.002<br>6.0                | 0.010<br>7.3                | 0.004<br>4.3                | 0.002<br>17.4               |
| Tyrosinase solubilisate  | < 0.001<br>2.0              | < 0.001<br>0.7              | < 0.001<br>2.1              | 0.001<br>2.3                | 0.003<br>2.3                | 0.002<br>1.7                | < 0.001<br>1.3              |
| Amylase / amyloglucosidase<br>solubilisate                                       | not<br>applied              | not applied                 | not<br>applied              | < 0.001<br>0.9              | 0.001<br>1.1                | 0.001<br>1.1                | 0.003<br>22.8               |
| Pepsin solubilisate  | < 0.001<br>2.0              | < 0.001<br>1.3              | < 0.001<br>1.3              | < 0.001<br>0.5              | 0.001<br>0.6                | 0.001<br>0.7                | < 0.001<br>0.5              |
| Pancreatin solubilisate  | < 0.001<br>0.5              | < 0.001<br>0.2              | < 0.001<br>0.7              | < 0.001<br>0.4              | 0.001<br>0.5                | < 0.001<br>0.3              | < 0.001<br>1.7              |
| <b>Sum of solubilised residue following further<br/>extraction</b>               | <b>0.004</b><br><b>37.6</b> | <b>0.003</b><br><b>28.1</b> | <b>0.004</b><br><b>28.5</b> | <b>0.004</b><br><b>14.5</b> | <b>0.022</b><br><b>16.4</b> | <b>0.012</b><br><b>13.4</b> | <b>0.009</b><br><b>63.1</b> |
| Final unextractable residue <sup>1</sup>   | 0.001<br>7.8                | < 0.001<br>3.8              | 0.001<br>5.7                | 0.009<br>32.7               | 0.044<br>33.8               | 0.039<br>44.8               | 0.001<br>8.1                |
| <i>Sum of solubilised radioactive<br/>residues + final unextractable residue</i> | <i>0.005</i><br><i>45.4</i> | <i>0.004</i><br><i>31.9</i> | <i>0.004</i><br><i>34.2</i> | <i>0.013</i><br><i>47.2</i> | <i>0.066</i><br><i>50.2</i> | <i>0.051</i><br><i>58.2</i> | <i>0.010</i><br><i>71.2</i> |
| <b>Cyclohexane label</b>   |                             |                             |                             |                             |                             |                             |                             |
| <i>RRR after solvent extraction</i>  | <i>0.007</i><br><i>54.9</i> | not<br>extracted            | <i>0.004</i><br><i>43.9</i> | <i>0.017</i><br><i>66.8</i> | <i>0.090</i><br><i>67.8</i> | <i>0.041</i><br><i>68.0</i> | <i>0.014</i><br><i>85.6</i> |
| Ammonia solubilisate   | < 0.001<br>3.4              |                             | 0.001<br>5.6                | < 0.001<br>1.7              | 0.002<br>1.5                | 0.003<br>5.2                | 0.003<br>19.3               |
| Macerozyme /<br>cellulase solubilisate   | 0.004<br>31.7               |                             | 0.001<br>13.2               | 0.003<br>11.8               | 0.009<br>6.8                | 0.002<br>3.9                | 0.003<br>17.5               |
| Tyrosinase solubilisate  | < 0.001<br>2.4              |                             | < 0.001<br>2.7              | 0.001<br>2.8                | 0.003<br>2.4                | 0.001<br>2.2                | < 0.001<br>1.9              |
| Amylase / amyloglucosidase<br>Solubilisate                                       | not<br>applied              |                             | not<br>applied              | < 0.001<br>1.1              | 0.002<br>1.6                | 0.001<br>1.2                | 0.005<br>29.2               |
| Pepsin solubilisate  | < 0.001<br>3.2              |                             | < 0.001<br>4.2              | < 0.001<br>0.8              | 0.001<br>0.8                | < 0.001<br>0.8              | < 0.001<br>0.5              |
| Pancreatin solubilisate  | < 0.001<br>0.5              |                             | < 0.001<br>0.9              | < 0.001<br>0.6              | 0.001<br>0.7                | < 0.001<br>0.4              | < 0.001<br>0.5              |
| <b>Sum of solubilised residue following further<br/>extraction</b>               | <b>0.005</b><br><b>41.2</b> |                             | <b>0.002</b><br><b>26.6</b> | <b>0.005</b><br><b>18.9</b> | <b>0.018</b><br><b>13.8</b> | <b>0.008</b><br><b>13.7</b> | <b>0.011</b><br><b>68.9</b> |
| Final unextractable residue <sup>1</sup>   | 0.001<br>8.8                |                             | 0.001<br>16.0               | 0.008<br>33.2               | 0.042<br>31.6               | 0.026<br>43.1               | 0.001<br>7.6                |
| <i>Sum of solubilised radioactive<br/>residues + final unextractable residue</i> | <i>0.006</i><br><i>50.0</i> |                             | <i>0.004</i><br><i>42.7</i> | <i>0.013</i><br><i>52.1</i> | <i>0.060</i><br><i>45.3</i> | <i>0.034</i><br><i>56.7</i> | <i>0.012</i><br><i>76.4</i> |

<sup>1</sup> The final residues are considered as not being bioavailable, since they are not released upon incubation with artificial gastric juice and artificial intestinal fluid.

Table 7-159: Residues released by further extraction (solubilisation) at 120 day PBI

| Fraction /<br>solubilisate   | Matrix                |                       |                       |
|--|-----------------------|-----------------------|-----------------------|
|  | Wheat<br>hay          | Wheat<br>straw        | Wheat<br>grain        |
|  | [mg eq/kg]<br>[% TRR] | [mg eq/kg]<br>[% TRR] | [mg eq/kg]<br>[% TRR] |
| Phenyl label   |                       |                       |                       |
| RRR after solvent extraction   | 0.017<br>29.2         | 0.014<br>23.9         | not extracted         |
| Ammonia solubilisate   | 0.002<br>2.9          | 0.002<br>3.5          |                       |
| Macerozyme /<br>cellulase solubilisate                                   | 0.004<br>7.4          | 0.001<br>2.2          |                       |
| Tyrosinase solubilisate  | < 0.001<br>0.9        | < 0.001<br>0.7        |                       |
| Amylase / amyloglucosidase<br>solubilisate                               | < 0.001<br>0.3        | < 0.001<br>0.3        |                       |
| Pepsin solubilisate  | < 0.001<br>0.2        | < 0.001<br>0.1        |                       |
| Pancreatin solubilisate  | < 0.001<br>0.3        | < 0.001<br>0.1        |                       |
| Sum of solubilised residue following<br>further extraction               | 0.007<br>11.9         | 0.004<br>6.8          |                       |
| Final unextractable residue  | 0.005<br>8.3          | 0.005<br>9.4          |                       |
| Sum of solubilised radioactive<br>residues + final unextractable residue | 0.012<br>20.3         | 0.009<br>16.2         |                       |
| Cyclohexane label  |                       |                       |                       |
| RRR after solvent extraction   | 0.013<br>70.9         | 0.010<br>51.7         | 0.009<br>66.2         |
| Ammonia solubilisate   | 0.001<br>5.1          | 0.001<br>4.0          | 0.001<br>7.3          |
| Macerozyme /<br>cellulase solubilisate                                   | 0.002<br>11.8         | 0.001<br>6.4          | 0.002<br>11.5         |
| Tyrosinase solubilisate  | < 0.001<br>0.8        | < 0.001<br>1.9        | < 0.001<br>2.3        |
| Amylase / amyloglucosidase<br>solubilisate                               | < 0.001<br>1.2        | < 0.001<br>0.6        | 0.003<br>24.5         |
| Pepsin solubilisate  | < 0.001<br>2.7        | < 0.001<br>0.6        | < 0.001<br>1.3        |
| Pancreatin solubilisate  | < 0.001<br>1.0        | < 0.001<br>0.2        | < 0.001<br>2.4        |
| Sum of solubilised residue following<br>further extraction               | 0.004<br>22.6         | 0.003<br>13.6         | 0.007<br>49.2         |
| Final unextractable residue  | 0.003<br>19.2         | 0.005<br>26.7         | 0.001<br>7.1          |
| Sum of solubilised radioactive<br>residues + final unextractable residue | 0.008<br>41.8         | 0.008<br>40.3         | 0.008<br>56.4         |

Table 7-160: Residues released by further extraction (solubilisation) at 365 day PBI

| Fraction /<br>solubilisate   | Matrix                      |                             |                             |                             |
|--|-----------------------------|-----------------------------|-----------------------------|-----------------------------|
|  | Radish<br>leaves            | Wheat<br>forage             | Wheat<br>hay                | Wheat<br>straw              |
|  | [mg eq/kg]<br>[% TRR]       | [mg eq/kg]<br>[% TRR]       | [mg eq/kg]<br>[% TRR]       | [mg eq/kg]<br>[% TRR]       |
| <b>Phenyl label</b>  |                             |                             |                             |                             |
| <i>RRR after solvent extraction</i>  | <i>0.002</i><br><i>23.4</i> | <i>0.004</i><br><i>33.0</i> | <i>0.023</i><br><i>38.3</i> | <i>0.012</i><br><i>49.2</i> |
| Ammonia solubilisate   | < 0.001<br>2.4              | < 0.001<br>2.2              | 0.004<br>5.8                | 0.002<br>9.4                |
| Macerozyme /<br>cellulase solubilisate   | < 0.001<br>4.6              | 0.001<br>5.3                | 0.002<br>2.9                | 0.001<br>2.2                |
| Tyrosinase solubilisate  | < 0.001<br>1.6              | < 0.001<br>1.1              | 0.001<br>0.9                | < 0.001<br>0.9              |
| Amylase / amyloglucosidase<br>solubilisate                                       | < 0.001<br>0.6              | < 0.001<br>0.8              | < 0.001<br>0.7              | < 0.001<br>0.6              |
| Pepsin solubilisate  | < 0.001<br>0.3              | < 0.001<br>0.2              | < 0.001<br>0.2              | < 0.001<br>0.1              |
| Pancreatin solubilisate  | < 0.001<br>0.5              | < 0.001<br>0.2              | < 0.001<br>0.1              | < 0.001<br>0.2              |
| <b>Sum of solubilised residue following<br/>further extraction</b>               | <b>0.001</b><br><b>10.1</b> | <b>0.001</b><br><b>9.7</b>  | <b>0.006</b><br><b>10.8</b> | <b>0.003</b><br><b>13.2</b> |
| Final unextractable residue  | < 0.001<br>4.3              | 0.002<br>15.0               | 0.011<br>18.8               | 0.006<br>26.6               |
| <i>Sum of solubilised radioactive<br/>residues + final unextractable residue</i> | <i>0.001</i><br><i>14.4</i> | <i>0.003</i><br><i>24.7</i> | <i>0.018</i><br><i>29.6</i> | <i>0.010</i><br><i>39.8</i> |
| <b>Cyclohexane label</b>   |                             |                             |                             |                             |
| not extracted  |                             |                             |                             |                             |

*Identification and characterisation*

A summary of identified and characterised radioactive residues is compiled in Table 7-161 to Table 7-163. **Error! Reference source not found.**

The only identified component was parent BAS 684 H, which was detected in immature spinach (30 DAT, phenyl label), radish leaves (30 DAT, phenyl label), wheat hay (30 DAT, phenyl label), wheat straw (30 DAT, both labels) and wheat straw (120 DAT, both labels). The unchanged parent compound was present at or below 0.002 mg eq/kg or 6.0% TRR.

A component denoted peak at 76 min (based on retention time in HPLC chromatograms used for quantification) was observed at a maximum of 38.3% TRR (0.022 mg eq/kg) in wheat straw at a PBI of 120 d (phenyl label). Significant attempts were made to identify the peak. Therefore, 1.7 kg of homogenised wheat straw (phenyl label, 120 DAT) was sequentially extracted to isolate and analyse the peak at 76 min. However, repeated structure elucidation attempts were of limited success. Comparison against reference standards BAS 684 H, M684H014, M684H059, Reg. Nos 545654 and 4108046 revealed no retention time matches. No significant ions could be detected by HPLC-MS. The isotopic pattern of the initially applied test item of BAS 684 H was not recoverable in the analysed sample hence the peak does not contain the parent molecular structure or fragments thereof. As neither a characteristic isotope pattern nor fragment ions were observed, comparison against identified primary crop metabolites was not performed, as these showed the isotope pattern and fragment ions.

After treatment with acetylating agents (using acetic anhydride and pyridine), no conversion of the peak at 76 min was observed. Enzymatic incubation with  $\beta$ -glucosidase produced a decrease in the peak at 76 min and a new peak at 39 min. Therefore the peak at 76 min may consist of glucosyl-related structures. Treatment of another aliquot with  $\alpha$ -amylase did not show significant conversions; many of the peaks also appear following treatment by buffer solution with no  $\alpha$ -amylase present. No further attempts were made to identify the peak at 39 min given its presence at low absolute levels (approx. 0.003 mg eq/kg).

In total, 0.007-0.059 mg eq/kg or 40.2-79.1% TRR (phenyl label) and 0.008-0.048 mg eq/kg or 36.7-83.2% TRR (cyclohexane label) were identified and characterised, whereby 1-17 peaks (excluding the peak at 76 min) were

characterised, the maximum peak individually accounting for 0.014 mg eq/kg (10.4 % TRR, wheat hay 30 d PBI cyclohexane label) or 29.2 % TRR (0.003 mg eq/kg radish leaves 30 d PBI cyclohexane label). The maximum peak at 0.014 mg eq/kg (10.4 % TRR) in wheat hay at 30 d PBI (cyclohexane label) was found in the water phase following partitioning of the methanol extract at a low retention time using a polar gradient elution (mobile phase A: water:formic acid (1000:1); mobile phase B: acetonitrile:formic acid (1000:1)) and so is concluded to be a polar metabolite. Given this was only observed in wheat hay; it is considered acceptable that further attempts to identify this peak have not been made.

Table 7-161: Summary of identified and characterised components at plant back interval of 30 days

| Designation   | Immature spinach<br>[mg eq/kg]<br>[% TRR] | Mature spinach<br>[mg eq/kg]<br>[% TRR] | Radish leaves<br>[mg eq/kg]<br>[% TRR] | Wheat forage<br>[mg eq/kg]<br>[% TRR] | Wheat hay<br>[mg eq/kg]<br>[% TRR] | Wheat straw<br>[mg eq/kg]<br>[% TRR] | Wheat grain<br>[mg eq/kg]<br>[% TRR] |
|---|---|---|--|---------------------------------------|------------------------------------|--------------------------------------|--------------------------------------|
| <b>Phenyl label</b>   |   |   |  |                                       |                                    |                                      |                                      |
| BAS 684 H   | 0.001<br>6.0                              | not<br>detected                         | < 0.001<br>1.5                         | not<br>detected                       | 0.002<br>1.4                       | 0.001<br>1.2                         | not<br>analysed<br>by HPLC           |
| Total identified  | 0.001<br>6.0                              | --                                      | < 0.001<br>1.5                         | --                                    | 0.002<br>1.4                       | 0.001<br>1.2                         |                                      |
| Peak at 76 min  | not<br>detected                           | not<br>detected                         | not<br>detected                        | not<br>detected                       | 0.002<br>1.2                       | 0.001<br>1.2                         |                                      |
| Maximum other peak  | 0.002<br>19.5                             | 0.002<br>17.3                           | 0.003<br>23.0                          | 0.003<br>9.1                          | 0.011<br>8.5                       | 0.008<br>8.9                         |                                      |
| (Number of other peaks)   | (5)                                       | (2)                                     | (7)                                    | (9)                                   | (9)                                | (17)                                 |                                      |
| [Number of other peaks $\geq$ 0.01 mg eq/kg]                                | [0]                                       | [0]                                     | [0]                                    | [0]                                   | [1]                                | [0]                                  |                                      |
| Total characterised<br>from ERR   | 0.004<br>35.5                             | 0.004<br>38.9                           | 0.006<br>44.1                          | 0.009<br>32.7                         | 0.036<br>27.1                      | 0.023<br>25.6                        | 0.001<br>10.9                        |
| Total characterised<br>from RRR   | 0.004<br>37.6                             | 0.003<br>28.1                           | 0.004<br>28.5                          | 0.004<br>14.5                         | 0.022<br>16.4                      | 0.012<br>13.4                        | 0.009<br>63.1                        |
| Total identified and<br>characterised                                       | 0.009<br>79.1                             | 0.008<br>66.9                           | 0.009<br>74.0                          | 0.013<br>47.2                         | 0.059<br>44.9                      | 0.035<br>40.2                        | 0.010<br>74.0                        |
| Final unextractable residue   | 0.001<br>7.8                              | <0.001<br>3.8                           | 0.001<br>5.7                           | 0.009<br>32.7                         | 0.044<br>33.8                      | 0.039<br>44.8                        | 0.001<br>8.1                         |
| Grand total of identified, characterised and final<br>unextractable residue | 0.010<br>86.9                             | 0.008<br>70.8                           | 0.010<br>79.7                          | 0.022<br>79.9                         | 0.104<br>78.7                      | 0.075<br>85.0                        | 0.011<br>82.2                        |
| <b>Cyclohexane label</b>  |   |   |  |                                       |                                    |                                      |                                      |
| BAS 684 H   | not<br>detected                           | not<br>extracted                        | not<br>detected                        | not<br>detected                       | not<br>detected                    | 0.001<br>1.0                         | not<br>analysed<br>by HPLC           |
| Total identified  | --  |   | --                                     | --                                    | --                                 | 0.001<br>1.0                         |                                      |
| Peak at 76 min  | not<br>detected                           |   | not<br>detected                        | not<br>detected                       | not<br>detected                    | 0.001<br>1.5                         |                                      |
| Maximum other peak  | 0.001<br>10.1                             |   | 0.003<br>29.2                          | 0.003<br>13.1                         | 0.014<br>10.4                      | 0.006<br>9.9                         |                                      |
| (Number of other peaks)   | (2)                                       |   | (1)                                    | (1)                                   | (1)                                | (6)                                  |                                      |
| [Number of other peaks $\geq$ 0.01 mg eq/kg]                                | [0]                                       |   | [0]                                    | [0]                                   | [1]                                | [0]                                  |                                      |
| Total characterised<br>from ERR   | 0.004<br>31.2                             |   | 0.005<br>55.4                          | 0.006<br>25.4                         | 0.030<br>22.9                      | 0.015<br>24.7                        | 0.002<br>14.4                        |
| Total characterised<br>from RRR   | 0.005<br>41.2                             |   | 0.002<br>26.6                          | 0.005<br>18.9                         | 0.018<br>13.8                      | 0.008<br>13.7                        | 0.011<br>68.9                        |
| Total identified and<br>characterised                                       | 0.009<br>72.4                             |   | 0.008<br>82.1                          | 0.011<br>44.3                         | 0.048<br>36.7                      | 0.024<br>39.4                        | 0.013<br>83.2                        |
| Final unextractable residue   | 0.001<br>8.8                              |   | 0.001<br>16.0                          | 0.008<br>33.2                         | 0.042<br>31.6                      | 0.026<br>43.1                        | 0.001<br>7.6                         |
| Grand total of identified, characterised and final<br>unextractable residue | 0.010<br>81.2                             |   | 0.009<br>98.1                          | 0.019<br>77.5                         | 0.090<br>68.2                      | 0.050<br>82.5                        | 0.014<br>90.8                        |

Table 7-162: Summary of identified and characterised components in rotational crop matrices at plant back interval of 120 days

| Designation  | Wheat hay            |         | Wheat straw |         | Wheat grain          |         |
|--|----------------------|---------|-------------|---------|----------------------|---------|
|  | [mg eq/kg]           | [% TRR] | [mg eq/kg]  | [% TRR] | [mg eq/kg]           | [% TRR] |
| Phenyl label   |                      |         |             |         |                      |         |
| BAS 684 H  | not detected         |         | 0.001       | 1.2     | not extracted        |         |
| Total identified   | --                   |         | 0.001       | 1.2     |                      |         |
| Peak at 76 min   | 0.013                | 23.3    | 0.022       | 38.3    |                      |         |
| Maximum other peak   | 0.006                | 9.8     | 0.005       | 9.0     |                      |         |
| (Number of other peaks)  | (10)                 | (10)    | (8)         | (8)     |                      |         |
| [Number of other peaks ≥ 0.01 mg eq/kg]                                  | [0]                  | [0]     | [0]         | [0]     |                      |         |
| Total characterised from ERR   | 0.033                | 58.5    | 0.041       | 70.0    |                      |         |
| Total characterised from RRR   | 0.007                | 11.9    | 0.004       | 6.8     |                      |         |
| Total identified and characterised                                       | 0.040                | 70.4    | 0.046       | 78.1    |                      |         |
| Final unextractable residue  | 0.005                | 8.3     | 0.005       | 9.4     |                      |         |
| Grand total of identified, characterised and final unextractable residue | 0.045                | 78.7    | 0.051       | 87.5    |                      |         |
| Cyclohexane label  |                      |         |             |         |                      |         |
| BAS 684 H  | not analysed by HPLC |         | < 0.001     | 1.3     | not analysed by HPLC |         |
| Total identified   |                      |         | < 0.001     | 1.3     |                      |         |
| Peak at 76 min   |                      |         | 0.002       | 7.6     |                      |         |
| Maximum other peak   |                      |         | 0.001       | 5.3     |                      |         |
| (Number of other peaks)  |                      |         | (2)         | (2)     |                      |         |
| [Number of other peaks ≥ 0.01 mg eq/kg]                                  | [0]                  | [0]     |             |         |                      |         |
| Total characterised from ERR   | 0.005                | 29.1    | 0.006       | 28.3    | 0.005                | 33.8    |
| Total characterised from RRR   | 0.004                | 22.6    | 0.003       | 13.6    | 0.007                | 49.2    |
| Total identified and characterised                                       | 0.009                | 51.7    | 0.009       | 43.2    | 0.011                | 83.0    |
| Final unextractable residue  | 0.003                | 19.2    | 0.005       | 26.7    | 0.001                | 7.1     |
| Grand total of identified, characterised and final unextractable residue | 0.013                | 70.9    | 0.014       | 69.9    | 0.012                | 90.2    |

Table 7-163: Summary of identified and characterised components in rotational crop matrices at plant back interval of 365 days

| Designation  | Radish leaves |         | Wheat forage |         | Wheat hay    |         | Wheat straw  |         |
|--|---------------|---------|--------------|---------|--------------|---------|--------------|---------|
|  | [mg eq/kg]    | [% TRR] | [mg eq/kg]   | [% TRR] | [mg eq/kg]   | [% TRR] | [mg eq/kg]   | [% TRR] |
| <b>Phenyl label</b>  |               |         |              |         |              |         |              |         |
| BAS 684 H  | not detected  |         | not detected |         | not detected |         | not detected |         |
| Total identified   | --            |         | --           |         | --           |         | --           |         |
| Peak at 76 min   | not detected  |         | not detected |         | 0.011        | 18.6    | 0.001        | 5.4     |
| Maximum other peak   | 0.002         | 24.3    | 0.002        | 13.2    | 0.008        | 12.6    | 0.003        | 12.0    |
| (Number of other peaks)  | (4)           | (4)     | (5)          | (5)     | (9)          | (9)     | (7)          | (7)     |
| [Number of other peaks $\geq$ 0.01 mg eq/kg]                             | [0]           | [0]     | [0]          | [0]     | [0]          | [0]     | [0]          | [0]     |
| Total characterised from ERR   | 0.006         | 62.2    | 0.006        | 53.8    | 0.034        | 55.8    | 0.011        | 44.4    |
| Total characterised from RRR   | 0.001         | 10.1    | 0.001        | 9.7     | 0.006        | 10.8    | 0.003        | 13.2    |
| Total identified and characterised                                       | 0.007         | 72.3    | 0.007        | 63.6    | 0.040        | 66.6    | 0.014        | 57.6    |
| Final unextractable residue  | <0.001        | 4.3     | 0.002        | 15.0    | 0.011        | 18.8    | 0.006        | 26.6    |
| Grand total of identified, characterised and final unextractable residue | 0.008         | 76.6    | 0.009        | 78.5    | 0.051        | 85.4    | 0.020        | 84.2    |
| <b>Cyclohexane label</b>   |               |         |              |         |              |         |              |         |
| not extracted  |               |         |              |         |              |         |              |         |

*Storage stability*

The rotational crop matrices were extracted 13 – 197 days after sampling and extracts analysed 7 – 696 days after extraction as summarised in Table 7-164. As the period between sampling and analysis was 32 – 715 days, matrix and extract stability experiments were performed for representative matrices of wheat hay 30 DAT (both labels) and wheat straw 30 DAT (both labels). Matrix storage for up to 737 days and extract storage up to 748 days were investigated.

Storage stability was not investigated for spinach and radish matrices. As the extractable residues were < 0.01 mg eq/kg, TRRs only exceeded 0.01 mg eq/kg (maximum TRR measured of 0.014 mg eq/kg for radish leaves at 30 DAT) and only parent BAS 684 H was identified, this is not considered a major deficiency.

Table 7-164: Storage stability durations

| Matrix                   | Samples used in rotational crop study |                                | Stability experiments         |                                |
|--------------------------|---------------------------------------|--------------------------------|-------------------------------|--------------------------------|
|                          | Maximum matrix storage (days)         | Maximum extract storage (days) | Maximum matrix storage (days) | Maximum extract storage (days) |
| <b>Phenyl label</b>      |                                       |                                |                               |                                |
| Immature spinach         | 195                                   | 510                            | Not investigated              |                                |
| Mature spinach           | 192                                   | 508                            |                               |                                |
| Radish leaves            | 84                                    | 642                            |                               |                                |
| Wheat forage             | 83                                    | 322                            |                               |                                |
| Wheat hay                | 34                                    | 370                            | 734                           | 725                            |
| Wheat straw              | 36                                    | 683                            | 731                           | 727                            |
| <b>Cyclohexane label</b> |                                       |                                |                               |                                |
| Immature spinach         | 197                                   | 512                            | Not investigated              |                                |
| Radish leaves            | 195                                   | 519                            |                               |                                |
| Wheat forage             | 57                                    | 652                            |                               |                                |
| Wheat hay                | 13                                    | 696                            | 737                           | 731                            |
| Wheat straw              | 61                                    | 343                            | 685                           | 733                            |

Liquid-liquid extractions of the methanol extracts were performed and the resulting ethyl acetate phases were purified by SPE resulting in purified ethyl acetate and water phases for investigation.

Chromatograms of water phases before and after storage of matrices or extracts were comparable.

Chromatograms of ethyl acetate phases generally showed inhomogeneous peak patterns before and after storage of matrices and extracts. However, some components (e.g. peak at 76 min and BAS 684 H) were detected in only one of the two labels even though label specificity is not expected (see wheat hay 30 DAT). Additionally, some components (e.g. peak at 76 min and BAS 684 H) were detected after storage but not in corresponding samples before storage (wheat hay 30 DAT, phenyl label) even though both components were detected in the initial extracts of the cyclohexane-labelled matrix (wheat hay 30 DAT, cyclohexane label).

The inhomogeneities may be due to the significant amount of extraction in the workup procedure, which will have had a pronounced effect due to the small quantities involved (Table 7-165), rather than degradation upon storage. The water phases were not purified by SPE prior to analysis and no inhomogeneity was observed before and after storage.

Table 7-165: Residues in ethyl acetate matrix and extract stability samples

| Matrix at 30 DAT  | Before or after storage | Ethyl acetate phase Matrix stability sample |       | Ethyl acetate phase Extract stability sample |       |
|-------------------|-------------------------|---|-------|--|-------|
|                   |                         | mg eq/kg                                    | % TRR | mg eq/kg                                     | % TRR |
| Phenyl label      |                         |   |       |  |       |
| Wheat hay         | Before                  | 0.006                                       | 4.3   | 0.006  | 4.3   |
|                   | After                   | 0.006                                       | 4.1   | 0.011  | 8.7   |
| Wheat straw       | Before                  | 0.009                                       | 10.5  | Not analysed                                 |       |
|                   | After                   | 0.006                                       | 6.1   |  |       |
| Cyclohexane label |                         |   |       |  |       |
| Wheat hay         | Before                  | 0.008                                       | 5.8   | 0.008  | 5.8   |
|                   | After                   | 0.006                                       | 4.6   | 0.012  | 8.9   |
| Wheat straw       | Before                  | 0.006                                       | 9.4   | Not analysed                                 |       |
|                   | After                   | 0.006                                       | 7.6   |  |       |

It was demonstrated for both the ethyl acetate and the water phases that the radioactivity is distributed among several components, each accounting for <0.01 mg eq/kg and <10% TRR.

Therefore it is concluded that sample integrity was maintained for the storage intervals in the study.

#### *Conclusion and metabolic pathway*

Low to moderate translocation of radioactive residues from soil into the plants was observed. The TRRs in rotational crop matrices showed similar levels for the two labels and was generally low for all samples being below or equal to 0.132 mg eq/kg for both labels.

For both labels, the extractability of radioactive residues with methanol and water ranged from 29.1 – 76.6% TRR (0.005 – 0.045 mg eq/kg) for immature and mature spinach, radish leaves and roots, wheat forage, hay and straw. For wheat grain, the extractability with methanol/water was lower with 10.9 – 14.4% TRR (0.001 – 0.002 mg eq/kg) at 30 DAT. At 120 DAT, the extractability of wheat grains increased to 33.8% TRR (0.005 mg eq/kg).

The residues after solvent extraction of all matrices were further characterised using a sequential solubilisation procedure applying ammonia treatment and enzymatic cleavage steps. Selected samples were also characterised by consecutive treatment with simulated gastric fluid (pepsin) and simulated intestinal fluid (pancreatin). In general, the amounts of released radioactive residues were similar in both labels and the main portions of radioactive residues were released in the macerozyme solubilisation steps. For wheat grain, the main portions were released with ammonia ( $\leq 19.4\%$  TRR, 0.003 mg eq/kg), macerozyme ( $\leq 17.5\%$  TRR, 0.003 mg eq/kg) and amylase ( $\leq 29.2\%$  TRR, 0.005 mg eq/kg). The final unextractable residues ( $\leq 0.044$  mg eq/kg (maximum: wheat hay 30 d PBI) or  $\leq 44.8\%$  TRR (maximum: wheat straw 30 d PBI)) are not considered bioavailable, since they are not released upon incubation with artificial gastric juice and artificial intestinal fluid.

The only identified component was parent BAS 684 H, which was detected in immature spinach (30 DAT, phenyl label), radish leaves (30 DAT, phenyl label), wheat hay (30 DAT, phenyl label), wheat straw (30 DAT, both labels)



and wheat straw (120 DAT, both labels). The unchanged parent compound was present at or below 0.002 mg eq/kg or 6.0% TRR.

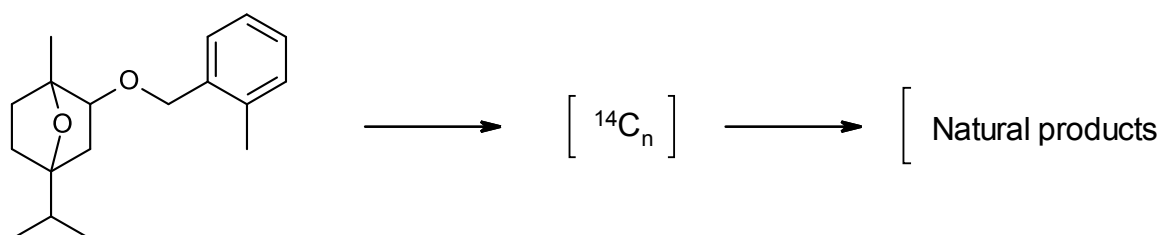
Other than a peak at 76 min (discussed below), the other peaks individually accounted for up to 0.014 mg eq/kg (10.4 % TRR, wheat hay 30 d PBI cyclohexane label) or 29.2 % TRR (0.003 mg eq/kg radish leaves 30 d PBI cyclohexane label). The maximum peak at 0.014 mg eq/kg (10.4 % TRR) in wheat hay at 30 d PBI (cyclohexane label) was found in the water phase following partitioning of the methanol extract at a low retention time using a polar gradient elution and so is concluded to be a polar metabolite. Given this was only observed in wheat hay; it is considered acceptable that further attempts to identify this peak have not been made.

A component denoted peak at 76 min (based on retention time in HPLC chromatograms used for quantification) was observed at a maximum of 38.3% TRR (0.022 mg eq/kg) in wheat straw at a PBI of 120 d (phenyl label). Significant attempts were made to identify the peak. Therefore, 1.7 kg of homogenised wheat straw (phenyl label, 120 DAT) was sequentially extracted to isolate and analyse the peak at 76 min. However, repeated structure elucidation attempts were of limited success. No significant ions could be detected by HPLC-MS. The isotopic pattern of the initially applied test item of BAS 684 H was not recoverable in the analysed sample hence the peak does not contain the parent molecular structure or fragments thereof. After treatment with acetylating agents (using acetic anhydride and pyridine), no conversion of the peak was observed. Enzymatic incubation with  $\beta$ -glucosidase produced a decrease in the peak at 76 min and a new peak at 39 min. Therefore the peak at 76 min may consist of glucosyl-related structures. Treatment of another aliquot with  $\alpha$ -amylase did not show significant conversions; many of the peaks also appear following treatment by buffer solution with no  $\alpha$ -amylase present.

Given the peak at 76 min does not share any MS fragments with the applied BAS 684 H test item, the aerobic soil metabolism study shows BAS 684 H is extensively degraded to numerous small polar molecules (Volume 3 CA B.8.1) and the peak is not observed in the primary crop metabolism studies, it is concluded that the peak at 76 min is likely to be formed in crops after uptake of small polar fragments from the soil and further metabolised in the crop e.g. conjugation with glucose. Therefore the peak at 76 min can be characterised as a natural endogenous compound (including glucosyl conjugates).

In conclusion, BAS 684 H is extensively metabolised in the rotational crop study after application to bare soil. The proposed metabolic pathway of BAS 684 H in rotational crops is shown in Figure 7-23.

Figure 7-23: Proposed metabolic pathway of BAS 684 H in rotational crops



#### B.7.6.2. Magnitude of residues in rotational crops

Studies investigation the magnitude of residues in rotational crops are not required as no components of the residue were identified at  $\geq 0.01$  mg/kg in the confined rotational crop study.

**B.7.7. OTHER STUDIES****B.7.7.1. Effect on the residue level in pollen and bee products**

At the date of submission (22/6/2018) there were no agreed EU guidance documents or test methods to address these data requirements. Since submission the Technical guidelines for determining the magnitude of pesticide residues in honey and setting Maximum Residue Levels in honey (SANTE/11956/2016 rev. 9) have been noted with an agreed implementation date of 1<sup>st</sup> January 2020.

The applicant has submitted the information below based on a draft version of the guidelines. The following use on oilseed rape is requested:

Table 7-166 Requested GAPs and critical GAP (in bold)

| Use-No.  | Member states/zones | Crop                       | Application   |                                    |                        |                                  | PHI (days)  |
|----------|---------------------|----------------------------|---------------|------------------------------------|------------------------|----------------------------------|-------------|
|          |                     |                            | Method / kind | Growth stage of crop               | Number of applications | Rate per application (g a.s./ha) |             |
| 1        | UK                  | Winter oilseed rape        | Spray         | Pre-emergence (BBCH 00-08)         | 1                      | 250                              | n.a.        |
| <b>2</b> | <b>UK</b>           | <b>Winter oilseed rape</b> | <b>Spray</b>  | <b>Post-emergence (BBCH 09-18)</b> | <b>1</b>               | <b>250</b>                       | <b>n.a.</b> |

HSE agrees with the applicant's statement that the future proposed use on oilseed rape (see Table 7-166) with application at post-emergence before BBCH 18 is considered to be the worst case GAP for honey residues. The information/data provided confirms that residues of sum of BAS 684 H, M684H005 and M684H006, expressed as BAS 684 H in aerial parts of the crop are likely to be <0.20 mg/kg at flowering based upon the proposed use on oilseed rape. Assuming a 1:1 transfer between aerial parts of the crop the worst case residues expected in honey are 0.20 mg/kg. Residues of BAS 684 H in the aerial parts of the crop are likely to be < 0.01 mg/kg at flowering based upon the future proposed use on oilseed rape.

The acute and chronic intakes based on these worst case residues are expected to be < 0.3 mg/kg bw (ARfD) and < 0.08 mg/kg bw/day (ADI) PRIMo. It is noted that the applicant has based the assessment on both NEU and SEU trials data, however risk assessment in other areas have only considered the NEU data to support the use in the UK.

As at the date of submission (22/6/2018) there were no agreed EU guidance documents or test methods to address these data requirements, the submission has not been critically evaluated, although it is noted that no significant risk to consumers based on the future proposed use on oilseed rape exists.

The trials on oilseed rape are considered worst-case compared to the representative uses on wheat and barley therefore residues of BAS 684 H in honey based on the representative uses are expected to be <0.01 mg/kg. Given a monitoring method for BAS 684 H in honey is available with an LOQ of 0.01 mg/kg (method L0337/03, Vol 3 CA B5.2.2), the MRL for BAS 684 H in honey is proposed at approval at 0.01\* mg/kg. This MRL would also accommodate the future proposed use on oilseed rape given residues of BAS 684 H in aerial parts of the crop are likely to be <0.01 mg/kg at flowering.

The applicant's submission is presented below.

*"The objective of these studies would be to determine the residue level in pollen and bee products important for human consumption. In principle, residues could be taken up by honeybees from crops during blossom. Of the representative uses supported in the present dossier, oilseed rape is generally important for honey production.*

*At the ~~moment~~ time of dossier submission, no validated test method or finalized guidance document was available for this data requirement, so in principal it could be waived in accordance with SANCO Guidance Document SANCO/10181/2013-rev 2.1 (13 May 2013); thus, no special study has been performed.*

*The only available document for addressing this requirement was the working document SANTE/11956/2016 rev. 6 from Nov 20<sup>th</sup>, 2017 "Draft Guidelines determining the magnitude of pesticide residues in bee products and setting*

specific Maximum Residue Levels in honey”, now replaced by the final document SANTE/11956/2016, rev.9 from Sep 14<sup>th</sup>, 2018 “Technical guidelines for determining the magnitude of pesticide residues in honey and setting Maximum Residue Levels in honey”. Based on the decision scheme in Appendix I of this document an assessment of the possible residues in bee products and the risk for the consumer by the intake of pollen or bee products such as honey is provided in this chapter.

#### **Step 1: Are residues expected in honey after pesticides application**

In this step the attractiveness to bees and melliferous capacity of the crops under consideration has to be considered: according to the table provided in Appendix II of the working document, of the representative uses wheat and oilseed rape, only oilseed rape is considered to have melliferous capacity.

Wheat is not considered as an attractive crop for bees (see also USDA, 2015, Attractiveness of Agricultural Crops to Pollinating Bees for the Collection of Nectar and/or Pollen). Wheat flowers do not produce nectar and consequently wheat is no nectar source for honey bees. Also, wheat is normally pollinated by wind and honey bees are usually not foraging cereal pollen as it is of minor nutritional value.

The other aspect to be considered here is the timepoint of application and possible systemicity of the active ingredient. The intended use of BAS 684 H in oilseed rape only foresees an application at a very early stage of leaf development (until max BBCH 18), well before flowering. However, as long as a translocation of residues into flowers cannot be fully excluded, the assessment proceeds to the next step.

#### **Step 2: Data on residue levels in aerial parts of the crop available?**

Residue data in aerial parts of oilseed rape is available from a complete data package with 8 trials for each EU-N and EU-S for samplings of whole plants without roots at the attractive period of the crop (i.e. flowering, BBCH 65). Residues of parent BAS 684 H, metabolites M684H005 and M684H006 and total residues of BAS 684 H plus M684H005 and M684H006 (expressed as BAS 684 H) are shown in [Table 7-167](#).

The data show that the residues according to the proposed risk assessment residue definition for plants (sum of BAS 684 H, M684H005, M684H006, expressed as BAS 684 H) range from <0.016 to 0.20 mg/kg, and thus are well below 0.5 mg/kg. Residues of BAS 684 H, the relevant compound for the residue definition for MRL setting, are consistently <0.01 mg/kg.

According to the decision scheme in Appendix I of the working document, a default MRL in honey can be set at the LOQ or 0.05 mg/kg, if residues in aerial part of the crop are <0.05 mg/kg.

When considering the proposed residue definition for MRL setting, a default MRL of 0.01 mg/kg or 0.05 mg/kg could be established. While applying the residue definition proposed for risk assessment would result in a calculation of a specific MRL of 0.3 mg/kg in honey based on the residues in aerial parts. As both versions result in MRL proposals <0.5 mg/kg, further calculations are based on the proposed residue definition for MRL setting.

*Table 7-167: Summary of residues in oilseed rape after application of BAS 684 02 H (EC) or BAS 684 03 H (EC)*

| <b>Trial Nos. (Country)</b> | <b>Matrix</b>            | <b>DALA</b> | <b>Growth Stage<sup>1</sup></b> | <b>BAS 684 H [mg/kg]</b> | <b>Sum of M684H005 and M684H006, expressed as M684H005 [mg/kg]</b> | <b>Sum, expressed as BAS 684 H<sup>3</sup> [mg/kg]</b> |
|-----------------------------|--------------------------|-------------|---------------------------------|--------------------------|--|--|
| <b>Oilseed rape</b>         |                          |             |                                 |                          |  |  |
| <b>North EU</b>             |                          |             |                                 |                          |  |  |
| L160024 (Germany)           | Whole plant <sup>2</sup> | 22          | 65                              | <0.01                    | 0.32   | 0.20   |
| L160025 (France)            | Whole plant <sup>2</sup> | 34          | 65                              | <0.01                    | <0.01  | <0.016   |
| L160026 (The Netherlands)   | Whole plant <sup>2</sup> | 36          | 65                              | <0.01                    | 0.11   | 0.077  |
| L160027 (United Kingdom)    | Whole plant <sup>2</sup> | 69          | 65                              | <0.01                    | <0.01  | <0.016   |
| L170029 (Germany)           | Whole plant <sup>2</sup> | 50          | 65                              | <0.01                    | 0.065  | 0.049  |
| L170030 (The Netherlands)   | Whole plant <sup>2</sup> | 49          | 65                              | <0.01                    | 0.043  | 0.036  |
| L170031 (France)            | Whole plant <sup>2</sup> | 40          | 65                              | <0.01                    | 0.015  | 0.019  |
| L170032 (Hungary)           | Whole plant <sup>2</sup> | 35          | 65                              | <0.01                    | <0.01  | <0.016   |
| <b>South EU</b>             |                          |             |                                 |                          |  |  |

Table 7-167: Summary of residues in oilseed rape after application of BAS 684 02 H (EC) or BAS 684 03 H (EC)

| Trial Nos. (Country) | Matrix                   | DALA | Growth Stage <sup>1</sup> | BAS 684 H [mg/kg] | Sum of M684H005 and M684H006, expressed as M684H005 [mg/kg] | Sum, expressed as BAS 684 H <sup>3</sup> [mg/kg] |
|----------------------|--------------------------|------|---------------------------|-------------------|---|--|
| L160028 (Italy)      | Whole plant <sup>2</sup> | 47   | 65                        | <0.01             | <0.01   | <0.016   |
| L160029 (Greece)     | Whole plant <sup>2</sup> | 44   | 65                        | <0.01             | 0.081   | 0.059  |
| L160030 (Italy)      | Whole plant <sup>2</sup> | 52   | 65                        | <0.01             | <0.01   | <0.016   |
| L160031 (Spain)      | Whole plant <sup>2</sup> | 32   | 65                        | <0.01             | 0.067   | 0.051  |
| L170033 (France)     | Whole plant <sup>2</sup> | 42   | 65                        | <0.01             | 0.020   | 0.022  |
| L170034 (Greece)     | Whole plant <sup>2</sup> | 37   | 65                        | <0.01             | 0.17  | 0.11   |
| L170035 (Italy)      | Whole plant <sup>2</sup> | 36   | 65                        | <0.01             | 0.025   | 0.025  |
| L170036 (Spain)      | Whole plant <sup>2</sup> | 35   | 65                        | <0.01             | 0.056   | 0.044  |

1 Growth stage at application

2 Without roots

3 For calculation of parent equivalents, M684H005 residue are multiplied with the conversion factor of 0.606.

The theoretical MRL calculation for honey was performed with the EU-OECD MRL calculator (2015) applying the total residue values from the proposed residue definition for MRL setting separately for the northern and southern residue zone.

Table 7-168: Theoretical MRL calculation for honey (according to proposed residue definition for MRL setting in plants)

| OECD Calculator          | BAS 684 H [mg/kg] |             |
|--------------------------|-------------------|-------------|
|                          | N-EU              | S-EU        |
| Total number of data (n) | 8                 | 8           |
| Highest residue          | 0.01              | 0.01        |
| Mean + 4 SD              | 0.01              | 0.01        |
| CF x 3 Mean              | 0.01              | 0.01        |
| <b>Rounded MRL</b>       | <b>0.01</b>       | <b>0.01</b> |
| STMR                     | 0.01              | 0.01        |

The theoretical MRL calculated for both zones was 0.01 mg/kg.

Consequently, an EU MRL of BAS 684 H for honey at the LOQ of 0.01 or 0.05 mg/kg - depending on the LOQ of the analytical method – could be proposed.

#### Estimation of the potential and actual exposure

To show that there is no risk for the consumer by the intake of pollen and bee products such as honey, a risk assessment was performed.

The Acceptable Daily Intake (ADI) is set to 0.08 mg/kg bw/day based on the 1-year dog study while the acute reference dose (ARfD) of 0.3 mg/kg bw has been derived based on the developmental toxicity study in rats.

Table 7-169: Toxicological endpoints - BAS 684 H

| Endpoint                      | Value           | Study                            | Safety factor | Reference       |
|-------------------------------|-----------------|----------------------------------|---------------|-----------------|
| Acceptable Daily Intake (ADI) | 0.08 mg/kg bw/d | 1-yr dog study                   | 100           | M-CA, Section 5 |
| Acute Reference Dose (ARfD)   | 0.3 mg/kg bw    | Rat developmental toxicity study | 100           |                 |

---

***Acceptable Daily Intake (ADI) and Dietary Exposure Calculation***

*The calculation of the Theoretical Maximum Daily Intake (TMDI) was performed considering the calculated STMR for honey (derived from aerial parts of oilseed rape whole plants at BBCH 65) of 0.035 mg/kg (sum of BAS 684 H and M684H005 (incl. M684H006), expressed as BAS 684 H).*

*With the current EFSA model (PRIMO, rev. 3.0 1) the chronic risk assessment is up to 0.004% of the ADI. The diet with the highest calculated long-term intake was the diet DE child. Details of TMDI calculations for BAS 684 H are presented in [Table 7-171](#).*

*It can be concluded that a long-term intake of BAS 684 H residues via honey is unlikely to present a public health concern.*

***Acute Reference Dose (ARfD) and Dietary Exposure Calculation***

*An assessment of the potential acute dietary consumer risk due to exposure to residues of BAS 684 H was performed with the EFSA model (PRIMO, rev. 3.0 1) using the highest residue (HR) for honey (derived from aerial parts of oilseed rape whole plants at BBCH 65) of 0.20 mg/kg.*

*The summary report on the IESTI calculations according to the EFSA model (PRIMO, rev. 3.0-1) is presented in*

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*Table 7-170. For honey using an HR of 0.20 mg/kg (derived from aerial parts of oilseed rape whole plants at BBCH 65), the ARfD utilization for children was 0.2%, while for adults the ARfD utilization was 0.09%.*

*Therefore, according to the presented IESTI calculation, an acute intake of BAS 684 H residues from honey is unlikely to present a public health concern.*

### **Conclusion**

*Of the representative crops wheat and oilseed rape, only oilseed rape is considered as attractive crop for bees and having melliferous capacity. Thus, the possibility of BAS 684 H residues in pollen and bee products such as honey has to be assessed. Following the available draft working document on residues in bee products and MRL setting in honey, an MRL based on the residues in aerial plant parts at the time of flowering was derived from a full set of residue trials over two seasons. Additionally, a dietary risk assessment was conducted demonstrating that the contribution to the overall consumer risk by BAS 684 H residues in honey is low and any risk can be excluded.*

*Table 7-170: IESTI calculation of BAS 684 H according to EFSA PRIMo (rev. 3.1) applying the HR*

Acute risk assessment /children

Acute risk assessment / adults / general population

Details - acute risk assessment /children

Details - acute risk assessment/adults

The acute risk assessment is based on the ARfD.

The calculation is based on the large portion of the most critical consumer group.

Show results for all crops

|                         |  |             |                            |                     |  |             |                            |                     |
|-------------------------|--|-------------|----------------------------|---------------------|--|-------------|----------------------------|---------------------|
| Unprocessed commodities | Results for children                                       |             |                            |                     | Results for adults   |             |                            |                     |
|                         | No. of commodities for which ARfD/ADI is exceeded (IESTI): |             |                            |                     | No. of commodities for which ARfD/ADI is exceeded (IESTI): |             |                            |                     |
|                         | ---  |             |                            |                     | ---  |             |                            |                     |
|                         | IESTI  |             |                            |                     | IESTI  |             |                            |                     |
|                         | Highest % of ARfD/ADI                                      | Commodities | MRL / input for RA (mg/kg) | Exposure (µg/kg bw) | Highest % of ARfD/ADI                                      | Commodities | MRL / input for RA (mg/kg) | Exposure (µg/kg bw) |
| 0.2%                    | Honey and other  | 0.01 / 0.2  | 0.72                       | 0.09%               | Honey and other  | 0.01 / 0.2  | 0.28                       |                     |

*Table 7-171: IEDI calculation of BAS 684 H according to EFSA PRIMo (rev. 3.0 1) applying the STMR*



EFSA PRIMo revision 3.0: 2017/12/11

| Cinmethylin (F)                |      |                     |                |
|--------------------------------|------|---------------------|----------------|
| LOQs (mg/kg) range from:       |      | to:                 |                |
| Toxicological reference values |      |                     |                |
| ADI (mg/kg bw/day):            | 0.08 | ARfD (mg/kg bw):    | 0.3            |
| Source of ADI:                 | M-CA | Source of ARfD:     | M-CA Section 5 |
| Year of evaluation:            |      | Year of evaluation: |                |

| Input values                             |   |
|--|---|
| Details - chronic risk assessment        | Supplementary results - chronic risk assessment |
| Details - acute risk assessment/children | Details - acute risk assessment/adults          |

|  |                                |                   |                             |  |                                     |  |                                  |  |                                  |                                   |  |
|--|--------------------------------|-------------------|-----------------------------|--|-------------------------------------|--|----------------------------------|--|----------------------------------|-----------------------------------|--|
| Chronic risk assessment: JMPR methodology (IEDI/TMDI)  |                                |                   |                             |  |                                     |  |                                  |  |                                  |                                   |  |
| Normal mode  |                                |                   |                             |  |                                     |  |                                  |  |                                  |                                   |  |
| Chronic risk assessment: JMPR methodology (IEDI/TMDI)  |                                |                   |                             |  |                                     |  |                                  |  |                                  |                                   |  |
| No of diets exceeding the ADI : ---  |                                |                   |                             |  |                                     |  |                                  |  |                                  |                                   |  |
|  | Calculated exposure (% of ADI) | MS Diet           | Exposure (µg/kg bw per day) | Highest contributor to MS diet (in % of ADI) | Commodity / group of commodities    | 2nd contributor to MS diet (in % of ADI) | Commodity / group of commodities | 3rd contributor to MS diet (in % of ADI) | Commodity / group of commodities | MRLs set at the LOQ (in % of ADI) | Exposure resulting from commodities under assessment (in % of ADI) |
| TMDI/NEDI/IEDI calculation (based on average food consumption)   | 0.004%                         | DE child          | 0.003                       | 0.004%                                       | Honey and other apiculture products |  | Grapefruits                      |  | Grapefruits                      |                                   | 0.004%   |
|  | 0.002%                         | UK infant         | 0.001                       | 0.002%                                       | Honey and other apiculture products |  | Grapefruits                      |  | Grapefruits                      |                                   | 0.002%   |
|  | 0.002%                         | NL toddler        | 0.001                       | 0.002%                                       | Honey and other apiculture products |  | Grapefruits                      |  | Grapefruits                      |                                   | 0.002%   |
|  | 0.002%                         | DE general        | 0.001                       | 0.002%                                       | Honey and other apiculture products |  | Grapefruits                      |  | Grapefruits                      |                                   | 0.002%   |
|  | 0.001%                         | FR toddler 2-3 yr | 0.001                       | 0.001%                                       | Honey and other apiculture products |  | Grapefruits                      |  | Grapefruits                      |                                   | 0.001%   |
|  | 0.001%                         | DE women 14-50 yr | 0.001                       | 0.001%                                       | Honey and other apiculture products |  | Grapefruits                      |  | Grapefruits                      |                                   | 0.001%   |
|  | 0.001%                         | SE general        | 0.001                       | 0.001%                                       | Honey and other apiculture products |  | Grapefruits                      |  | Grapefruits                      |                                   | 0.001%   |
|  | 0.001%                         | FR child 3-15 yr  | 0.001                       | 0.001%                                       | Honey and other apiculture products |  | Grapefruits                      |  | Grapefruits                      |                                   | 0.001%   |
|  | 0.001%                         | UK toddler        | 0.001                       | 0.001%                                       | Honey and other apiculture products |  | Grapefruits                      |  | Grapefruits                      |                                   | 0.001%   |
|  | 0.001%                         | NL child          | 0.001                       | 0.001%                                       | Honey and other apiculture products |  | Grapefruits                      |  | Grapefruits                      |                                   | 0.001%   |
|  | 0.001%                         | FR adult          | 0.001                       | 0.001%                                       | Honey and other apiculture products |  | Grapefruits                      |  | Grapefruits                      |                                   | 0.001%   |
|  | 0.001%                         | ES child          | 0.001                       | 0.001%                                       | Honey and other apiculture products |  | Grapefruits                      |  | Grapefruits                      |                                   | 0.001%   |
|  | 0.001%                         | ES adult          | 0.001                       | 0.001%                                       | Honey and other apiculture products |  | Grapefruits                      |  | Grapefruits                      |                                   | 0.001%   |
|  | 0.001%                         | RO general        | 0.001                       | 0.001%                                       | Honey and other apiculture products |  | Grapefruits                      |  | Grapefruits                      |                                   | 0.001%   |
|  | 0.001%                         | UK adult          | 0.000                       | 0.001%                                       | Honey and other apiculture products |  | Grapefruits                      |  | Grapefruits                      |                                   | 0.001%   |
|  | 0.000%                         | NL general        | 0.000                       | 0.000%                                       | Honey and other apiculture products |  | Grapefruits                      |  | Grapefruits                      |                                   | 0.000%   |
|  | 0.000%                         | FI 3-yr           | 0.000                       | 0.000%                                       | Honey and other apiculture products |  | Grapefruits                      |  | Grapefruits                      |                                   | 0.000%   |
|  | 0.000%                         | FR infant         | 0.000                       | 0.000%                                       | Honey and other apiculture products |  | Grapefruits                      |  | Grapefruits                      |                                   | 0.000%   |
|  | 0.000%                         | FI 6-yr           | 0.000                       | 0.000%                                       | Honey and other apiculture products |  | Grapefruits                      |  | Grapefruits                      |                                   | 0.000%   |
|  | 0.000%                         | IE child          | 0.000                       | 0.000%                                       | Honey and other apiculture products |  | Grapefruits                      |  | Grapefruits                      |                                   | 0.000%   |
|  |                                | DK child          |                             |  | Grapefruits                         |  | Grapefruits                      |  | Grapefruits                      |                                   |  |
|  |                                | DK child          |                             |  | Grapefruits                         |  | Grapefruits                      |  | Grapefruits                      |                                   |  |
|  |                                | DK child          |                             |  | Grapefruits                         |  | Grapefruits                      |  | Grapefruits                      |                                   |  |
|  |                                | DK child          |                             |  | Grapefruits                         |  | Grapefruits                      |  | Grapefruits                      |                                   |  |
|  |                                | DK child          |                             |  | Grapefruits                         |  | Grapefruits                      |  | Grapefruits                      |                                   |  |
|  |                                | DK child          |                             |  | Grapefruits                         |  | Grapefruits                      |  | Grapefruits                      |                                   |  |
|  |                                | DK child          |                             |  | Grapefruits                         |  | Grapefruits                      |  | Grapefruits                      |                                   |  |
|  |                                | DK child          |                             |  | Grapefruits                         |  | Grapefruits                      |  | Grapefruits                      |                                   |  |
|  | DK child                       |                   |                             | Grapefruits                                  |                                     | Grapefruits                              |                                  | Grapefruits                              |                                  |                                   |  |
|  | DK child                       |                   |                             | Grapefruits                                  |                                     | Grapefruits                              |                                  | Grapefruits                              |                                  |                                   |  |
|  | DK child                       |                   |                             | Grapefruits                                  |                                     | Grapefruits                              |                                  | Grapefruits                              |                                  |                                   |  |
|  | DK child                       |                   |                             | Grapefruits                                  |                                     | Grapefruits                              |                                  | Grapefruits                              |                                  |                                   |  |
| Conclusion:<br>The estimated long-term dietary intake (TMDI/NEDI/IEDI) was below the ADI.<br>The long-term intake of residues of Cinmethylin (F) is unlikely to present a public health concern. |                                |                   |                             |  |                                     |  |                                  |  |                                  |                                   |  |



## Literature search

[illegible]

- 
- [REDACTED]  
[REDACTED]  
[REDACTED]

[REDACTED]  
[REDACTED]

[REDACTED]

[REDACTED]  
[REDACTED]  
[REDACTED]  
[REDACTED]

| Data Point  | Author(s)                           | Year   | Title<br>Company Report No.<br>Source (where different from company)<br>GLP or GEP status<br>Published or not  | Vertebrate<br>study<br>Y/N | Data<br>protection<br>claimed<br>Y/N | Justification<br>if data<br>protection is<br>claimed | Owner | Previous<br>evaluation |
|-------------|-------------------------------------|--------|--|----------------------------|--------------------------------------|--|-------|------------------------|
| KCA 6.1/1   | Spangler C.                         | 2018 a | Investigation of the storage stability of BAS 684 H in plant matrices<br>2016/1029128<br>BASF SE, Limburgerhof, Germany Fed.Rep.<br>yes<br>Unpublished             | No                         | Yes                                  | Data for first approval                              | BASF  | None                   |
| CA 6.1/3    | Eilers B.                           | 2020 a | Investigation of the storage stability of M684H005 in plant matrices<br>2020/2005975<br>BASF SE, Limburgerhof, Germany Fed.Rep.<br>yes<br>Unpublished              | No                         | Yes                                  | Data for first approval                              | BASF  | None                   |
| KCA 6.2.1/1 | Rosenbaum-Stieber C.,<br>Kessler M. | 2018 a | Metabolism of 14C-BAS 684 H in wheat<br>2017/1004405<br>BASF SE, Limburgerhof, Germany Fed.Rep.<br>yes<br>Unpublished  | No                         | Yes                                  | Data for first approval                              | BASF  | None                   |
| KCA 6.2.1/2 | Rabe U.,<br>Forieri I.              | 2018 a | Metabolism of 14C-BAS 684 H in oilseed rape<br>2017/1110861<br>BASF SE, Limburgerhof, Germany Fed.Rep.<br>yes<br>Unpublished                                       | No                         | Yes                                  | Data for first approval                              | BASF  | None                   |
| KCA 6.2.1/3 | Schweda Z.,<br>Forieri I.           | 2018 a | Metabolism of [14C]-BAS 684 H in carrots<br>2017/1186063<br>BASF SE, Limburgerhof, Germany Fed.Rep.<br>yes<br>Unpublished  | No                         | Yes                                  | Data for first approval                              | BASF  | None                   |
| KCA 6.2.1/4 | Woodward M.D.                       | 1984 a | Metabolism of sd95481 in soybeans 1. quantitation and fractionation of residues<br>CI-640-001<br>Shell Development Co., Modesto CA, United States of America<br>no | No                         | No                                   | Not applicable                                       | BASF  | None                   |

|             |             |        |  |    |    |                |      |      |
|-------------|-------------|--------|--|----|----|----------------|------|------|
|             |             |        | Unpublished  |    |    |                |      |      |
| KCA 6.2.1/5 | Woodward M. | 1984 a | Metabolism of sd95481 in soybeans 2. characterisation and identification of the principal metabolites from foliage<br>CI-640-002<br>Shell Development Co., Modesto CA, United States of America<br>no<br>Unpublished     | No | No | Not applicable | BASF | None |
| KCA 6.2.1/6 | Woodward M. | 1983 a | Identification of the principal metabolites of sd95481 from the hydroponic growth medium of soybean plants<br>CI-640-003<br>Shell Development Co., Modesto CA, United States of America<br>no<br>Unpublished             | No | No | Not applicable | BASF | None |
| KCA 6.2.1/7 | Woodward M. | 1984 b | Characterization and identification of the principal metabolites of sd95481 in soybean plants<br>CI-640-004<br>Shell Development Co., Modesto CA, United States of America<br>no<br>Unpublished                          | No | No | Not applicable | BASF | None |
| KCA 6.2.1/8 | Woodward M. | 1984 c | Metabolism of sd95481 in soybeans 3. characterisation and identification of the principal metabolites in a pilot study<br>CI-640-015<br>Shell Development Co., Modesto CA, United States of America<br>no<br>Unpublished | No | No | Not applicable | BASF | None |
| KCA 6.2.1/9 | Woodward M. | 1984 e | Metabolism of sd95481 in peanuts 1. quantitation and fractionation of residues<br>CI-640-008<br>Shell Development Co., Modesto CA, United States of America<br>no<br>Unpublished   | No | No | Not applicable | BASF | None |

**Substance name**
**Volume 3 – B.7 (AS)**

|              |                              |        |   |     |     |                         |      |      |
|--------------|------------------------------|--------|---|-----|-----|-------------------------|------|------|
| KCA 6.2.1/10 | Woodward M.                  | 1984 f | Metabolism of sd95481 in peanuts 2. characterization and identification of the principal metabolites from foliage<br>CI-640-009<br>Shell Development Co., Modesto CA, United States of America<br>no<br>Unpublished | No  | No  | Not applicable          | BASF | None |
| KCA 6.2.1/11 | Woodward M.                  | 1984 d | Metabolism of sd95481 in peanuts 3. characterization and identification of the principal metabolites in a pilot study<br>CI-640-016<br>no<br>Unpublished  | No  | No  | Not applicable          | BASF | None |
| KCA 6.2.1/12 | Edwards V.T.                 | 1988 a | The metabolism of 14C w195481 in rice outdoors<br>CI-640-011<br>Shell Research Ltd., Sittingbourne Kent ME9 8AG, United Kingdom<br>yes<br>Unpublished   | No  | Yes | Data for first approval | BASF | None |
| KCA 6.2.1/13 | Croucher A.,<br>Edwards V.T. | 1989 a | The distribution and metabolism of 14c w195481 in rice under controlled environmental conditions<br>CI-640-012<br>Shell Research Ltd., Sittingbourne Kent ME9 8AG, United Kingdom<br>yes<br>Unpublished             | No  | Yes | Data for first approval | BASF | None |
| KCA 6.2.2/1  | ██████<br>██████             | 2018 a | The metabolism of (14C)-Reg. No 900202 (BAS 684 H) in laying hens<br>2017/1068568<br>████████████████████<br>yes<br>Unpublished   | Yes | Yes | Data for first approval |      | None |
| KCA 6.2.2/2  | ██████<br>██████             | 2018 a | Metabolism of two metabolites of 14C-BAS 684 H (M684H005 und M684H006) in hen intestine<br>2017/1140183<br>████████████████████   | Yes | Yes | Data for first approval |      | None |

|             |                  |        |  |     |     |                         |      |      |
|-------------|------------------|--------|--|-----|-----|-------------------------|------|------|
|             |                  |        | yes<br>Unpublished   |     |     |                         |      |      |
| KCA 6.2.3/1 | ██████<br>██████ | 2018 a | The metabolism of (14C)-Reg.No. 900202 (BAS 684 H) in lactating goats<br>2017/1037602<br>████████████████████<br>yes<br>Unpublished  | Yes | Yes | Data for first approval |      | None |
| KCA 6.2.3/2 | ██████<br>██████ | 2018 b | Metabolism of two metabolites of 14C-BAS 684 H (M684H005 and M684H006) in rumen fluid<br>2017/1140182<br>████████████████████<br>yes<br>Unpublished  | Yes | Yes | Data for first approval |      | None |
| KCA 6.2.3/3 | ██████<br>██████ | 1983 a | Use of goats for ruminant metabolism studies. part 1 an exploratory study<br>CI-440-013<br>████████████████████<br>██████<br>no<br>Unpublished   | Yes | Yes | Data for first approval | BASF | None |
| KCA 6.2.3/4 | ████████<br>███  | 1984 a | Use of goats for ruminant metabolism studies. Part 2 characterization and identification of sd95481 metabolites in urine and faeces<br>CI-440-014<br>████████████████████<br>██████<br>no<br>Unpublished | Yes | Yes | Data for first approval | BASF | None |
| KCA 6.2.3/5 | █████<br>████    | 1989 a | Metabolic fate of cinmethylin in goat<br>CI-905-008<br>no<br>Unpublished   | Yes | Yes | Data for first approval | BASF | None |
| KCA 6.3.2/1 | Ale E.           | 2017 a | Residue study (Decline) with BAS 684 02 H applied to wheat in Northern and Southern Europe in 2015<br>2016/1118116<br>Envigo CRS Ltd. Sucursal en Espana, Valencia, Spain<br>yes                         | No  | Yes | Data for first approval | BASF | None |

|             |                         |        |   |    |     |                         |      |      |
|-------------|-------------------------|--------|---|----|-----|-------------------------|------|------|
|             |                         |        | Unpublished   |    |     |                         |      |      |
| KCA 6.3.2/2 | Mahlo C.,<br>Vagt I.    | 2017 a | Study on the residue behaviour of BAS 684 H in spring wheat after treatment with BAS 684 02 H under field conditions in Germany, Denmark, Northern France, Belgium, Southern France Greece, Italy and Spain, 2016<br>2017/1198202<br>SGS Institut Fresenius GmbH, Taunusstein, Germany Fed. Rep.<br>yes<br>Unpublished              | No | Yes | Data for first approval | BASF | None |
| KCA 6.3.2/3 | Mahlo C.                | 2018 a | Amendment 1: Study on the residue behaviour of BAS 684 H in spring wheat after treatment with BAS 684 02 H under field conditions in Germany, Denmark, Northern France, Belgium, Southern France Greece, Italy and Spain, 2016<br>2018/1030172<br>SGS Institut Fresenius GmbH, Taunusstein, Germany Fed. Rep.<br>yes<br>Unpublished | No | Yes | Data for first approval | BASF | None |
| KCA 6.3.2/4 | Martin T.,<br>Ruiz E.   | 2018 a | Study on the residue behavior of BAS 684 H in wheat after the application of BAS 684 03 H under field conditions in Germany, Netherlands, Austria, France (North and South), Greece, Italy and Spain, 2017<br>2017/1202170<br>Agrologia SLU, Utrera, Spain<br>yes<br>Unpublished  | No | Yes | Data for first approval | BASF | None |
| KCA 6.3.1/1 | Klimmek S.,<br>Bruhn F. | 2017 a | Study on the residue behaviour of BAS 684 H in oilseed rape after one application with BAS 684 02 H under field conditions in Germany, Northern France, The Netherlands, United Kingdom, Greece, Italy and Spain, 2016<br>2017/1219191<br>Eurofins Agrosience Services Chem GmbH, Hamburg, Germany Fed.Rep.<br>yes<br>Unpublished   | No | Yes | Data for first approval | BASF | None |
| KCA 6.3.1/2 | Klimmek S.,<br>Bruhn F. | 2018 a | Amendment No.1, study on the residue behaviour of BAS 684 H in oilseed rape after one application with BAS 684 02 H under field conditions in Germany,  | No | Yes | Data for first approval | BASF | None |

|             |                      |        |   |    |     |                         |      |      |
|-------------|----------------------|--------|---|----|-----|-------------------------|------|------|
|             |                      |        | Northern France, The Netherlands, United Kingdom, Greece, Italy and Spain, 2016<br>2018/1028316<br>Eurofins Agrosience Services Chem GmbH, Hamburg, Germany Fed.Rep.<br>yes<br>Unpublished  |    |     |                         |      |      |
| KCA 6.3.1/3 | Klimmek S., Bruhn F. | 2018 b | Study on the residue behaviour of BAS 684 H in oilseed rape after one application with BAS 684 03 H under field conditions in Germany, Northern France<br>2017/1219684<br>Eurofins Agrosience Services Chem GmbH, Hamburg, Germany Fed.Rep.<br>yes<br>Unpublished | No | Yes | Data for first approval | BASF | None |
| KCA 6.5.1/1 | Wijntjes C. et al.   | 2016 a | 14C BAS 684 H: Simulated processing - Hydrolysis at 90 C, 100 C and 120 C<br>2015/1198477<br>IES - Innovative Environmental Services Ltd., Witterswil, Switzerland<br>yes<br>Unpublished  | No | Yes | Data for first approval | BASF | None |
| KCA 6.6.1/1 | Wenzel N. et al.     | 2018 a | Confined rotational crop study with [14C]-BAS 684 H<br>2016/1321090<br>BASF SE, Limburgerhof, Germany Fed.Rep.<br>yes<br>Unpublished  | No | Yes | Data for first approval | BASF | None |