Draft Assessment Report

Evaluation of Active Substances

Plant Protection Products

Prepared according to **assimilated Regulation No 1107/2009**
as it applies in Great Britain

Inpyrfluxam

Volume 3 – B.5 (AS)

Methods of Analysis

Great Britain

March 2026

Version History

When	What
November 2025	Initial DAR
March 2026	Updates made after ECP

Contents

B.5. Methods of Analysis

B.5.1. Methods used for the generation of pre-authorisation data

B.5.1.1. Methods for the analysis of the active substance as manufactured

Method for the determination of inpyrfluxam in active substance as manufactured.

Reference:	KCA 4.1.1/01
Report Title:	Enforcement Analytical Methods for S-2399 Technical Grade.
Author(s) & Year:	[redacted]
Document No, Authority registration No	Report No.:TPA-0019 Study ID: GP16057
Guideline(s):	SANCO/3030/99 rev.5
Deviations:	No
GLP or GEP:	Yes
Acceptability:	None
Study relied upon:	Yes

The purpose of the study was to demonstrate the validity of a high-performance liquid chromatographic (HPLC) method for the determination of active ingredient in inpyrfluxam technical.

Principle of the method

100 mg of test sample was weighed accurately into a 50 mL volumetric flask. The test sample was dissolved and diluted to the 50 mL mark using acetonitrile. 5.0 mL of the

sample solution was pipetted out into a 20 mL volumetric flask and diluted to mark with acetonitrile. The nominal concentration of test item is approximately 0.5 mg/mL.

Method conditions:

Instrument	HPLC-UV
Column	CHIRALPALK ID 5 µm, 4.6 mm i.d. x 15 cm or equivalent
Mobile Phase	Water/acetonitrile (65:35 (v/v))
Flow Rate	Adjust flow rate so retention time of inpyrfluxam is about 16 mins (between 15 to 17 mins, about 1.0 mL/min)
Oven temperature	40°C
Detector	UV absorption photometer (wavelength: 240 nm)
Run Time	25 minutes
Retention time	~16 mins

Table B.5.1.1-1: Analytical validation data for the determination of the active in the technical material

Matrix	Analyte	LOQ (%w/w)	Recovery	Repeatability % RSD (n)	Linearity
Inpyrfluxam technical	Inpyrfluxam	Not required		0.153 @ 96.4 %w/w (n=6) Modified Horwitz= 1.35 Hr- 0.11	0.35 – 0.625 mg/mL (~70-125% of the nominal content in test item solution) N=6 r = 1.000 y= 108678x +12668

Specificity

Specificity was demonstrated by retention time match with a reference standard and the absence of interfering peaks in the chromatogram of a blank solvent sample

Linearity

Linearity was demonstrated by the analysis of six standards, in duplicate, of increasing concentration. The range of standard concentrations used was 0.35 – 0.625 mg/mL, equivalent to approximately 70 – 125% of the nominal content in the test item solutions. The linear range extends over the lowest and highest nominal concentration of the active substance in the test solutions with an appropriate range of ±at least 20%. The response was linear with a correlation coefficient of 1.000. This linear range is sufficient to cover the 5-batch analysis data for inpyrfluxam.

Recovery/repeatability

6 determinations of the test sample were analysed using the method described above, and the %RSD was calculated. The relative standard deviation obtained was within the guideline requirements of a HORRAT (Hr) of ≤ 1.

Conclusion

The method is acceptably validated in accordance with SANCO/3030/99 rev.5 and is suitable for the determination of inpyrfluxam in the technical material.

B.5.1.2. Methods for risk assessment

B.5.1.2.1. Methods in soil, water, sediment, air and any additional matrices used in support of environmental fate studies

Table B.5.1.2.1-1: Summary of risk assessment methods supporting environmental fate.

Data point	Study	Conclusion	Studies relied on
KCA 4.1.2/01	S-2399: Validation of Valent's Method RM-50S "Determination of Residues of S-2399, 3'-OH-S-2840, 1'-COOH-S-2840-A and 1'-COOH-S-2840-B in	HPLC-MS/MS Analyte: Inpyrfluxam LOQ: 0.01 mg/kg in soil.	KCA 7.1.2.2.1/01 Report no: TPR-0031
			KCA 7.1.2.2.1/09 Report no.: TPR-0064

	Soil. [redacted] 2017a Report No.: TPA-0028	Acceptable.	
KCA 4.1.2/02	S-2399: Validation of Valent's Method RM-50V, "Determination of Residues of S-2399 on Application Verification Pads" [redacted] 2017b Report No.: TPA-0029	HPLC-MS/MS Analyte: Inpyrfluxam LOQ: 0.5 µg/cm^2 on verification pads Acceptable.	KCA 7.1.2.2.1/01 Report no: TPR-0031
KCA 4.1.2/03	Validation Study for the Determination of S2399 and its Metabolites 3'-OH-S-2840, 1'-COOH-S-2840A and 1'-COOH-S-2840B in Soil. [redacted] 2017 Report No.: TPA-0043	HPLC-MS/MS Analyte: Inpyrfluxam, 3'-OH-S-2840 LOQ: 0.002 mg/kg in soil. Analyte: 1'-COOH-S-2840A, 1'-COOH-S-2840B LOQ: 0.001 mg/kg in soil. Acceptable.	KCA 7.1.2.2.1/07 Report no.: TPR-0085. KCA 7.1.2.2.1/10 Report no.:TPR-0088 KCA 6.6.2/01 Report number: TPR-0080

Reference:	KCA 4.1.2/01
Report Title:	S-2399: Validation of Valent's Method RM-50S "Determination of Residues of S-2399, 3'-OH-S-2840, 1'-COOH-S-2840-A and 1'-COOH-S-2840-B in Soil.
Author(s) & Year:	[redacted] 2017a
Document No, Authority registration No	Report No.: TPA-0028

Guideline(s):	OCSPP 860.1340
Deviations:	No
GLP or GEP:	Yes
Acceptability:	Yes
Study relied upon:	Yes

The purpose of the study was to demonstrate the validity of a HPLC-MS/MS method for the determination of inpyrfluxam 3'-OH-S-2840, 1'-COOH-S-2840-A and 1'-COOH-S-2840-B in soil.

Principle of the method

Mix by hand or homogenize the bulk sample in the presence of dry ice to obtain a homogenous sample. If homogenized, allow the dry ice to sublime from the sample before taking a subsample for analysis.

Weigh 10.0 g (±0.1 g) of the homogenized sample into a 50 mL polypropylene centrifuge tube. Add 25 mL of acetone/water (4/1, v/v) to the centrifuge tube containing the sample and shake on a reciprocating shaker for 30 minutes. Centrifuge the sample for approximately 5 minutes at approximately 2000 rpm or as needed to separate the solids from the extraction solvent. Decant the sample extract into a stoppered 100 mL graduate cylinder. The next step must be conducted in a timely manner due to the acid instability of 3'-OH-S-2840. Add 25 mL of acetone/0.5 M HCl (4/1, v/v) to the centrifuge tube containing the sample and shake on a reciprocating shake for 30 minutes. Immediately centrifuge the sample for approximately 5 minutes and decant the sample extract into the 100 mL graduated cylinder containing the first two extracts. Immediately add 2 mL of 0.5 M sodium acetate solution to the graduated cylinder and mix. Add 25 mL of 5% sodium chloride solution to the 250 mL separatory funnel. Remix the contents of the graduated cylinder just prior to taking an aliquot. Add 2.5 mL of extract from the just mixed contents in the graduated cylinder and 50 mL dichloromethane to the separatory funnel. Immediately shake for 1 minute and allow to separate. Drain the lower dichloromethane layer through a funnel containing approximately 50 g of sodium sulphate (suspended on a plug of glass wool) and collect into a 250 mL or 500 mL round bottom flask. Add an additional 50 mL dichloromethane to the separatory funnel. Shake for one minute and allow to separate. Drain the lower dichloromethane layer through the funnel containing sodium sulphate into

the round bottom flask containing the first extract. Evaporate to dryness using a rotary-evaporator and water bath set to ≤40 °C or other evaporator system. Re-dissolve the extract in 5.0 mL of methanol/water (1/1, v/v) (or internal standard final volume solution, 1 µg/L if using optional internal standards), sonicate, then transfer the extracts to a screw cap vial. The sample was then analysed by HPLC-MS/MS using the conditions shown below.

HPLC-MS/MS conditions

Column Eclipse XDB-C8, 5 µm, 150 mm x 4.6 mm, Agilent part #993967-906

Mobile phases Eluent A: 5 mM ammonium acetate in HPLC water
Eluent B: 5 mM ammonium acetate in methanol

Flow rate 700 µL/min

Gradient

Time (min)	%A	%B
0	65	35
1.0	65	35
6.0	10	90
7.0	35	65
10.0	35	65
11.0	65	35
15.0	65	35

Column oven temperature 40 °C

Injection volume 25 µL

Retention times 1'-COOH-S-2840A approx. 5.9 minutes
1'-COOH-S-2840B approx. 6.3 minutes
3'-OH-S-2840 approx. 9.0 minutes
Inpyrfluxam approx. 9.4 minutes

MS/MS periods one and two conditions Negative ion mode
MRM scanning
Electrospray ionisation

MS/MS period three conditions Positive ion mode
MRM scanning
Electrospray ionisation

MS/MS period one ion transitions monitored (m/z) 1'-COOH-S-2840A and 1'-COOH-S-2840B:
362 → 318*
362 → 131

MS/MS period two ion transitions monitored (m/z) 3'-OH-S-2840
348 → 175*
348 → 130

MS/MS period three ion transitions monitored (m/z) Inpyrfluxam
334 → 238*

334 → 258

*Ion transition used for the quantification

Table B.5.1.2.1-2: Summary of method validation for the determination of inpyrfluxam, 3'-OH-S-2840, 1'-COOH-S-2840A and 1'-COOH-S-2840B in soil

Analyte	LOQ (mg/kg)	Recovery fortification level (mg/kg)	Recoveries % range (mean)	Repeatability % RSD (n)	Linearity	Specificity
Inpyrfluxam 334 → 238 m/z	0.01	0.01	90 – 99 (93) N = 5	3.9 @ ~0.01 mg/kg (N = 5)	0.25 – 10.0 µg/L (Equivalent to 0.005-0.2 mg/kg) (N = 6) y = 3.28E-03x^2 + 1.02x - 2.58E-02 R^2 = 0.99976	Retention time match to reference standard. No significant interfering peaks observed in the blank matrix.
		0.1	101 – 107 (104) N = 5	2.1 @ ~0.1 mg/kg (N = 5)		
3'-OH-S-2840 348 → 175 m/z	0.01	0.01	87 – 108 (95) N = 5	8.8 @ ~0.01 mg/kg (N = 5)	0.25 – 10.0 µg/L (Equivalent to 0.005-0.2 mg/kg) (N = 6) y = 1.53E-04x^2 + 0.781x – 8.76E-03 R^2 = 0.99976	
		0.1	99 – 110 (104) N = 5	4.0 @ ~0.1 mg/kg (N = 5)		
1'-COOH-S-2840A 362 → 318 m/z	0.01	0.01	71 – 89 (83) N = 5	8.6 @ ~0.01 mg/kg (N = 5)	0.25 – 10.0 µg/L (Equivalent to 0.005-0.2 mg/kg) (N = 6) y = 1.49E-03x^2 +	
		0.1	77 – 88 (82) N = 5	5.4 @ ~0.1 mg/kg (N = 5)		

					0.848x + 1.94E-02 R^2 = 0.99976	
1'-COOH-S-2840B 362 → 318 m/z	0.01	0.01*	74 – 84 (78) N = 4	5.9 @ ~0.01 mg/kg (N = 4)	0.25 – 10.0 µg/L (Equivalent to 0.005-0.2 mg/kg) (N = 6) y = 2.42E-02x^2 + 0.894x + 3.34E-02 R^2 = 0.99976	
		0.1	72 – 77 (74) N = 5	2.7 @ ~0.1 mg/kg (N = 5)		

* The recoveries for three of the samples were determined twice. The mean of these samples has been reported above and used for the calculation of the mean recoveries and the %RSD.

Matrix effects

The matrix effects have not been addressed.

Specificity

Specificity was demonstrated by retention time match with a calibration standard and the absence of significant (>30% LOQ) interfering peaks in the chromatogram of a matrix blank sample for all analytes. A retention time match was observed between the calibration standard and the fortified test item for all analytes.

Linearity

Linearity was demonstrated by the analysis of six standards of increasing concentration for each analyte. The range of standard concentrations used was 0.25 – 10.0 µg/L. The concentrations extend over an appropriate range. A non-linear calibration was used as it provided a better fit than a linear model, as this has been justified, it is acceptable. The co-efficient of determination was 0.99976 for all analytes.

Precision (repeatability)

The precision of the method was assessed via analysis of the accuracy samples for the analytes. The reported %RSDs were all <20%.

Accuracy (recovery)

Inpyrfluxam, 3'-OH-S-2840 and 1'-COOH-S-2840A
The accuracy of the method was assessed by analysing five samples fortified with the analyte at concentrations of 0.01 and 0.1 mg/kg. Recovery values were calculated as a percentage of measured concentration relative to fortified concentration. Acceptable mean recovery levels were obtained (within the range 70 to 120%).

1'-COOH-S-2840B
The accuracy of the method was assessed by analysing four samples fortified with the analyte at concentrations of 0.01 and 0.1 mg/kg. Recovery values were calculated as a percentage of measured concentration relative to fortified concentration. Acceptable mean recovery levels were obtained (within the range 70 to 120%).

One of the 0.01 mg/kg fortification samples for 1'-COOH-S-2840B was not within the 70-120% recovery range and a second smaller set consisting of one untreated control and three untreated controls fortified at the LOQ (0.01 mg/kg) was analysed for 1'-COOH-S-2840B only. Acceptable mean recovery levels were obtained (within the range 70 to 120%).

LOQ

The limit of quantitation (LOQ), defined as the lowest fortification level where acceptable precision and accuracy data were obtained, has been demonstrated to be 0.01 mg/kg for all analytes.

Procedural recoveries

In report no: TPR-0031, procedural recoveries were conducted for all analytes using fortification levels of 0.01 and 0.1 mg/kg The mean recoveries were within the acceptable range of 70-120%. The procedural recoveries are acceptable for all analytes at both fortification levels.

In report no: TPR-0064, procedural recoveries were conducted for all analytes using fortification levels of 0.1 mg/kg The mean recoveries were within the acceptable range of 70-120%. The procedural recoveries are acceptable for all analytes.

Stability of standards and extracts

The stability of standards and extracts has not been addressed.

Conclusion

The analytical method is not acceptably validated according to SANTE/2020/12830 rev. 1 for the determination of inpyrfluxam, 3'-OH-S-2840, 1'-COOH-S-2840A and 1'-COOH-S-2840B in soil as the matrix effects have not been determined and the stability of standards and extracts has not been addressed. However, the studies were generated prior to the implementation of SANTE 2020/12830 rev.1 and SANCO 3029/99 rev.4 did not require matrix effects and the stabilities of standards to be addressed. As all other validation

requirements have been met (including the minimum validation requirements outlined in SANTE 2020/12830 rev.1), the method is considered to be fit for purpose.

Reference:	KCA 4.1.2/02
Report Title:	S-2399: Validation of Valent's Method RM-50V, "Determination of Residues of S-2399 on Application Verification Pads"
Author(s) & Year:	[redacted] 2017b
Document No, Authority registration No	Report No.: TPA-0029
Guideline(s):	OCSPP 860.1340
Deviations:	No
GLP or GEP:	Yes
Acceptability:	Yes
Study relied upon:	Yes

The purpose of the study was to demonstrate the validity of a HPLC-MS/MS method for the determination of inpyrfluxam on verification pads.

Principle of the method

Place two solvent saturation pads side by side on a piece of aluminium foil. Fortify the two pads evenly using the fortifying solution. Flip one of the pads on top of the other pad. Fold the two saturation pads together in half. Starting from the shorter end, roll the two pads together and place in 250 mL glass jar. Discard the aluminium foil. Add 200 mL of acetone to each sample jar. Tightly cap the jar and place the jar on its side in a reciprocating shaker for 10 minutes. Place 100 µL of sample extract into a 100 mL graduated cylinder and bring up to volume with 0.05% formic acid in methanol/ 0.05% formic acid in water (1/1,v/v). Mix the sample. Transfer a portion of the final volume extract to an autosampler vial. The sample was then analysed by HPLC-MS/MS under the following conditions.

Column Eclipse XDB-C8, 5 µm, 150 mm x 4.6 mm, Agilent part #993967-906

Mobile phases Eluent A: 0.05% formic acid in HPLC water
Eluent B: 0.05% formic acid in methanol

Flow rate 400 µL/min

Gradient

Time (min)	%A	%B
0	50	50
1.0	50	50
6.0	10	90
14.0	10	90
14.5	50	50
18.0	50	50

Column oven temperature 20 °C

Injection volume 25 µL

Retention time Approx. 12.7 minutes

Ionisation mode Positive electrospray ionisation

Ion transitions monitored (m/z) 334 → 258*
334 → 294

Table B.5.1.2.1-3: Summary of method validation for the determination of inpyrfluxam on application verification pads

Analyte	LOQ (µg/cm^2)	Recovery fortification level (µg/cm^2)	Recoveries % range (mean)	Repeatability % RSD (n)	Linearity	Specificity
Inpyrfluxam 334 → 258 m/z	0.5	0.5	83 – 91 (87) N = 5	3.5 @ ~0.5 µg/cm^2 (N = 5)	0.1 – 10.0 µg/L 0.2 (equivalent to 0.025-2.5 µg/cm^2) (N = 5) y = 7.046E-14x^2 + 1.947E-06x – 8.796E-04 R^2 = 0.99919	Retention time match to reference standard. No significant interfering peaks observed in the blank matrix.
		1.68	88 – 95 (91) N = 5	3.2 @ ~1.68 µg/cm^2 (N = 5)		

Matrix effects

The matrix effects have not been addressed.

Specificity

Specificity was demonstrated by retention time match with a calibration standard and the absence of significant (>30% LOQ) interfering peaks in the chromatogram of a matrix blank sample. A retention time match was observed between the calibration standard and the fortified test item.

Linearity

Linearity was demonstrated by the analysis of five standards of increasing concentration. The range of standard concentrations used was 0.1 – 10.0 µg/L. The concentrations extend over an appropriate range. A non-linear calibration was used as it provided a better fit than a linear model, as this has been justified, it is acceptable. The co-efficient of determination was 0.99919.

Precision (repeatability)

The precision of the method was assessed via analysis of the accuracy samples. The reported %RSDs were all <20%.

Accuracy (recovery)

The accuracy of the method was assessed by analysing five samples fortified with inpyrfluxam at concentrations of 0.5 and 1.68 µg/cm^2. Recovery values were calculated as a percentage of measured concentration relative to fortified concentration. Acceptable mean recovery levels were obtained (within the range 70 to 120%).

LOQ

The limit of quantitation (LOQ), defined as the lowest fortification level where acceptable precision and accuracy data were obtained, has been demonstrated to be 0.5 µg/cm^2.

Procedural recoveries

In study ID: TPR-031***,*** Procedural recoveries were conducted using fortification levels of 0.5 and 1.68 µg/cm^2. The mean recoveries were within the acceptable range of 70-120%. The procedural recoveries are acceptable at both fortification levels.

Stability of standards and extracts

Samples were extracted and analysed on the same day therefore extract stability is not required

The stability of standards and stocks has not been addressed.

Conclusion

The analytical method is not acceptably validated according to SANTE/2020/12830 rev. 1 for the determination of inpyrfluxam in application validation pads as the matrix effects have not been determined and the stability of standards and stocks has not been addressed. However, the studies were generated prior to the implementation of SANTE 2020/12830 rev.1 and SANCO 3029/99 rev.4 did not require matrix effects and the stabilities of standards to be addressed. As all other validation requirements have been met (including the minimum validation requirements outlined in SANTE 2020/12830 rev.1), the method is considered to be fit for purpose.

Reference:	KCA 4.1.2/03
Report Title:	Validation Study for the Determination of S-2399 and its Metabolites 3'-OH-S-2840, 1'-COOH-S-2840A and 1'-COOH-S-2840B in Soil
Author(s) & Year:	[redacted] 2017
Document No, Authority registration No	Report No.: TPA-0043
Guideline(s):	SANCO/825/00 rev. 8.1
Deviations:	No
GLP or GEP:	Yes
Acceptability:	Yes
Study relied upon:	Yes

The purpose of the study was to demonstrate the validity of a HPLC-MS/MS method for the determination of inpyrfluxam 3'-OH-S-2840, 1'-COOH-S-2840-A and 1'-COOH-S-2840-B in soil.

Principle of the method

5.0 g of a homogenized soil was weighed into a 50 mL centrifuge tube. A 12.5 mL aliquot of acetone/water (80/20, v/v) was added, and the sample was agitated on a horizontal flatbed shaker for 10 minutes. The tube was then centrifuged at 4000 rpm for 5 minutes. The supernatant was transferred into a separate 50 mL centrifuge tube. A further 12.5 mL aliquot of acetone/water (80/20, v/v) was added to the sample (in the first centrifuge tube). The sample was then agitated on a horizontal flatbed for 10 minutes. The tube was then centrifuged at 4000 rpm for 5 minutes. The supernatant was collected and combined with the supernatant from the first extraction. Then 12.5 mL acetone/0.5 M HCl (80/20, v/v) was added to the sample (in the first centrifuge tube). The sample was then agitated on a horizontal flatbed for 10 minutes. The sample must not be agitated for more than 10 minutes due to acid instability of 3'-OH-S-2840. Then 1 mL of 5 M NaOH was immediately added to the sample which was then shaken by hand. The supernatant of this sample was filtered

through filter paper cover with small amounts of celite into a previous 50 mL tube. The supernatants were combined, and the volume was adjusted to 50 mL with acetone/water (80/20, v/v) as required.

10 mL of the above solution was transferred into a 100 mL Schott bottle. 32 mL of ethyl acetate was added, and the sample was agitated for 10 minutes on a horizontal flatbed shaker. Solid NaCl was then added, and the sample was shaken for a further 2 minutes on a horizontal flatbed shaker. Na_2SO_4 was added and a further agitation step for 2 minutes followed. 20 mL of the resulting supernatant was transferred into a pear-shaped flask and evaporated to dryness by rotary evaporation (water bath set to 40 °C). Dried residues were reconstituted with 0.125 mL of LC-MS grade MeOH followed by 0.375 mL of HPLC grade water (using an ultrasonic bath to aid dissolution). The sample was then analysed by HPLC-MS/MS under the following conditions.

HPLC-MS/MS conditions

HPLC System 1290 Infinity II Binary LC System, Agilent Technologies (HPLC, ≤ 1200 bar)

Column Luna 2.5µ C18(2)-HAST (100 x 2 mm, 2.5 µm, Phenomenex, Art. No. 00D-4446-B0)

Mobile phases Eluent A: Water + 0.1% formic acid
Eluent B: Methanol + 0.1% formic acid

Flow rate 400 µL/min

Gradient

Time (min)	%A	%B
0.00	55	45
6.00	5	95
7.00	5	95
7.10	55	45
8.00	55	45

Column oven temperature 40 °C

Injection volume 10 µL

Retention times 1'-COOH-S-2840A approx. 3.2 minutes
1'-COOH-S-2840B approx. 3.5 minutes
3'-OH-S-2840 approx. 4.9 minutes
Inpyrfluxam approx. 5.4 minutes

MS System SCIEX QTRAP 6500+ System

Ionisation type Electrospray ionization (ESI)

Scan type MS/MS, Multiple Reaction Monitoring (MRM)

Polarity Positive ion mode

Ion mass transition monitored (m/z) Inpyrfluxam:
334 → 238*
334 → 258

3'-OH-S-2840:
348 → 175*
348 → 131

1'-COOH-S-2840A and 1'-COOH-S-2840B
362 → 318*
362 → 298

*Proposed for quantification but both ion mass transitions can be used for quantification.

Table B.5.1.2.1-4: Summary of method validation for the determination of inpyrfluxam, 3'-OH-S-2840, 1'-COOH-S-2840A and 1'-COOH-S-2840B in soil

Analyte	Matrix	LOQ (mg/kg)	Recovery fortification level (mg/kg)	Recoveries % range (mean)	Repeatability % RSD (n)	Linearity	Specificity
Inpyrfluxam 334 → 238 m/z (Quantification)	ATC F6 (Czech Republic)	0.002	0.002	97 – 111 (102) N = 5	5 @ ~0.002 mg/kg (N = 5)	0.4 – 100 ng/mL (equivalent to 0.0004 to 0.1 mg/kg) (N = 8) y = 763000x + 1770 r = 0.9999	Retention time match to reference standard. No significant interfering peaks observed in the blank matrix.
			0.2	90 – 101 (94) N = 5	4 @ ~0.2 mg/kg (N = 5)		
	TRI F5 (Spain)	0.002	0.002	102 – 108 (105) N = 3	3 @ ~0.002 mg/kg (N = 3)		
			0.2	88 – 96 (93) N = 3	4 @ ~0.2 mg/kg (N = 3)		
	AGR F7 (Italy)	0.002	0.002	87 – 112 (103) N = 3	13 @ ~0.002 mg/kg (N = 3)		
			0.2	95 – 99 (97) N = 3	2 @ ~0.2 mg/kg (N = 3)		

	BIO F3 (Germany)	0.002	0.002	96 – 105 (100) N = 3	5 @ ~0.002 mg/kg (N = 3)		
			0.2	93 – 96 (94) N = 3	2 @ ~0.2 mg/kg (N = 3)		
Inpyrfluxam 334 → 258 m/z (Confirmation)	ATC F6 (Czech Republic)	0.002	0.002	95 – 114 (103) N = 5	7 @ ~0.002 mg/kg (N = 5)	0.4 – 100 ng/mL (equivalent to 0.0004 to 0.1 mg/kg) (N = 8) y = 237000x – 11900 r = 0.9999	
			0.2	89 – 100 (93) N = 5	4 @ ~0.2 mg/kg (N = 5)		
	TRI F5 (Spain)	0.002	0.002	107 – 108 (108) N = 3	1 @ ~0.002 mg/kg (N = 3)		
			0.2	88 – 96 (93) N = 3	5 @ ~0.2 mg/kg (N = 3)		
	AGR F7 (Italy)	0.002	0.002	90 – 112 (104) N = 3	12 @ ~0.002 mg/kg (N = 3)		
			0.2	96 – 97 (96) N = 3	1 @ ~0.2 mg/kg (N = 3)		
	BIO F3 (Germany)	0.002	0.002	95 – 104 (99) N = 3	5 @ ~0.002 mg/kg (N = 3)		
			0.2	93 – 96 (94) N = 3	2 @ ~0.2 mg/kg (N = 3)		

3'-OH-S-2840 348 → 175 m/z (Quantification)	ATC F6 (Czech Republic)	0.002	0.002	82 – 113 (99) N = 5	13 @ ~0.002 mg/kg (N = 5)	0.4 – 100 ng/mL (equivalent to 0.0004 to 0.1 mg/kg) (N = 8) y = 601000x + 138000 r = 0.9996	
			0.2	102 – 108 (104) N = 5	2 @ ~0.2 mg/kg (N = 5)		
	TRI F5 (Spain)	0.002	0.002	100 – 101 (101) N = 3	1 @ ~0.002 mg/kg (N = 3)		
			0.2	87 – 93 (90) N = 3	3 @ ~0.2 mg/kg (N = 3)		
	AGR F7 (Italy)	0.002	0.002	82 – 108 (99) N = 3	15 @ ~0.002 mg/kg (N = 3)		
			0.2	92 – 98 (95) N = 3	3 @ ~0.2 mg/kg (N = 3)		
	BIO F3 (Germany)	0.002	0.002	101 – 108 (104) N = 3	4 @ ~0.002 mg/kg (N = 3)		
			0.2	102 – 106 (103) N = 3	2 @ ~0.2 mg/kg (N = 3)		
3'-OH-S-2840 348 → 131 m/z (Confirmation)	ATC F6 (Czech Republic)	0.002	0.002	82 – 114 (100) N = 5	13 @ ~0.002 mg/kg (N = 5)	0.4 – 100 ng/mL (equivalent to 0.0004 to 0.1 mg/kg)	
			0.2	102 – 108 (105) N = 5	2 @ ~0.2 mg/kg (N = 5)		

	TRI F5 (Spain)	0.002	0.002	100 – 100 (100) N = 3	0 @ ~0.002 mg/kg (N = 3)	(N = 8) y = 502000 + 99500 r = 0.9998	
			0.2	86 – 95 (91) N = 3	5 @ ~0.2 mg/kg (N = 3)		
	AGR F7 (Italy)	0.002	0.002	80 – 111 (99) N = 3	17 @ ~0.002 mg/kg (N = 3)		
			0.2	92 – 98 (95) N = 3	3 @ ~0.2 mg/kg (N = 3)		
	BIO F3 (Germany)	0.002	0.002	103 – 111 (106) N = 3	4 @ ~0.002 mg/kg (N = 3)		
			0.2	99 – 102 (101) N = 3	2 @ ~0.2 mg/kg (N = 3)		
1'-COOH-S-2840A 362 → 318 m/z (Quantification)	ATC F6 (Czech Republic)	0.001	0.001	84 – 92 (88) N = 5	3 @ ~0.001 mg/kg (N = 5)	0.2 – 50 ng/mL (equivalent to 0.0002 to 0.05 mg/kg) (N = 8)	
			0.1	78 – 94 (84) N = 5	7 @ ~0.1 mg/kg (N = 5)		
	TRI F5 (Spain)	0.001	0.001	107 – 110 (108) N = 3	1 @ ~0.001 mg/kg (N = 3)		
			0.1	86 – 95 (90) N = 3	5 @ ~0.1 mg/kg (N = 3)		

	AGR F7 (Italy)	0.001	0.001	90 – 102 (98) N = 3	7 @ ~0.001 mg/kg (N = 3)	y = 335000 + 5930 r = 0.9999	
			0.1	95 – 98 (96) N = 3	2 @ ~0.1 mg/kg (N = 3)		
	BIO F3 (Germany)	0.001	0.001	76 – 82 (80) N = 3	4 @ ~0.001 mg/kg (N = 3)		
			0.1	74 – 77 (76) N = 3	2 @ ~0.1 mg/kg (N = 3)		
1'-COOH-S-2840A 362 → 298 m/z (Confirmation)	ATC F6 (Czech Republic)	0.001	0.001	81 – 90 (86) N = 5	5 @ ~0.001 mg/kg (N = 5)	0.2 – 50 ng/mL (equivalent to 0.0002 to 0.05 mg/kg) (N = 8) y = 17300 + 510 r = 0.9999	
			0.1	77 – 92 (83) N = 5	7 @ ~0.1 mg/kg (N = 5)		
	TRI F5 (Spain)	0.001	0.001	105 – 112 (107) N = 3	4 @ ~0.001 mg/kg (N = 3)		
			0.1	84 – 92 (88) N = 3	5 @ ~0.1 mg/kg (N = 3)		
	AGR F7 (Italy)	0.001	0.001	86 – 96 (91) N = 3	6 @ ~0.001 mg/kg (N = 3)		
			0.1	95 – 99 (96) N = 3	2 @ ~0.1 mg/kg (N = 3)		

	BIO F3 (Germany)	0.001	0.001	70 – 81 (75) N = 3	8 @ ~0.001 mg/kg (N = 3)		
			0.1	76 – 78 (77) N = 3	1 @ ~0.1 mg/kg (N = 3)		
1'-COOH-S-2840B 362 → 318 m/z (Quantification)	ATC F6 (Czech Republic)	0.001	0.001	89 – 94 (92) N = 5	2 @ ~0.001 mg/kg (N = 5)	0.2 – 50 ng/mL (equivalent to 0.0002 to 0.05 mg/kg) (N = 8) y = 424000 – 4080 r = 1.0000	
			0.1	85 – 99 (91) N = 5	6 @ ~0.1 mg/kg (N = 5)		
	TRI F5 (Spain)	0.001	0.001	100 – 107 (104) N = 3	3 @ ~0.001 mg/kg (N = 3)		
			0.1	85 – 95 (89) N = 3	6 @ ~0.1 mg/kg (N = 3)		
	AGR F7 (Italy)	0.001	0.001	91 – 102 (98) N = 3	6 @ ~0.001 mg/kg (N = 3)		
			0.1	92 – 97 (94) N = 3	3 @ ~0.1 mg/kg (N = 3)		
	BIO F3 (Germany)	0.001	0.001	87 – 88 (87) N = 3	1 @ ~0.001 mg/kg (N = 3)		
			0.1	81 – 84 (83) N = 3	2 @ ~0.1 mg/kg (N = 3)		

1'-COOH-S-2840B 362 → 298 m/z (Confirmation)	ATC F6 (Czech Republic)	0.001	0.001	87 – 98 (95) N = 5	5 @ ~0.001 mg/kg (N = 5)	0.2 – 50 ng/mL (equivalent to 0.0002 to 0.05 mg/kg) (N = 8) y = 23200 – 9.48 r = 0.9999	
			0.1	83 – 101 (90) N = 5	8 @ ~0.1 mg/kg (N = 5)		
	TRI F5 (Spain)	0.001	0.001	106 – 113 (109) N = 3	3 @ ~0.001 mg/kg (N = 3)		
			0.1	83 – 94 (88) N = 3	6 @ ~0.1 mg/kg (N = 3)		
	AGR F7 (Italy)	0.001	0.001	99 – 101 (100) N = 3	1 @ ~0.001 mg/kg (N = 3)		
			0.1	94 – 98 (96) N = 3	2 @ ~0.1 mg/kg (N = 3)		
	BIO F3 (Germany)	0.001	0.001	83 – 94 (89) N = 3	6 @ ~0.001 mg/kg (N = 3)		
			0.1	81 – 84 (83) N = 3	2 @ ~0.1 mg/kg (N = 3)		

Matrix effects

The matrix effects were determined to be significant (>20%) so matrix-matched standards were used for the calibration. No further consideration is required.

Specificity

Specificity was demonstrated by retention time match with a calibration standard and the absence of significant (>30% LOQ) interfering peaks in the chromatogram of a matrix blank sample for all analytes. A retention time match was observed between the calibration standard and the fortified test item for all analytes.

Two mass transitions were validated for the method. Product ion spectrum was provided demonstrating the acceptability of the mass transitions used

Linearity

Inpyrfluxam and 3'-OH-S-2840 (Both quantification and confirmation)

Linearity was demonstrated by the analysis of eight matrix-matched standards of increasing concentration. The range of standard concentrations used was 0.4 – 100 ng/mL, corresponding to 0.0004 mg/kg to 0.1 mg/kg for inpyrfluxam and 3'-OH-S-2840. The concentrations extend over an appropriate range, and the response was linear with a correlation co-efficient of at least 0.9996. If necessary, samples were diluted to within linear range.

1'-COOH-S-2840A and 1'-COOH-S-2840B (Both quantification and confirmation)

Linearity was demonstrated by the analysis of eight matrix-matched standards of increasing concentration. The range of standard concentrations used was 0.2 – 50 ng/mL, corresponding to 0.0002 mg/kg to 0.05 mg/kg for 1'-COOH-S-2840A and 1'-COOH-S-2840B. The concentrations extend over an appropriate range, and the response was linear with a correlation co-efficient of at least 0.9999. If necessary, samples were diluted to within linear range.

Accuracy

Inpyrfluxam and 3'-OH-S-2840 (Both quantification and confirmation)

The accuracy of the method for ATC F6 soil was assessed by analysing five samples fortified with the analyte at concentrations of 0.001 and 0.1 mg/kg. The accuracy of the method for TRI F5, AGR F7 and BIO F3 soil was assessed by analysing three samples fortified with the analyte at concentrations of 0.001 and 0.1 mg/kg. The applicant confirmed that separately weighed samples were used to determine the recovery in response to a request for additional information. Recovery values were calculated as a percentage of measured concentration relative to fortified concentration. Acceptable mean recovery levels were obtained.

1'-COOH-S-2840A and 1'-COOH-S-2840B (Both quantification and confirmation)

The accuracy of the method for ATC F6 soil was assessed by analysing five samples fortified with the analyte at concentrations of 0.002 and 0.2 mg/kg. The accuracy of the method for TRI F5, AGR F7 and BIO F3 soil was assessed by analysing three samples fortified with the analyte at concentrations of 0.002 and 0.2 mg/kg. Recovery values were calculated as a percentage of measured concentration relative to fortified concentration. Acceptable mean recovery levels were obtained (within the range 70 to 120%).

Procedural recoveries

In study report no.:TPR-0088, procedural recoveries were conducted for all analytes (Inpyrfluxam, 3'-OH-S-2840, 1'-COOH-S-2840A and 1'-COOH-S-2840B) using a fortification level 10xLOQ. The mean recoveries were within the acceptable range of 70-120%. The procedural recoveries are acceptable for all analytes.

In study report no TPR-0080, procedural recoveries were conducted for inpyrfluxam using a fortification level 0.002, 0.02 and 0.2 mg/kg (0.2 mg/kg fortification level was diluted to within calibration range). The mean recoveries were within the acceptable range of 70-120%. The procedural recoveries are acceptable for the analyte at both fortification levels

Precision (repeatability)

The precision of the method was assessed via analysis of the accuracy samples. The reported %RSDs were all <20%.

LOQ

The limit of quantitation (LOQ), defined as the lowest fortification level where acceptable precision and accuracy data were obtained, has been demonstrated to be 0.002 mg/kg for Inpyrfluxam and 3'-OH-S-2840 (both quantification and confirmation) and 0.001 mg/kg 1'-COOH-S-2840A and 1'-COOH-S-2840B (both quantification and confirmation).

Stability of standards and extracts

Stability of inpyrfluxam, 3'-OH-S-2840, 1'-COOH-S-2840A and 1'-COOH-S-2840B in sample extracts were tested after storage in the dark at 1 – 10 °C for at least 8 days. The recoveries of the fortified samples were measured against freshly prepared standards. All recoveries were within the acceptable range of 70 – 120 %. The stability of inpyrfluxam, 3'-OH-S-2840, 1'-COOH-S-2840A and 1'-COOH-S-2840B in sample extracts was sufficiently demonstrated after storage in the dark at 1 – 10 °C for at least 8 days.

Additionally, the maximum storage interval of the final sample extracts at 1 to 10 °C from extraction to injection to LC-MS/MS was 1 day.

Inpyrfluxam and 3'-OH-S-2840 standards and stock solutions was demonstrated to be stable for 62 days in methanol when stored in the dark between 1 and 10 °C as the peak areas of the stored then diluted stock solutions were within ±20% of the peak areas of the freshly prepared standards.

1'-COOH-S-2840A and 1'-COOH-S-2840B standards and stock solutions was demonstrated to be stable for 46 days in methanol when stored in the dark between 1 and

10 °C as the peak areas of the stored then diluted stock solutions were within ±20% of the peak areas of the freshly prepared standards.

It is noted that the standards during method validation were frozen, but this considered acceptable with the above data.

Conclusion

The analytical method is validated according to SANTE/2020/12830 rev. 1 for the determination of inpyrfluxam, 3'-OH-S-2840, 1'-COOH-S-2840A and 1'-COOH-S-2840B in soil.

Use of an alternative HPLC column

From the 24th of April 2017 onwards, a different column (Agilent Zorbax Eclipse XDB-C18, 50 x 4.6 mm, 1.8 µm) was used for the quantification of inpyrfluxam, 3'-OH-S-2840, 1'-COOH-S-2840A and 1'-COOH-S-2840B in soil. Validation for this alternate column was provided in report no.: TPR-0085.

The same sample preparation and HPLC-MS/MS conditions were used. The use of a different column resulted in different retention times to the original column:

- 1'-COOH-S-2840A approximately 3.2 minutes
- 1'-COOH-S-2840B approximately 3.5 minutes
- 3'-OH-S-2840 approximately 4.9 minutes
- Inpyrfluxam approximately 5.4 minutes

Only one ion transition was monitored using the alternative column.

Table B.5.1.2.1-5: Summary of method validation for the determination of inpyrfluxam, 3'-OH-S-2840, 1'-COOH-S-2840A and 1'-COOH-S-2840B in soil using an alternative column

Analyte	LOQ (mg/kg)	Recovery fortification level (mg/kg)	Recoveries % range (mean)	Repeatability % RSD (n)	Linearity	Specificity
Inpyrfluxam 334 → 238 m/z	0.002	0.002	100 – 110 (104) N = 5	4 @ ~0.002 mg/kg (N = 5)	0.4 – 50 ng/mL (equivalent to 0.0004 to 0.05 mg/kg) (N = 7) y = 272000x + 31200	Retention time match to reference standard. No significant interfering peaks observed in the blank matrix.
		0.1	87 – 105 (93) N = 5	7 @ ~0.1 mg/kg (N = 5)		

					r = 0.9988	
3'-OH-S-2840 348 → 175 m/z	0.002	0.002	93 – 114 (102) N = 5	9 @ ~0.002 mg/kg (N = 5)	0.4 – 50 ng/mL (equivalent to 0.0004 to 0.05 mg/kg) (N = 7) y = 369000x + 76400 r = 0.9973	
		0.1	91 – 104 (98) N = 5	5 @ ~0.2 mg/kg (N = 5)		
1'-COOH-S-2840A 362 → 318 m/z	0.001	0.001	98 – 113 (104) N = 5	6 @ ~0.001 mg/kg (N = 5)	0.2 – 25 ng/mL (equivalent to 0.0002 to 0.025 mg/kg) (N = 7) y = 137000 + 4760 r = 0.9994	
		0.05	91 – 104 (97) N = 5	5 @ ~0.05 mg/kg (N = 5)		
1'-COOH-S-2840B 362 → 318 m/z	0.001	0.001	95 – 112 (104) N = 5	6 @ ~0.001 mg/kg (N = 5)	0.2 – 25 ng/mL (equivalent to 0.0002 to 0.025 mg/kg) (N = 7) y = 175000 + 3220 r = 0.9993	
		0.05	90 – 104 (95) N = 5	6 @ ~0.05 mg/kg (N = 5)		

Matrix effects

The matrix effects were determined to be significant (>20%) so matrix-matched standards were used for the calibration. No further consideration is required.

Specificity

Specificity was demonstrated by retention time match with a calibration standard and the absence of significant (>30% LOQ) interfering peaks in the chromatogram of a matrix blank sample for all analytes. A retention time match was observed between the calibration standard and the fortified test item for all analytes.

Linearity

Inpyrfluxam and 3'-OH-S-2840
Linearity was demonstrated by the analysis of seven matrix-matched standards of increasing concentration. The range of standard concentrations used was 0.4 – 50 ng/mL. The concentrations extend over an appropriate range, and the response was linear with a correlation co-efficient of at least 0.9973. If necessary, samples were diluted to within linear range.

1'-COOH-S-2840A and 1'-COOH-S-2840B
Linearity was demonstrated by the analysis of seven matrix-matched standards of increasing concentration. The range of standard concentrations used was 0.2 – 25 ng/mL. The concentrations extend over an appropriate range, and the response was linear with a correlation co-efficient of at least 0.9993. If necessary, samples were diluted to within linear range.

Precision (repeatability)

The precision of the method was assessed via analysis of the accuracy samples for all the analytes. The reported %RSDs were all <20%.

Accuracy (recovery)

Inpyrfluxam and 3'-OH-S-2840
The accuracy of the method was assessed by analysing five samples fortified with the analyte at concentrations of 0.002 and 0.1 mg/kg. Recovery values were calculated as a percentage of measured concentration relative to fortified concentration. Acceptable mean recovery levels were obtained (within the range 70 to 120%).

1'-COOH-S-2840A and 1'-COOH-S-2840B
The accuracy of the method was assessed by analysing five samples fortified with the analyte at concentrations of 0.001 and 0.05 mg/kg. Recovery values were calculated as a percentage of measured concentration relative to fortified concentration. Acceptable mean recovery levels were obtained (within the range 70 to 120%).

Procedural recoveries

*In report no.: TPR-0085, p*rocedural recoveries were conducted for inpyrfluxam and 3'-OH-S-2840 using fortification levels of 0.002, 0.02, 0.05, 0.1 and 1 (and 10 mg/kg for inpyrfluxam). Procedural recoveries were conducted for 1'-COOH-S-2840A and 1'-COOH-

S-2840B using fortification levels of 0.001, 0.01, 0.025, 0.05 and 0.5 mg/kg. The mean recoveries were within the range 86-102%. It is unclear which of these results were obtained using which column but as the results are acceptable, further information is not required. The procedural recoveries are acceptable for all analytes at all fortification levels.

LOQ

The limit of quantitation (LOQ), defined as the lowest fortification level where acceptable precision and accuracy data were obtained, has been demonstrated to be 0.002 mg/kg for inpyrfluxam and 3'-OH-S-2840 and 0.001 mg/kg 1'-COOH-S-2840A and 1'-COOH-S-2840B.

Stability of standards and extracts

The stability of standards and extracts was demonstrated for the method using the initial column. No further consideration is required.

Conclusion

The analytical method is validated according to SANTE/2020/12830 rev. 1 for the determination of inpyrfluxam, 3'-OH-S-2840, 1'-COOH-S-2840A and 1'-COOH-S-2840B in soil using the alternative column.

B.5.1.2.2. Methods in soil, water and any additional matrices used in support of efficacy studies

No methods of analysis to support efficacy studies for the active substance have been submitted.

B.5.1.2.3. Methods in feed, body fluids and tissues, air and any additional matrices used in support of toxicological studies

Table B.5.1.2.3-1: Summary of risk assessment methods supporting toxicological studies

Data point	Study	Conclusion	Studies relied on
KCA 4.1.2/05	Validation of analytical method for S-2399 Technical Grade (Polyethylene Glycol 400 Solution) on glass filters.	HPLC-UV Analyte: Inpyrfluxam LOQ: 0.500 mg/filter	KCA 5.2.3/01 Report no. TPT-0015

Data point	Study	Conclusion	Studies relied on
	[redacted] 2015 Report No.: TPA-0044	Acceptable.	
KCA 4.1.2/07	Validation of Analytical Method for S-2399 TG in the Diet for Rodents. [redacted] 2013 Report No.: TPA-0002	HPLC-UV Analyte: Inpyrfluxam LOQ: 8 mg/kg in rodent diet Acceptable.	KCA 5.3.2/01 Report no.: TPT-0048
			KCA 5.3.2/02 Report no.: TPT-0050
			KCA 5.5/01 Report no.: TPT-0090
			KCA 5.5/03 Report no.: TPT-0089
			KCA 5.6.1/02 Report no.: TPT-0088
			KCA 5.7.1/03 Report no.: TPT-0058
KCA 4.1.2/08	Validation of Analytical Method for S-2399 TG in Dog Plasma, Final Report Amendment. [redacted] 2014b/2020 Report No.: TPA-0008	LC-MS/MS Analyte: Inpyrfluxam LOQ: 0.004 mg/L in dog plasma Acceptable.	KCA 5.3.2/04 Report no.: TPT-0076.
KCA 4.1.2/12	Validation of Analytical Method for S-2399 in Rat Plasma, Final Report Amendment.	LC-MS/MS Analyte: Inpyrfluxam LOQ: 0.01 mg/L in rat plasma	KCA 5.5/01 Report no.: TPT-0090

Data point	Study	Conclusion	Studies relied on
	[redacted] 2014a/2020 Report No.: TPA-0005	Acceptable.	
KCA 4.1.2/11	Validation of Analytical Method for S-2399 in Mouse Plasma, Final Report Amendment. [redacted] 2015/2020 Report No.: TPA-0009	LC-MS/MS Analyte: Inpyrfluxam LOQ: 0.004 mg/L in mouse plasma Acceptable.	KCA 5.5/03 Report no.: TPT-0089
KCA 4.1.2/15	3'-OH-S-2840: Validation of Analytical Method in the Diet for Rodents [redacted] 2017 Report No.: TPA-0032	HPLC/UV Analyte: 3'-OH-S-2840 LOQ: 8 mg/kg in rodent diet Acceptable.	KCA 5.8.1.1/02 Report no.: TPT-0127
KCA 4.1.2/44	Reg. No. 5069089 (metabolite of BAS 700 F) Repeated dose 90-day oral toxicity study in Wistar rats; Administration in the diet. [redacted] 2009 Report ID: 2009/1072503	*Stability analysis* HPLC/UV LOQ: N/A Not acceptable but some confidence in results. *Homogeneity and concentration control.* HPLC/UV LOQ: N/A Not acceptable but some confidence in results.	Report ID: 2009/1072503

Data point	Study	Conclusion	Studies relied on
KCA 4.1.2/45	Reg. No. 5069089 (metabolite of BAS 700 F) Prenatal developmental toxicity study in New Zealand white rabbits Oral administration (gavage). [redacted] 2009 Report ID: 2009/1072507	*Stability analysis* HPLC/UV LOQ: N/A Not acceptable but some confidence in results. *Homogeneity and concentration control* HPLC/UV LOQ: N/A Not acceptable but some confidence in results. Note: Two further HPLC/UV methods were summarised in this report, both used in homogeneity and concentration control tests. These were also not acceptable but there is some confidence in results.	Report ID: 2009/1072507
KCA 4.1.2/46	Reg. No. 5435595 (metabolite of BAS 700 F) Repeated dose 90-day oral toxicity study in Wistar rats; Administration in the diet	*Stability analysis* HPLC/UV LOQ: N/A	Report ID: 2009/1012026

Data point	Study	Conclusion	Studies relied on
	[redacted]. 2009 Report ID: 2009/1012026	Not acceptable but some confidence in results. *Homogeneity and concentration control* HPLC/UV LOQ: N/A Not acceptable but some confidence in results.	
KCA 4.1.2/47	Reg. No. 5435595 (metabolite of BAS 700 F) Prenatal developmental toxicity study in New Zealand white rabbits Oral administration (gavage) [redacted]. 2009 Report ID: 2009/1072509	*Stability analysis* HPLC/UV LOQ: N/A Not acceptable but some confidence in results. *Homogeneity and concentration control* HPLC/UV LOQ: N/A Not acceptable but some confidence in results.	Report ID: 2009/1072509

Reference:	KCA 4.1.2/05
Report Title:	Validation of analytical method for S-2399 Technical Grade (Polyethylene Glycol 400 Solution) on glass filters
Author(s) & Year:	[redacted] 2015
Document No, Authority registration No	Study No.: 14002VAL Amendment No. 1 Report No.: TPA-0044
Guideline(s):	Not confirmed
Deviations:	No
GLP or GEP:	Yes
Acceptability:	Yes
Study relied upon:	Yes

The purpose of the study was to demonstrate the validity of a HPLC-UV method for the determination of inpyrfluxam on glass filters.

Principle of the method

One glass filter and 50 mL of acetonitrile were added to a 200 mL glass bottle and sealed with a screw cap. The bottle was put into an ultrasonic bath and inpyrfluxam (on the glass filter) was extracted by ultrasonication for 10 minutes at room temperature. If necessary, the sample was further diluted with acetonitrile. The samples were diluted to be in the range between five times of the lowest concentration of the calibration curve and two thirds of the highest concentration of the calibration curve. The sample is then filtered with a disk filter. The sample is analysed by HPLC-UV under the following conditions.

HPLC-UV conditions

Column	Mightysil RP-18 GP, 5 µm, 4.6 mm x 150 mm
Mobile phase	Trifluoroacetic acid aqueous solution (0.1% v/v): acetonitrile (45:55, v/v)
Column temperature	40 °C

Injection volume	10 µL
Flow rate	1 mL/min
Detector	UV 240 nm
Retention time	~8.0 minutes

Table B.5.1.2.3-2: Summary of method validation for the determination of inpyrfluxam on glass filters

Analyte	LOQ (mg/filter)	Recovery fortification level (mg/filter)	Recoveries % range (mean)	Repeatability % RSD (n)	Linearity	Specificity
Inpyrfluxam	0.500	0.500	98.0 – 98.8 (98.5) N = 5	0.308 @ ~0.500 mg/filter (n=5)	0.002 – 0.1 mg/mL (n = 5) y = 19063418x + 433 R^2 = 1.0	Retention time match to reference standard. No significant interfering peaks observed in the blank matrix.
		20.0	101 – 102 (101) N = 5	0.442 @ ~20.0 mg/filter (n=5)		

Matrix effects

Matrix effects have not been addressed.

Specificity

Specificity was demonstrated by retention time match with a calibration standard and the absence of significant (>30% LOQ) interfering peaks in the chromatogram of a matrix blank sample. A retention time match was observed between the calibration standard and the fortified test item.

Linearity

Linearity was demonstrated by the analysis of five standards of increasing concentration. The range of standard concentrations used was 0.002 – 0.1 mg/mL. The concentrations extend over an appropriate range, and the response was linear with a coefficient of determination (R^2) of 1.0. If necessary, samples were diluted to within linear range. The calibration curve was replotted as no y-intercept was reported. Furthermore, the response of the solvent blank was excluded from the calibration curve.

Precision (repeatability)

The precision of the method was assessed via analysis of the accuracy samples. The reported %RSDs were <20%. It is noted that the system precision was determined by injecting the same sample six times, this has been excluded from the table above.

Accuracy (recovery)

The accuracy of the method was assessed by analysing five samples fortified with inpyrfluxam at concentrations of 0.500 and 20.0 mg/filter. The higher fortification level was diluted by a factor of 10 to fit within the linear range. Recovery values were calculated as a percentage of measured concentration relative to fortified concentration. Acceptable mean recovery levels are within the range 70 to 120%.

Procedural recoveries

In Report TPT-0015*,* the recovery of the method was also determined on the day of analysis of the test samples. One sample was fortified with inpyrfluxam at 6.0 mg/filter. Recovery values were calculated as a percentage of measured concentration relative to fortified concentration. The recovery level was 100% which is acceptable.

LOQ

The limit of quantitation (LOQ), defined as the lowest fortification level where acceptable precision and accuracy data were obtained, has been demonstrated to be 0.5 mg/filter.

Stability of standards and extracts

Stability of inpyrfluxam in sample extracts were tested after storage at room temperature for 24 hours. Five replicate measurements from each level (0.5 and 20 mg/filter) were compared against the initial recovery measurements. All recoveries were within the acceptable range of 70 – 120 %. The stability of inpyrfluxam in sample extracts was sufficiently demonstrated. However, the length of storage of the extracts in the study was not confirmed.

The stability of inpyrfluxam in standards and stock solutions were not addressed.

Conclusion

The analytical method is not acceptably validated according to SANTE/2020/12830 rev. 1 for the determination of the active substance inpyrfluxam on glass filters as the matrix effects have not been determined and the stability of standards and stock solutions have not been addressed. However, the studies were generated prior to the implementation of SANTE 2020/12830 rev.1 and SANCO 3029/99 rev.4 did not require matrix effects and the stabilities of standards to be addressed. As all other validation requirements have been

met (including the minimum validation requirements outlined in SANTE 2020/12830 rev.1), the method is considered to be fit for purpose.

Reference:	KCA 4.1.2/07
Report Title:	Validation of Analytical Method for S-2399 TG in the Diet for Rodents
Author(s) & Year:	[redacted] 2013
Document No, Authority registration No	Study No.: IET 13-5030 Report No.: TPA-0002
Guideline(s):	Not confirmed
Deviations:	No
GLP or GEP:	Yes
Acceptability:	Yes
Study relied upon:	Yes

The purpose of the study was to demonstrate the validity of a HPLC-UV method for the determination of inpyrfluxam in rodent diet.

Principle of the method

Samples of rodent diet (5.00 g) were weighed into a 200 mL Erlenmeyer flask. 100 mL of acetonitrile was added to the flask. The flask was shaken using a reciprocal shaker for 20 minutes. 10 mL of the supernatant was filtered through a disposable disk filter. 4 mL of the filtrate was taken and diluted with 4 mL of acetonitrile: water (50:50, v/v) to prepare the sample solution. Further dilution with acetonitrile: water (50:50, v/v) were performed as necessary. The samples were analysed by HPLC-UV under the following conditions.

HPLC-UV conditions

HPLC System	1260 Infinity (Agilent Technologies, CA, USA)
Column	Symmetry C_{18} (5 µm), 250 mm x 4.6 mm
Mobile phase	Acetonitrile: water: phosphoric acid (65:35:0.1, v/v/v)
Column temperature	40 °C
Injection volume	20 µL
Flow rate	1.0 mL/min
Detector	UV 254 nm
Retention time	~7.0 minutes

Table B.5.1.2.3-3: Summary of method validation for the determination of inpyrfluxam in rodent diet

Analyte	LOQ (mg/kg)	Recovery fortification level (mg/kg)	Recoveries % range (mean)	Repeatability % RSD (n)	Linearity	Specificity
Inpyrfluxam	8	8	105 - 105 (105) N = 5	0 @ ~8 mg/kg (n=5)	0.02 – 1.0 mg/L (n = 5) y = 1.813x – 0.01279 r = 1.0	Retention time match to reference standard. No significant interfering peaks observed in the blank matrix.
		10000	Analyst 1 99 – 100 (99) N = 5	Analyst 1 0.4 @ ~10000 ppm (n=5)		
			Analyst 2 102 – 103 (102) N = 5	Analyst 2 0.5 @ ~10000 ppm (n=5)		

Matrix effects

The matrix effects have not been addressed.

Specificity

Specificity was demonstrated by retention time match with a calibration standard and the absence of significant (>30% LOQ) interfering peaks in the chromatogram of a matrix blank sample. A retention time match was observed between the calibration standard and the fortified test item.

Linearity

Linearity was demonstrated by the analysis of five standards of increasing concentration. The range of standard concentrations used was 0.02 – 1.0 mg/L. The concentrations extend over an appropriate range, and the response was linear with a correlation coefficient (r) of 1.0. If necessary, samples were further diluted to within linear range.

Precision (repeatability)

The precision of the method was assessed via analysis of the accuracy samples. The reported %RSDs were <20%. The precision for the higher fortification level was determined by two different analysts, these data sets have been kept separate.

Accuracy (recovery)

The accuracy of the method was assessed by analysing five samples fortified with inpyrfluxam at concentrations of 8 and 10000 mg/kg. The samples were diluted as necessary. Recovery values were calculated as a percentage of measured concentration relative to fortified concentration. Acceptable mean recovery levels are within the range 70 to 120%.

Procedural recoveries

Recovery tests were performed in reports where this analytical method was used (See Table B.5.1.2.3-1). Recoveries using different concentrations of fortified diet were analysed. Recovery values were calculated as a percentage of measured concentration relative to fortified concentration. Mean recoveries were within from 70-120% and therefore acceptable.

LOQ

The limit of quantitation (LOQ), defined as the lowest fortification level where acceptable precision and accuracy data were obtained, has been demonstrated to be 8 mg/kg.

Stability of standards and extracts

Stability of inpyrfluxam in sample extracts were tested after storage at 4 °C for 24 hours. Five replicate measurements from each level (8 and 10000 mg/kg) were compared against the initial recovery measurements. All recoveries were within the acceptable range of 70 –

120 %. The stability of inpyrfluxam in sample extracts was sufficiently demonstrated. However, the length of storage of the extracts in the study was not confirmed.

The stability of inpyrfluxam in standards and stock solutions were not addressed

Conclusion

The analytical method is not acceptably validated according to SANTE/2020/12830 rev. 1 for the determination of the active substance inpyrfluxam in rodent diet as the matrix effects and have not been determined and the stability of standards and stock solutions have not been addressed. However, the studies were generated prior to the implementation of SANTE 2020/12830 rev.1 and SANCO 3029/99 rev.4 did not require matrix effects and the stabilities of standards to be addressed. As all other validation requirements have been met (including the minimum validation requirements outlined in SANTE 2020/12830 rev.1), the method is considered to be fit for purpose.

Reference:	KCA 4.1.2/08
Report Title:	Validation of Analytical Method for S-2399 TG in Dog Plasma, Final Report Amendment
Author(s) & Year:	[redacted] 2014b/2020
Document No, Authority registration No	Study No.: IET 14-5074 Report No.: TPA-0008
Guideline(s):	SANCO/3029/99 rev.4
Deviations:	No
GLP or GEP:	Yes
Acceptability:	Yes
Study relied upon:	Yes

The purpose of the study was to demonstrate the validity of a LC-MS/MS method for the determination of inpyrfluxam in dog plasma.

Principle of the method

An aliquot (0.25 mL) of plasma was taken into a test tube. 0.1 mL of acetonitrile: water (5:5, v/v) was added to the test tube. The sample was mixed to prepare a sample solution of plasma: acetonitrile: water (5:1:1, v/v/v). A C_{18} cartridge was pre-conditioned by sequential washing with 5 mL of acetonitrile and 5 mL of water. 5 mL of water was added to the sample solution and mixed. The mixed solution is passed through the C_{18} cartridge. The inside of the test tube was washed with 5 mL of acetonitrile/water (4:6, v/v). The washed solution was passed through the C_{18} cartridge. These eluates were discarded. 7 mL of acetonitrile/water (6:4, v/v) was passed through the C_{18} cartridge. This eluate was collected in a test tube. The collected eluate was made up to 10 mL by adding acetonitrile/water (6:4, v/v). This solution

was further diluted with acetonitrile/water (6:4, v/v) if needed. The sample is then analysed by LC-MS/MS under the following conditions.

LC-MS/MS Conditions

Column	ZORBAX Eclipse Plus C18 (Agilent Technologies), Inner diameter 2.1 mm, length 150 mm, particle size 3.5 µm
Mobile phase	Acetonitrile – 5 mmol/L ammonium acetate (6:4, v/v)
Column temperature	40 °C
Injection volume	5 µL
Flow rate	0.2 mL/min
Retention time	~6.1 minutes
Ionization method	Electrospray ionization (ESI) Positive mode
Monitoring Ion	Precursor ion: m/z 334.2 Product ion: m/z 238.1
Ion detection method	MRM

Table B.5.1.2.3-4: Summary of method validation for the determination of inpyrfluxam in dog plasma

Analyte	LOQ (mg/L)	Recovery fortification level (mg/L)	Recoveries % range (mean)	Repeatability % RSD (n)	Linearity	Specificity
Inpyrfluxam	0.004	0.004	93 - 96 (95) N = 5	1.2 @ ~0.004 mg/L (n=5)	0.05 – 2.0 µg/L (n = 5) y = 1411.6x + 2.62 r = 0.999998	Retention time match to reference standard. No significant interfering peaks observed in the blank matrix.
		0.4	95 – 96 (95) N = 5	0.6 @ ~0.4 mg/L (n=5)		

Matrix effects

Matrix effects have not been addressed.

Specificity

Specificity was demonstrated by retention time match with a calibration standard and the absence of significant (>30% LOQ) interfering peaks in the chromatogram of a matrix blank sample. A retention time match was observed between the calibration standard and the fortified test item.

Linearity

Linearity was demonstrated by the analysis of five standards of increasing concentration. The range of standard concentrations used was 0.05 – 2.0 µg/L. The concentrations extend over an appropriate range, and the response was linear with a correlation coefficient (r) of 0.999998. If necessary, samples were further diluted to within linear range.

Precision (repeatability)

The precision of the method was assessed via analysis of the accuracy samples. The reported %RSDs were <20%.

Accuracy (recovery)

The accuracy of the method was assessed by analysing five samples fortified with inpyrfluxam at concentrations of 0.004 and 0.4 mg/L. The samples were diluted as necessary. Recovery values were calculated as a percentage of measured concentration relative to fortified concentration. Acceptable mean recovery levels are within the range 70 to 120%.

Procedural recoveries

In study TPT-0076, the recovery of the method was also determined on the day of analysis of the test samples. On each day of analysis of the test items, samples were fortified with 0.04 µg/L of inpyrfluxam 0, 2, 4, 7 and 24 hours after administration of the dose. Recovery values were calculated as a percentage of measured concentration relative to fortified concentration. The recovery levels were all within the range 85-102% which is acceptable.
A recovery test was also performed, with five determinations of sample fortified at 2 µg/L. This resulted in a mean recovery of 93% which is acceptable.

LOQ

The limit of quantitation (LOQ), defined as the lowest fortification level where acceptable precision and accuracy data were obtained, has been demonstrated to be 0.004 mg/L.

Stability of standards and extracts

Stability of inpyrfluxam in sample solutions were tested after storage at ≤ -20 °C for 20 days. The recoveries of three replicate samples (fortification level 0.4 mg/L) were determined and compared to the initial fortification level. All recoveries were within the acceptable range of 70 – 120 %. However, this was conducted before the extraction of the analyte and therefore extract stability has not been fully addressed.

The stability of standards and stock solutions have not been addressed.

Conclusion

The analytical method is not acceptably validated according to SANTE/2020/12830 rev. 1 for the determination of the active substance inpyrfluxam in dog plasma as the matrix effects have not been determined and the stability of extracts, standards and stock solutions have not been addressed. However, the studies were generated prior to the implementation of SANTE 2020/12830 rev.1 and SANCO 3029/99 rev.4 did not require matrix effects and the stabilities of extracts, stocks and standards to be addressed. As all other validation requirements have been met (including the minimum validation requirements outlined in SANTE 2020/12830 rev.1), the method is considered to be fit for purpose.

Reference:	KCA 4.1.2/12
Report Title:	Validation of Analytical Method for S-2399 in Rat Plasma, Final Report Amendment
Author(s) & Year:	[redacted] 2014a/2020
Document No, Authority registration No	Study No.: IET 14-5051 Report No.: TPA-0005
Guideline(s):	SANCO/3029/99 rev.4
Deviations:	No
GLP or GEP:	Yes
Acceptability:	Yes
Study relied upon:	Yes

The purpose of the study was to demonstrate the validity of a LC-MS/MS method for the determination of inpyrfluxam in rat plasma.

Principle of the method

An aliquot (0.1 mL) of plasma was taken into a test tube. 0.1 mL of acetonitrile: water (5:5, v/v) was added to the test tube. The sample was mixed to prepare a sample solution of plasma: acetonitrile: water (10:5:5, v/v/v). A C_{18} cartridge was pre-conditioned by sequential washing with 5 mL of acetonitrile and 5 mL of water. 5 mL of water was added to the sample

solution and mixed. The mixed solution is passed through the C_{18} cartridge. The inside of the test tube was washed with 5 mL of acetonitrile/water (4:6, v/v). The washed solution was passed through the C_{18} cartridge. These eluates were discarded. 7 mL of acetonitrile/water (6:4, v/v) was passed through the C_{18} cartridge. This eluate was collected in a test tube. The collected eluate was made up to 10 mL by adding acetonitrile/water (6:4, v/v). This solution was further diluted with acetonitrile/water (6:4, v/v) if needed. The sample is then analysed by LC-MS/MS under the following conditions.

LC-MS/MS Conditions

Column	ZORBAX Eclipse Plus C18 (Agilent Technologies), Inner diameter 2.1 mm, length 150 mm, particle size 3.5 µm
Mobile phase	Acetonitrile – 5 mmol/L ammonium acetate (6:4, v/v)
Column temperature	40 °C
Injection volume	5 µL
Flow rate	0.2 mL/min
Retention time	~6.2 minutes
Ionization method	Electrospray ionization (ESI) Positive mode
Monitoring Ion	Precursor ion: m/z 334.2 Product ion: m/z 238.1
Ion detection method	MRM

Table B.5.1.2.3-5: Summary of method validation for the determination of inpyrfluxam in rat plasma

Analyte	LOQ (mg/L)	Recovery fortification level (mg/L)	Recoveries % range (mean)	Repeatability % RSD (n)	Linearity	Specificity
Inpyrfluxam	0.01	0.01	85 – 91 (89) N = 5	2.8 @ ~0.01 mg/L (n=5)	0.05 – 2.0 µg/L (n = 5) y = 1074.4x + 70.0 r = 0.99986	Retention time match to reference standard. No significant interfering peaks observed in the blank matrix.
		1	93 – 95 (94) N = 5	0.8 @ ~1 mg/L (n=5)		

Matrix effects

Matrix effects have not been addressed.

Specificity

Specificity was demonstrated by retention time match with a calibration standard and the absence of significant (>30% LOQ) interfering peaks in the chromatogram of a matrix blank sample. A retention time match was observed between the calibration standard and the fortified test item.

Linearity

Linearity was demonstrated by the analysis of five standards of increasing concentration. The range of standard concentrations used was 0.05 – 2.0 µg/L. The concentrations extend over an appropriate range, and the response was linear with a correlation coefficient (r) of 0.99986. If necessary, samples were further diluted to within linear range.

Precision (repeatability)

The precision of the method was assessed via analysis of the accuracy samples. The reported %RSDs were <20%.

Accuracy (recovery)

The accuracy of the method was assessed by analysing five samples fortified with inpyrfluxam at concentrations of 0.01 and 1 mg/L. The samples were diluted as necessary. Recovery values were calculated as a percentage of measured concentration relative to fortified concentration. Acceptable mean recovery levels are within the range 70 to 120%.

Procedural recoveries

In study TPT-0090, the recovery of the method was also determined on the day of analysis of the test samples. On each day of analysis of the test items, samples were fortified with 0.1 mg/L of inpyrfluxam for each dose level. Recovery values were calculated as a percentage of measured concentration relative to fortified concentration. The recovery levels were all within the range 90-108% which is acceptable.

LOQ

The limit of quantitation (LOQ), defined as the lowest fortification level where acceptable precision and accuracy data were obtained, has been demonstrated to be 0.01 mg/L.

Stability of standards and extracts

Stability of inpyrfluxam in sample solutions were tested after storage at ≤ -20 °C for 30 days. The recoveries of three replicate samples (fortification level 1 mg/L) were determined and compared to the initial fortification level. All recoveries were within the acceptable range of

70 – 120 %. However, this was conducted before the extraction of the analyte and therefore extract stability has not been fully addressed.

The stability of standards and stock solutions have not been addressed.

Conclusion

The analytical method is not acceptably validated according to SANTE/2020/12830 rev. 1 for the determination of the active substance inpyrfluxam in rat plasma as the matrix effects have not been determined and the stability of extracts, standards and stock solutions have not been addressed. However, the studies were generated prior to the implementation of SANTE 2020/12830 rev.1 and SANCO 3029/99 rev.4 did not require matrix effects and the stabilities of extracts, standards and stock solutions to be addressed. As all other validation requirements have been met (including the minimum validation requirements outlined in SANTE 2020/12830 rev.1), the method is considered to be fit for purpose.

Reference:	KCA 4.1.2/11
Report Title:	Validation of Analytical Method for S-2399 in Mouse Plasma, Final Report Amendment
Author(s) & Year:	[redacted] 2015/2020
Document No, Authority registration No	Study No.: IET 14-5052 Report No.: TPA-0009
Guideline(s):	SANCO/3029/99 rev.4
Deviations:	No
GLP or GEP:	Yes
Acceptability:	Yes
Study relied upon:	Yes

The purpose of the study was to demonstrate the validity of a LC-MS/MS method for the determination of inpyrfluxam in mouse plasma.

Principle of the method

An aliquot (0.25 mL) of plasma was taken into a test tube. 0.1 mL of acetonitrile: water (5:5, v/v) was added to the test tube. The sample was mixed to prepare a sample solution of plasma: acetonitrile: water (5:1:1, v/v/v). A C_{18} cartridge was pre-conditioned by sequential washing with 5 mL of acetonitrile and 5 mL of water. 5 mL of water was added to the sample solution and mixed. The mixed solution is passed through the C_{18} cartridge. The inside of the test tube was washed with 5 mL of acetonitrile/water (4:6, v/v). The washed solution was passed through the C_{18} cartridge. These eluates were discarded. 7 mL of acetonitrile/water (6:4, v/v) was passed through the C_{18} cartridge. This eluate was collected in a test tube. The collected eluate was made up to 10 mL by adding acetonitrile/water (6:4, v/v). This solution

was further diluted with acetonitrile/water (6:4, v/v) if needed. The sample is then analysed by LC-MS/MS under the following conditions.

LC-MS/MS Conditions

Column	ZORBAX Eclipse Plus C18 (Agilent Technologies), Inner diameter 2.1 mm, length 150 mm, particle size 3.5 µm
Mobile phase	Acetonitrile – 5 mmol/L ammonium acetate (6:4, v/v)
Column temperature	40 °C
Injection volume	5 µL
Flow rate	0.2 mL/min
Retention time	~6.1 minutes
Ionization method	Electrospray ionization (ESI) Positive mode
Monitoring Ion	Precursor ion: m/z 334.2 Product ion: m/z 238.1
Ion detection method	MRM

Table B.5.1.2.3-6: Summary of method validation for the determination of inpyrfluxam in mouse plasma

Analyte	LOQ (mg/L)	Recovery fortification level (mg/L)	Recoveries % range (mean)	Repeatability % RSD (n)	Linearity	Specificity
Inpyrfluxam	0.004	0.004	94 – 96 (95) N = 5	0.9 @ ~0.004 mg/L (n=5)	0.05 – 2.0 µg/L (n = 5) y = 2136.6 + 6.56 r = 0.99992	Retention time match to reference standard. No significant interfering peaks observed in the blank matrix.
		0.4	98 – 101 (100) N = 5	1.3 @ ~0.4 mg/L (n=5)		

Matrix effects

The matrix effects have not been addressed.

Specificity

Specificity was demonstrated by retention time match with a calibration standard and the absence of significant (>30% LOQ) interfering peaks in the chromatogram of a matrix blank sample. A retention time match was observed between the calibration standard and the fortified test item.

Linearity

Linearity was demonstrated by the analysis of five standards of increasing concentration. The range of standard concentrations used was 0.05 – 2.0 µg/L. The concentrations extend over an appropriate range, and the response was linear with a correlation coefficient (r) of 0.99992. If necessary, samples were further diluted to within linear range.

Precision (repeatability)

The precision of the method was assessed via analysis of the accuracy samples. The reported %RSDs were <20%.

Accuracy (recovery)

The accuracy of the method was assessed by analysing five samples fortified with inpyrfluxam at concentrations of 0.004 and 0.4 mg/L. The samples were diluted as necessary. Recovery values were calculated as a percentage of measured concentration relative to fortified concentration. Acceptable mean recovery levels are within the range 70 to 120%.

Procedural recoveries

In study TPT-0089, the recovery of the method was also determined on the day of analysis of the test samples. On each day of analysis of the test items, samples were fortified with 0.04 mg/L of inpyrfluxam for each dose level. Recovery values were calculated as a percentage of measured concentration relative to fortified concentration. The recovery levels were all within the range 97-100% which is acceptable.

LOQ

The limit of quantitation (LOQ), defined as the lowest fortification level where acceptable precision and accuracy data were obtained, has been demonstrated to be 0.004 mg/L.

Stability of standards and extracts

Stability of inpyrfluxam in sample solutions were tested after storage at ≤ -20 °C for 30 days. The recoveries of three replicate samples (fortification level 0.4 mg/L) were determined and compared to the initial fortification level. All recoveries were within the acceptable range of 70 – 120 %. However, this was conducted before the extraction of the analyte and therefore extract stability has not been fully addressed.

The stability of standards and stock solutions has not been addressed.

Conclusion

The analytical method is not acceptably validated according to SANTE/2020/12830 rev. 1 for the determination of the active substance inpyrfluxam in mouse plasma as the matrix effects have not been determined and the stability of extracts, standards and stock solutions have not been addressed. However, the studies were generated prior to the implementation of SANTE 2020/12830 rev.1 and SANCO 3029/99 rev.4 did not require matrix effects and the stabilities of extract, stock and standard solutions to be addressed. As all other validation requirements have been met (including the minimum validation requirements outlined in SANTE 2020/12830 rev.1), the method is fit for purpose.

Reference:	KCA 4.1.2/15
Report Title:	3'-OH-S-2840: Validation of Analytical Method in the Diet for Rodents
Author(s) & Year:	[redacted] 2017
Document No, Authority registration No	Study No.: IET 17-5003 Report No.: TPA-0032
Guideline(s):	SANCO/3029/99 rev.4
Deviations:	No
GLP or GEP:	Yes
Acceptability:	Yes
Study relied upon:	Yes

The purpose of the study was to demonstrate the validity of a HPLC-UV method for the determination of 3'-OH-S-2840 in rodent diet.

Principle of the method

Samples of rodent diet (5.00 g) were weighed into a 200 mL Erlenmeyer flask. 100 mL of acetonitrile was added to the flask. The flask was shaken using a reciprocal shaker for 20 minutes. 10 mL of the supernatant was filtered through a disposable disk filter. The samples were analysed by HPLC-UV under the following conditions.

HPLC-UV conditions

HPLC System	1260 Infinity (Agilent Technologies, CA, USA)
Column	L-column ODS (5 µm), inner diameter 4.6 mm, length 250 mm

Mobile phase	Acetonitrile: water: phosphoric acid (60:40:0.1, v/v/v)
Column temperature	40 °C
Injection volume	10 µL
Flow rate	1.0 mL/min
Detector	UV 265 nm
Retention time	~7.0 minutes

Table B.5.1.2.3-7: Summary of method validation for the determination of 3'-OH-S-2840 in rodent diet

Analyte	LOQ (mg/kg)	Recovery fortification level (mg/kg)	Recoveries % range (mean)	Repeatability % RSD (n)	Linearity	Specificity
3'-OH-S-2840	8	8	100 - 100 (100) N = 5	0 @ ~8 ppm (n=5)	0.04 – 2.0 mg/L (n = 5) y = 2.52077x – 0.0124617 r = 1.0	Retention time match to reference standard. No significant interfering peaks observed in the blank matrix.
		24000	Analyst 1 99 – 101 (100) N = 5	Analyst 1 0.7 @ ~24000 ppm (n=5)		
			Analyst 2 98 – 100 (99) N = 5	Analyst 2 0.8 @ ~24000 ppm (n=5)		

Matrix effects

Matrix effects have not been addressed.

Specificity

Specificity was demonstrated by retention time match between a calibration standard and the fortified test item, and the absence of significant (>30% LOQ) interfering peaks in the chromatogram of a matrix blank sample.

Linearity

Linearity was demonstrated by the analysis of five standards of increasing concentration. The range of standard concentrations used was 0.04 – 2.0 mg/L. The concentrations extend over an appropriate range, and the response was linear with a correlation coefficient (r) of 1.0. If necessary, samples were further diluted to within linear range.

Precision (repeatability)

The precision of the method was assessed via analysis of the accuracy samples. The reported %RSDs were <20%. The precision for the higher fortification level was determined by two different analysts, these data sets have been kept separate.

Accuracy (recovery)

The accuracy of the method was assessed by analysing five samples fortified with 3'-OH-S-2840 at concentrations of 8 and 24000 mg/kg. The samples were diluted as necessary. Recovery values were calculated as a percentage of measured concentration relative to fortified concentration. Acceptable mean recovery levels are within the range 70 to 120%.

Procedural recoveries

The recovery was also determined in study TPT-0127 at 500, 2000 and 4000 mg/kg (3 samples at each level). The mean recovery levels were within the acceptable range 70 to 120%.

LOQ

The limit of quantitation (LOQ), defined as the lowest fortification level where acceptable precision and accuracy data were obtained, has been demonstrated to be 8 mg/kg.

Stability of standards and extracts

After the first injections of 8 and 24000 mg/kg samples solutions prepared by the first analyst, the sample solutions in the vials were left in the rack of automatic sample injector (set at 4°C) for about 24 hours, and then second injections were performed. Recoveries, mean recoveries and the RSDs for the second measurement were calculated. The differences of the mean recoveries between the first and second measurements were 1% and therefore acceptable.

The stability of standards and stocks solutions has not been addressed.

Conclusion

The analytical method is not acceptably validated according to SANTE/2020/12830 rev. 1 for the determination of 3'-OH-S-2840 in rodent diet as the matrix effects have not been determined and the stability of standards and stock solutions has not been addressed. However, the studies were generated prior to the implementation of SANTE 2020/12830 rev.1 and SANCO 3029/99 rev.4 did not require matrix effects and the stabilities of standards and stock solutions to be addressed. As all other validation requirements have been met (including the minimum validation requirements outlined in SANTE 2020/12830 rev.1), the method is fit for purpose.

Reference:	KCA 4.2.1/44
Report Title:	Reg. No. 5069089 (metabolite of BAS 700 F) Repeated dose 90-day oral toxicity study in Wistar rats; Administration in the diet.
Author(s) & Year:	[redacted] 2009
Document No, Authority registration No	[redacted] DocID 2009/1072503
Guideline(s):	Not confirmed
Deviations:	None
GLP or GEP:	Yes
Acceptability:	No
Study relied upon:	Yes

This study includes two analytical methods of analysis. One was used to analyse the stability of the metabolite in kliba lab diet mouse/rat and the other was to analyse the homogeneity and concentration control of the metabolite in kliba lab diet mouse/rat.

Stability analysis

Principle of method

A 10g aliquot of each sample was weighed directly into an extraction thimble. The extraction thimble was placed in a Soxhlet extractor. The sample was extracted for 5 hours using 250 mL round bottomed flask containing 100 mL methanol as the extractant heated using a heating mantle. The extracts were transferred to a 100 mL volumetric flask and made up to the mark with methanol. One aliquot of these extracts was then filtered and injected directly into the HPLC equipment.

Method conditions

Column	Phenomenex Luna 3µ C18 (250x3mm)
Eluent	15% acetonitrile + 0.5 M sulphuric acid (1000 + 5 ml) 85% doubly distilled water + 0.5 sulphuric acid (1000 + 5 ml)
Flow rate	0.6 ml/min
Column temperature	Ambient
Injection volume	5 µl

Detection	UV, 220 nm

Conclusion

Demonstration of linearity has been provided in the study in the form of a linear plot, covering the range which is suitable for the determination of the levels required. The report demonstrates some degree of selectivity in the form of chromatograms of a blank and reference standard. However, it's not been fully addressed due to an unexplained shift in retention time of the metabolite in the test sample at timepoint 0.
Acceptable recovery has not been demonstrated. The results of the test samples indicate that the level found is in line with the applied dose but this isn't enough to address the lack of acceptable recoveries.

The minimum validation requirements set out in section 4.2 of SANTE/2020/12830, Rev.1 have not been meet and therefore this method cannot be considered acceptably validated but there is some confidence in the results based of the data provided.

Homogeneity and concentration control

Principle of the method

A 10g or 5g aliquots of each sample was weighed directly into an extraction thimble. The extraction thimble was placed in a Soxhlet extractor. The sample was extracted for 5 hours using 250 mL round bottomed flask containing 100 mL methanol as the extractant heated using a heating mantle. The extracts were transferred to a 100 mL volumetric flask and made up to the mark with methanol. Some samples were further diluted with methanol. One aliquot of these extracts was then filtered and injected directly into the HPLC equipment.

Method conditions:

Column	Phenomenex Luna 3µ C18 (250x3mm) (used on 16 March 2009) Phenomenex Luna 5µ C18 (250x3mm) (used for all other analysis)
Eluent	15% acetonitrile + formic acid (1000 + 5 ml) 85% doubly distilled water + formic acid (1000 + 5 ml)
Flow rate	0.6 ml/min
Column temperature	Ambient

Injection volume	2 µl, 5 µl
Detection	UV, 220 nm

Conclusion

Demonstration of linearity has been provided in the study in the form of a linear plot, covering the range which is suitable for the determination of the levels required. The report demonstrates some degree of selectivity in the form of chromatograms of a blank and reference standard.
Acceptable recovery has not been demonstrated. The results of the test samples indicate that the level found is in line with the applied dose but this isn't enough to address the lack of acceptable recoveries.

The minimum validation requirements set out in section 4.2 of SANTE/2020/12830, Rev.1 have not been meet and therefore this method cannot be considered acceptably validated but there is some confidence in the results based of the data provided.

Reference:	KCA 4.1.2/45
Report Title:	Reg. No. 5069089 (metabolite of BAS 700 F) Prenatal developmental toxicity study in New Zealand white rabbits Oral administration (gavage)
Author(s) & Year:	[redacted] 2009
Document No, Authority registration No	[redacted] DocID 2009/1072507
Guideline(s):	Not confirmed
Deviations:	No
GLP or GEP:	Yes
Acceptability:	No
Study relied upon:	Yes

This study includes two analytical methods of analysis. One was used to analyse the stability of the metabolite in drinking water with 1% carboxymethyl cellulose and the other was to analyse the homogeneity and concentration control of the metabolite in drinking water with 1% carboxymethyl cellulose. There are also two summary reports in the appendix that also rely on analytical methods for homogeneity and concentration control test in 1% carboxymethyl cellulose in drinking water:

- Reg. No. 5069089 (metabolite of 700F). Maternal toxicity study in Himalayan Rabbits (Range-Finding) Oral Administration (Gavage). Project No.: 20R0451/07114.
- Reg. No. 5069089 (metabolite of 700F). Maternal toxicity study in New Zealand White Rabbits (range-finding) Oral administration (gavage). Project No: 20R0451/07120.

Stability analysis

Principle of method

The test substance was diluted in drinking water with 1% carboxymethyl cellulose under stirring (2 min) and subsequent sonification (5 min). The sample was split into two parts, one was stored at ambient temperature and the other one stored in the refrigerator. To the given time points aliquots were diluted with acetonitrile/highly deionized water 1/1 (v/) at a ratio of 1:5 and used for HPLC-UV analysis (wit external calibration).

Method conditions

Column	Phenomenex Luna 3µ C18 (250x3mm)
Eluent	15% acetonitrile + 0.5 M sulphuric acid (1000 + 5 ml) 85% doubly distilled water + 0.5 sulphuric acid (1000 + 5 ml)
Flow rate	0.6 ml/min
Column temperature	Ambient
Injection volume	5 µl
Detection	UV, 220 nm

Conclusion

Demonstration of linearity has been provided in the study in the form of a linear plot, covering the range which is suitable for the determination of the levels required. The report demonstrates some degree of selectivity in the form of chromatograms of a blank and reference standard.
Acceptable recovery has not been demonstrated. The results of the test samples indicate that the level found is in line with the applied dose but this isn't enough to address the lack of acceptable recoveries.

The minimum validation requirements set out in section 4.2 of SANTE/2020/12830, Rev.1 have not been meet and therefore this method cannot be considered acceptably validated but there is some confidence in the results based of the data provided.

Homogeneity and concentration control analysis

Principle of method

Samples were diluted with acetonitrile/highly deionized water 1/1 (v/v) into measuring flasks and filled up to a total volume of 100 ml. Aliquots of the samples were used for HPLC-UV analysis (with external calibrations).

Method conditions

Column	Phenomenex Luna 5µ C18 (250x3mm) (06 March 2009 and 07 May 2009) Phenomenex Luna 3µ C18 (250x3mm) (used on 13 March 2009)
Eluent	15% acetonitrile + formic acid (1mL/L) 85% doubly distilled water + formic acid (1mL/L)
Flow rate	0.6 ml/min
Column temperature	Ambient
Injection volume	2 µl, 5 µl
Detection	UV, 220 nm

Conclusion

Demonstration of linearity has been provided in the study in the form of a linear plot, covering the range which is suitable for the determination of the levels required. The report demonstrates some degree of selectivity in the form of chromatograms of a blank and reference standard.
Acceptable recovery has not been demonstrated. The results of the test samples indicate that the level found is in line with the applied dose but this isn't enough to address the lack of acceptable recoveries.

The minimum validation requirements set out in section 4.2 of SANTE/2020/12830, Rev.1 have not been meet and therefore this method cannot be considered acceptably validated but there is some confidence in the results based of the data provided.

Reg. No. 5069089 (metabolite of 700F). Maternal toxicity study in Himalayan Rabbits (Range-Finding) Oral Administration (Gavage). Project No.: 20R0451/07114.

Homogeneity and concentration control analysis.

Principle of method

Samples were transferred with acetonitrile/highly deionized water 1/1 (v/v) into 100 ml or 200 mL measuring flasks and filled up to the calibration mark. Some samples were further diluted with acetonitrile/highly deionized water 1/1 (v/v) at a ratio of 1:1 (v/v). Aliquots were used for HPLC-UV analysis (with external calibration).

Method conditions:

Column	Phenomenex Luna 3µ C18 (250x3mm)
Eluent	15% acetonitrile + 0.5 M sulphuric acid (5ml/L) 85% doubly distilled water + 0.5 sulphuric acid (5ml/L)
Flow rate	0.6 ml/min
Column temperature	Ambient
Injection volume	5 µl
Detection	UV, 220 nm

Conclusion

Demonstration of linearity has been provided in the study in the form of a linear plot, covering the range which is suitable for the determination of the levels required. The report demonstrates some degree of selectivity in the form of chromatograms of a blank and reference standard.
Acceptable recovery has not been demonstrated. Acceptable recovery has not been demonstrated. The results of the test samples indicate that the level found is in line with the applied dose but this isn't enough to address the lack of acceptable recoveries.

The minimum validation requirements set out in section 4.2 of SANTE/2020/12830, Rev.1 have not been meet and therefore this method cannot be considered acceptably validated but there is some confidence in the results based of the data provided.

Reg. No. 5069089 (metabolite of 700F). Maternal toxicity study in New Zealand White Rabbits (range-finding) Oral administration (gavage). Project No: 20R0451/07120

Homogeneity and concentration control analysis.

Principle of method

Samples were diluted with acetonitrile/highly deionized water 1/1 (v/v) into measuring flasks and filled up to a total volume of 100 ml or 200 mL. Some samples were further diluted with acetonitrile/highly deionized water 1/1 (v/v) at a ratio of 1:5 (v/v). Aliquots were used for HPLC-UV analysis (with external calibration)

Method conditions:

Column	Phenomenex Luna 5µ C18 (250x3mm) (06 March 2009) Phenomenex Luna 3µ C18 (250x3mm) (used on 11 March 2009)
Eluent	15% acetonitrile + formic acid (1mL/L) 85% doubly distilled water + formic acid (1mL/L)
Flow rate	0.6 ml/min
Column temperature	Ambient
Injection volume	2 µl, 5 µl
Detection	UV, 220 nm

Conclusion

Demonstration of linearity has been provided in the study in the form of a linear plot, covering the range which is suitable for the determination of the levels required. The report demonstrates some degree of selectivity in the form of chromatograms of a blank and reference standard.
Acceptable recovery has not been demonstrated. The results of the test samples indicate that the level found is in line with the applied dose but this isn't enough to address the lack of acceptable recoveries.

The minimum validation requirements set out in section 4.2 of SANTE/2020/12830, Rev.1 have not been meet and therefore this method cannot be considered acceptably validated but there is some confidence in the results based of the data provided.

Reference:	KCA 4.1.2/46
Report Title:	Reg. No. 5435595 (metabolite of BAS 700 F) Repeated dose 90-day oral toxicity study in Wistar rats; Administration in the diet
Author(s) & Year:	[redacted]. 2009
Document No, Authority registration No	[redacted] DocID 2009/1012026
Guideline(s):	Not confirmed
Deviations:	No
GLP or GEP:	Yes
Acceptability:	No
Study relied upon:	Yes

This study includes two analytical methods of analysis. One was used to analyse the stability of the metabolite in kliba lab diet mouse/rat and the other was to analyse the homogeneity and concentration control of the metabolite in kliba lab diet mouse/rat.

Stability analysis

Principle of method

A 10g aliquot of each sample was weighed directly into an extraction thimble. The extraction thimble was placed in a Soxhlet extractor. The sample was extracted for 5 hours using 250 mL round bottomed flask containing 100 mL methanol as the extractant heated using a heating mantle. The extracts were transferred to a 100 mL volumetric flask and made up to the mark with methanol. One aliquot of these extracts was then filtered and injected directly into the HPLC equipment.

Method conditions

Column:	Symmetry Shield RP 18, 250 x 4.6 mm
Eluent:	A: Doubly distilled water + HCOOH (1000+1 ml)
	B: Acetonitrile + HCOOH (1000 + 1 mL)

	Time (min)	A (%)	B (%)
	0	100	0
	15	50	50
	16	10	90
	35	10	90
	45	100	0
Flow rate:	1.2 mL/min		
Column temp:	Ambient		
Injection volume:	10µL		
Detection:	UV, 220 nm		

Conclusion

Demonstration of linearity has been provided in the study in the form of a linear plot, covering the range which is suitable for the determination of the levels required. The report demonstrates some degree of selectivity in the form of chromatograms of a blank and reference standard.
Acceptable recovery has not been demonstrated. The results of the test samples indicate that the level found is in line with the applied dose but this isn't enough to address the lack of acceptable recoveries.

The minimum validation requirements set out in section 4.2 of SANTE/2020/12830, Rev.1 have not been meet and therefore this method cannot be considered acceptably validated but there is some confidence in the results based of the data provided.

Homogeneity and concentration control

Principle of method

A 10g aliquot of each sample was weighed directly into an extraction thimble. The extraction thimble was placed in a Soxhlet extractor. The sample was extracted for 5 hours using 250 mL round bottomed flask containing 100 mL methanol as the extractant heated using a heating mantle. The extracts were transferred to a 100 mL volumetric flask and made up to the mark with methanol. One aliquot of these extracts was then filtered and injected directly into the HPLC equipment.

Method conditions

Column:	Symmetry Shield RP 18, 250 x 4.6 mm
Eluent:	A: Acetonitrile + formic acid (1000 + 1 mL)
	B: Doubly distilled water + formic acid (1000+1 ml)
	(For samples from beginning of the study)

Time (min)	A (%)	B (%)
0	0	100
15	50	50
16	90	10
35	90	10
45	0	100
50	0	100

(For samples from beginning of the study)

Eluent:	20% acetonitrile + sulfuric acid 0.5M (1000+5 ml)
	80% doubly distilled water + sulphuric acid 0.5 M (1000+5 ml)
	(for samples from the end of the study)
Flow rate:	1.2 mL/min
Column temp:	Ambient
Injection volume:	1, 5, 10µL
Detection:	UV, 220 nm

Conclusion

Demonstration of linearity has been provided in the study in the form of a linear plot, covering the range which is suitable for the determination of the levels required. The report demonstrates some degree of selectivity in the form of chromatograms of a blank and reference standard.
Acceptable recovery has not been demonstrated. The results of the test samples indicate that the level found is in line with the applied dose but this isn't enough to address the lack of acceptable recoveries.

The minimum validation requirements set out in section 4.2 of SANTE/2020/12830, Rev.1 have not been meet and therefore this method cannot be considered acceptably validated but there is some confidence in the results based of the data provided.

Reference:	KCA 4.1.2/47
Report Title:	Reg. No. 5435595 (metabolite of BAS 700 F) Prenatal developmental toxicity study in New Zealand white rabbits Oral administration (gavage)
Author(s) & Year:	[redacted] 2009
Document No, Authority registration No	[redacted] DocID 2009/1072509
Guideline(s):	Not confirmed
Deviations:	No
GLP or GEP:	Yes
Acceptability:	No
Study relied upon:	Yes

This study includes two analytical methods of analysis. One was used to analyse the stability of the metabolite in drinking water with 1% carboxymethyl cellulose and the other was to analyse the homogeneity and concentration control of the metabolite in drinking water with 1% carboxymethyl cellulose.

Stability analysis

Principle of method

Test substance was diluted in drinking water with 1% carboxymethyl cellulose under stirring for 15 min and subsequent sonification (5 min). The sample was split into two parts, one part was stored at room temperature and the othe rone in the refridgerator. After the requested timepoints, aliquots were diluted with acetonitrile/highly deionized water 1/1 (v/v) at a ratio of 1:5 and used for HPLC-UV analysis.

Method conditions

Column:	Symmetry Shield RP 18, 250 x 4.6 mm
Eluent:	20% acetonitrile + sulfuric acid 0.5M (1L + 5 mL)
	80% doubly distilled water + sulphuric acid 0.5 M (1L+5 ml)
Flow rate:	1.2 mL/min
Column temp:	Ambient
Injection volume:	10µL
Detection:	UV, 220 nm

Conclusion

Demonstration of linearity has been provided in the study in the form of a linear plot, covering the range which is suitable for the determination of the levels required. The report demonstrates some degree of selectivity in the form of chromatograms of a blank and reference standard.
Acceptable recovery has not been demonstrated. The results of the test samples indicate that the level found is in line with the applied dose but this isn't enough to address the lack of acceptable recoveries.

The minimum validation requirements set out in section 4.2 of SANTE/2020/12830, Rev.1 have not been meet and therefore this method cannot be considered acceptably validated but there is some confidence in the results based of the data provided.

Homogeneity and concentration control

Principle of method

Samples were diluted with acetonitrile/highly deionised water 1/1 v/v. Aliquots of the dilutions were used for the HPLC analysis.

Method conditions

Column:	Gemini C18 5µm, 50 x 3 mm
Eluent:	A: acetonitrile + formic acid (1000 mL + 1ml)

	B: highly deionized water + formic acid (1000 mL + 1mL)
Flow rate:	0.4 mL/min
Injection volume:	2 µL + 5 µL
Detection:	UV, 220 nm

Conclusion

Demonstration of linearity has been provided in the study in the form of a linear plot, covering the range which is suitable for the determination of the levels required. The report demonstrates some degree of selectivity in the form of chromatograms of a blank and reference standard.
Acceptable recovery has not been demonstrated. The results of the test samples indicate that the level found is in line with the applied dose but this isn't enough to address the lack of acceptable recoveries.

The minimum validation requirements set out in section 4.2 of SANTE/2020/12830, Rev.1 have not been meet and therefore this method cannot be considered acceptably validated but there is some confidence in the results based of the data provided.

B.5.1.2.4. Methods in body fluids, air and any additional matrices used in support of operator, worker, resident and bystander exposure studies

No methods of analysis to support operator exposure studies for the active substance have been submitted.

B.5.1.2.5. Methods in or on plants, plant products, processed food commodities, food of plant and animal origin, feed and any additional matrices used in support of residues studies

Table B.5.1.2.5-1: Summary of risk assessment methods supporting residue studies

Data point	Study	Conclusion	Matrix	Studies relied on
KCA 4.1.2/19	Validation of an Analytical Method for the Determination of three Metabolites of S-2399 (N-des-Me-S-2840, N-des-Me-1'-CH_2OH-s-2840A and N-des-Me-1'-CH_2OH-s-2840B) in Cereal (Whole Plant and Grain), Potato (Tubers), Grapes and Soybeans (Seeds). [redacted] 2017a Study No.: TPA-0053	HPLC-MS/MS Analyte: N-des-Me-1'-CH_2OH-S-2840A, N-des-Me-1'-CH_2OH-S-2840B, N-des-Me-S-2840 LOQ: 0.01 mg/kg (N-des-Me-S-2840) 0.005 mg/kg (N-des-Me-1'-CH_2OH-S-2840 A and B) Acceptable	barley (whole plant), wheat (grain), potato (tubers), grapes, soybean (seeds),	KCA 6.1/01 (TPR-0075) KCA 6.6.2/01 (TPR-0080)
KCA 4.1.2/20	Validation of an Analytical Method for the Determination of S-2399 and its Metabolites in Cereal (Whole Plant and Grain), Potato (Tubers), Grapes and Soybean (Seeds) – Amendment No. 1 to Final Report [redacted] 2017 Study No.: TPA-0057	HPLC-MS/MS Analytes: Inpyrfluxam, 3´-OH-S-2840, DFPA-$CONH_2$, N-des-Me-DFPA, DFPA, 1´-COOH-S-2840A 1´-COOH-S-2840B 1´-CH_2OH-S-2840A 1´-CH_2OH-S-2840B LOQ:	wheat (whole plant), wheat (grain), potato (tubers), grapes soybean (seeds).	KCA 6.1/02 (TPR-0093) KCA 6.1/03 (TPR-0101) KCA 6.3.1/01 (TPR-0137) KCA 6.3.2/01 (TPR-0129) KCA 6.3.4/01 (TPR-0076) KCA 6.3.5/01 (TPR-0074) KCA 6.6.2/01 (TPR-0139) KCA 6.5.3/01 (TPR-0130)

		0.01 mg/kg (inpyrfluxam, 3'-OH-S-2840, DFPA-$CONH_2$, N-des-Me-DFPA, DFPA) 0.005 mg/kg (1'-COOH-S-2840A , 1'-COOH-S-2840B, 1´-CH_2OH-S-2840A, 1´-CH_2OH-S-2840B) Acceptable		KCA 6.3.7/01 (TPR-0072) KCA 6.3.7/02 (TPR-0077) KCA 6.5.3/01 (TPR-0081) KCA 6.5.3/02 (TPR-0082) KCA 6.5.3/03 (TPR-0084) KCA 6.6.2/01 (TPR-0080)
KCA 4.1.2/21	S-2399: Validation of Valent's Method RM-50C-1, Determination of Residues of S-2399, 3′-OHS-2840, 1′-CH_2OH-S-2840-A,1′-CH_2OH-S-2840-B, DFPA-$CONH_2$, 1′-COOH-S-2840-A, and 1′-COOH-S-2840-B in Crops (including Conjugated Forms of the Metabolites Converted to their Aglycones) [redacted] 2017. Study No.: 201700135	HPLC- MS/MS Free Compounds: Inpyrfluxam, 3'-OH-S-2840, 1'-CH_2OH-S-2840A, 1'-CH_2OH-S-2840B DFPA-$CONH_2$ Conjugated: 1'-COOH-S-2840A, 1'-COOH-S-2840B, 1-CH_2OH-S-2840-A, 1-CH_2OH-S-2840-B LOQ: 0.01 mg/kg 0.02 mg/kg (corn stover)	Apples, Corn grain Soybean seeds Corn stover	KCA 6.1/04 (TPR-0067) KCA 6.1/05 (TPR-0065)

KCA 4.1.2/28	Magnitude of S-2399 and Metabolites 1'-CH_2OH-S-2840 A&B (including conjugate) and 1'-COOH-S-2840 A&B Residues in Laying Hen Tissues and Eggs from a 28-Day Feeding Study [redacted] 2017 Study No.: TPR-0013	HPLC-MS/MS Analyte: Inpyrfluxam, 1'-COOH-S-2840A, 1'-COOH-S-2840B, 1'-CH_2OH-S-2840A 1'-CH_2OH-S-2840B LOQ 0.01 mg/kg (inpyrfluxam) 0.005 mg/kg (1'-COOH-S-2840A, 1'-COOH-S-2840B, 1'-CH_2OH-S-2840A, 1'-CH_2OH-S-2840B) Acceptable	Egg, Muscle, Liver, Fat	KCA 6.4.1/01 (TPR-0015)
KCA 4.1.2/29	Magnitude of S-2399 and Metabolites 1'-CH_2OH-S-2840 A&B (including conjugate) and 1'-COOH-S-2840 A&B Residues in Bovine Tissues and Milk from a 28-Day Feeding Study [redacted] [redacted] 2016 Study No.: TPR-0013	HPLC-MS/MS Analyte: Inpyrfluxam, 1'-COOH-S-2840A, 1'-COOH-S-2840B, 1'-CH_2OH-S-2840A 1'-CH_2OH-S-2840B LOQ: 0.01 mg/kg (inpyrfluxam) 0.005 mg/kg (1'-COOH-S-2840A, 1'-COOH-S-2840B, 1'-	Milk, Muscle, Liver, Kidney Fat	CA 6.4.2/01 (TPR-0013)

		CH_2OH-S-2840A, 1'-CH_2OH-S-2840B) Acceptable.		
KCA 4.1.2/30	Magnitude of the Residue of S-2399 and its Metabolites in Wheat Processed Fractions in Northern and Southern Europe – 2016 [redacted] 2018d Study No.: TPR-0081	HPLC-MS/MS Analyte: Inpyrfluxam, 3'-OH-S-2840, DFPA-$CONH_2$, N-des-Me-DFPA, DFPA, 1'-COOH-S-2840A, 1'-COOH-S-2840B, 1'-CH_2OH-S-2840A 1'-CH_2OH-S-2840B LOQ: 0.01 mg/kg (inpyrfluxam, 3'-OH-S-2840, DFPA-$CONH_2$, N-des-Me-DFPA and DFPA) 0.005 mg/kg (1'-COOH-S-2840A, 1'-COOH-S-2840B, 1'-CH_2OH-S-2840A and 1'-CH_2OH-S-2840B) Acceptable.	Wheat (flour) Wheat (whole-grain bread)	KCA 6.5.3/02 (TPR-0081)
KCA 4.1.2/31	Magnitude of residues of S-2399 and its metabolites in barley processed fractions in Northern and Southern Europe – 2016	HPLC-MS/MS Analyte Inpyrfluxam,	Beer	CA 6.5.3/02 (TPR-0082)

	[redacted] 2018b Study No.: TPR-0082	3´-OH-S-2840, DFPA-$CONH_2$, N-des-Me-DFPA, DFPA, 1´-COOH-S-2840A 1´-COOH-S-2840B 1´-CH_2OH-S-2840A 1´-CH_2OH-S-2840B LOQ 0.01 mg/kg (inpyrfluxam, 3'-OH-S-2840, DFPA-$CONH_2$, N-des-Me-DFPA and DFPA) 0.005 mg/kg (1'-COOH-S-2840A, 1'-COOH-S-2840B, 1'-CH_2OH-S-2840A and 1'-CH_2OH-S-2840B) Acceptable.		

Reference:	KCA 4.1.2/19
Report Title:	Validation of an Analytical Method for the Determination of three Metabolites of S-2399 (N-des-Me-S-2840, N-des-Me-1'-CH_2OH-S-2840A and N-des-Me-1'-CH_2OH-S-2840B) in Cereal (Whole Plant and Grain), Potato (Tubers), Grapes and Soybeans (Seeds)
Author(s) & Year:	[redacted] 2017a
Document No, Authority registration No	Study No.: S17-00277 (SUM-1701V) Report No.: TPA-0053
Guideline(s):	SANCO/3029/99 rev. 4
Deviations:	No
GLP or GEP:	Yes
Acceptability:	Yes
Study relied upon:	Yes

The following HPLC-MS/MS method was validated for grapes, potato (tuber), soybean (seeds), barley (whole plant) and wheat (grain) for N-des-Me-S-2840, N-des-Me-1'-CH_2OH-S-2840A and B and used in studies to support the residue data requirements.

Samples for analysis of N-des-Me-1'-CH_2OH-S-2840 A and B residues are subjected to acid hydrolysis. Therefore, the quantified residue levels will include both free and conjugated forms.

Principle of the method

Samples of grapes, potato (tuber), soybean (seeds), barley (whole plant) and wheat (grain) were homogenised before analysis by HPLC-MS/MS.

Samples (20 g) of grapes, potato (tuber), soybean (seeds), barley (whole plant) and wheat (grain) are weighed into 250 mL bottles and 50 mL (60 mL for wheat (grain) and soybean (seeds)) of acetonitrile/water (1:1 v/v) is added. The samples are homogenised using a high-speed homogeniser (8000 rpm) for 2 minutes and then mechanically shaken for 30 minutes. The extracts are filtered through filter paper and Celite (40 g suspended in

methanol (80 mL)) in a Buchner funnel. The extraction/filtration process is repeated twice more and the extracts combined and made up to 200 mL with acetonitrile/water (1:1, v/v).

Different procedures for the clean-up of sample extracts were necessary and different HPLC conditions were used. These are described below

N-des-Me-S-2840

An aliquot (1.0 mL) of the combined extract is transferred into a test tube and evaporated to aqueous remainder under nitrogen at 40ºC and reconstituted in 10 mL of water/methanol (3:1 v/v). The resulting sample concentration is 0.01 g sample/mL.

N-des-Me-1'-CH_2OH-S-2840A and B

An aliquot (2 mL) of the combined extract is hydrolysed with 2 mL of 4M hydrochloric acid for 4 hours at 100°C, cooled to room temperature and cleaned up using an Oasis HLB cartridge (conditioned with 5 mL of methanol followed by 10 mL of water). The extract is loaded onto the cartridge and the analytes are eluted with 10 mL of methanol. The sample is evaporated to 5 mL under nitrogen at 40°C. The sample is diluted with water to 20 mL prior to analysis. The resulting sample concentration is 0.01 g sample/mL.

All samples are analysed by high performance liquid chromatography with tandem mass specific detection (HPLC-MS/MS) in both positive and negative ion modes. The analytical parameters are shown below:

HPLC conditions (N-des-Me-S-2840)

Column Accucore Phenyl-Hexyl, 2.6 µm, 4.6 mm x 50 mm

Mobile phase A = Methanol containing 0.1% (v/v) formic acid

B = Water containing 0.1% (v/v) formic acid

Time (minutes)	Flow rate (µL/min)	%A	%B
0.0	1000	20.0	80.0
5.5	1000	90.0	10.0
6.0	1000	90.0	10.0
6.1	1000	20.0	80.0

7.5	1000	20.0	80.0

Column temperature 40.0 °C

Injection volume 10.0 µL

Retention time ~4.6 minutes

MS conditions

Instrument SCIEX TripleQuad 6500 System, SCIEX (Triple quadrupole mass spectrometer)

Ionisation Type Electrospray ionisation (ESI, Turbulon Spray)

Polarity Positive/negative ion switching mode

Scan Type MRM

Capillary voltage 5000 V/ - 4500 V

Mass transition monitored (*m/z*) 318 → 278* (neg)

320 → 280 (pos)

Ion spray turbo heater (TEM) 550 °C

* Proposed for quantification

HPLC conditions (N-des-Me-1'-CH_2OH-S-2840A and B)

Column Accucore Phenyl-Hexyl, 2.6 µm, 4.6 mm x 50 mm.

Mobile phase A = Methanol containing 0.05% (v/v) acetic acid

B = Water containing 0.05% (v/v) acetic acid

Time (minutes)	Flow rate (µL/min)	%A	%B

0.0	1000	20.0	80.0
4.5	1000	60.0	40.0
4.6	1000	90.0	10.0
5.0	1000	90.0	10.0
5.1	1000	20.0	80.0
6.5	1000	20.0	80.0

Column temperature 60.0 °C

Injection volume 30.0 µL

Retention time ~3.2 minutes (N-des-Me-1'-CH_2OH-S-2840A)

~3.5 minutes (N-des-Me-1'-CH_2OH-S-2840B)

MS conditions

Instrument SCIEX TripleQuad 6500 System, SCIEX (Triple quadrupole mass spectrometer)

Ionisation Type Electrospray ionisation (ESI, Turbulon Spray)

Polarity Positive/negative ion switching mode

Scan Type MRM

Capillary voltage 5500 V/ - 4500 V

Mass transition monitored (*m/z*) 334 → 294* (neg)

334 → 314 (neg)

Ion spray turbo heater (TEM) 550 °C

* Proposed for quantification

Table B.5.1.2.5-2: Analytical validation data for the determination of N-des-Me-S-2840 in barley (whole plant), wheat (grain), potato (tubers), grapes and soybeans (seeds)

Analyte	Matrix	LOQ (mg/kg)	Recovery fortification level (mg/kg)	Recoveries % range (mean)	Repeatability % RSD (n)	Linearity
N-des-Me-S-2840	**No group:** Barley (whole plant)	0.01 (*318 → 278 m/z*)	0.01	102 – 108 (105)	2.5 (5)	0.02 – 2.0 ng/mL (0.002 – 0.20 mg/kg). r = 0.998 (n = 6) Y = 294366x - 2472
			0.1	91 – 107 (100)	6.3 (5)	
		0.01 (*320 → 280 m/z*)	0.01	99 – 118 (107)	6.7 (5)	0.02 – 2.0 ng/mL (0.002 – 0.20 mg/kg). r = 0.996 (n = 8) Y = 77622x - 608
			0.1	94 – 105 (101)	4.3 (5)	
	High starch/dry group:	0.01 (*318 → 278 m/z*)	0.01	74 – 100 (87)	12 (5)	0.02 – 2.0 ng/mL (0.002 – 0.20 mg/kg). r = 0.9958

	Wheat (grain)		0.1	67 – 96 (83)	14 (5)	(n = 8) Y = 114507x + 358
		0.01 (*320 → 280 m/z*)	0.01	73 – 94 (84)	11 (5)	0.02 – 2.0 ng/mL (0.002 – 0.20 mg/kg). r = 0.995 (n = 8) Y = 78019x + 807
			0.1	70 – 91 (83)	11 (5)	
	High water group: Potato (tubers)	0.01 (*318 → 278 m/z*)	0.01	82 – 114 (102)	12 (5)	0.02 – 2.0 ng/mL (0.002 – 0.20 mg/kg). r = 0.9994 (n = 8) Y = 82572x - 190
			0.1	88 – 101 (94)	6.1 (5)	
		0.01 (*320 → 280 m/z*)	0.01	79 – 112 (101)	13 (5)	0.02 – 2.0 ng/mL (0.002 – 0.20 mg/kg). r = 0.9998

			0.1	88 – 103 (96)	7.0 (5)	(n = 8) Y = 146192x - 238
	High acid group: Grapes	0.01 (*318 → 278 m/z*)	0.01	93 – 110 (103)	6.9 (5)	0.02 – 2.0 ng/mL (0.002 – 0.20 mg/kg). r = 0.9987 (n = 8) Y = 69725x - 738
			0.1	83 – 93 (89)	4.3 (5)	
		0.01 (*320 → 280 m/z*)	0.01	102 – 118 (107)	6.1 (5)	0.02 – 2.0 ng/mL (0.002 – 0.20 mg/kg). r = 0.9983 (n = 8) Y = 108232x - 1172
			0.1	84 – 97 (91)	5.7 (5)	
	High oil group: Soybeans (seeds)	0.01 (*318 → 278 m/z*)	0.01	97 – 114 (106)	6.0 (5)	0.02 – 2.0 ng/mL (0.002 – 0.20 mg/kg). r = 0.9982 (n = 8)

			0.1	82 – 98 (91)	7.0 (5)	Y = 274428x - 2021
		0.01 (*320 → 280 m/z*)	0.01	86 – 106 (93)	9.4 (5)	0.02 – 2.0 ng/mL (0.002 – 0.20 mg/kg). r = 0.9989 (n = 8) Y = 62300x + 210
			0.1	85 – 101 (92)	7.0 (5)	

Table B.5.1.2.5-3: Analytical validation data for the determination of N-des-Me-1'-CH_2OH-S-2840 A in barley (whole plant), wheat (grain), potato (tubers), grapes and soybeans (seeds)

Analyte	Matrix	LOQ (mg/kg)	Recovery fortification level (mg/kg)	Recoveries % range (mean)	Repeatability % RSD (n)	Linearity
N-des-Me-1'-CH_2OH-S-2840 A	**No group:** Barley (whole plant)	0.005 (*334 → 294 m/z*)	0.005	94 – 108 (100)	5.4 (5)	0.01 – 1.0 ng/mL (0.001 – 0.10 mg/kg). r = 0.9958 (n = 7) Y = 591363x - 2918
			0.05	80 – 90 (86)	4.5 (5)	
		0.005 (*334 → 314 m/z*)	0.005	98 – 112 (104)	5.3 (5)	0.01 – 1.0 ng/mL (0.001 – 0.10 mg/kg). r = 0.9964 (n = 7) Y = 456294x - 2673
			0.05	84 – 89 (86)	2.8 (5)	
	High starch/dry group:	0.005 (*334 → 294 m/z*)	0.005	60 – 80 (72)	10 (5)	0.01 – 1.0 ng/mL (0.001 – 0.10 mg/kg).

	Wheat (grain)		0.05	67 – 77 (72)	5.8 (5)	r = 0.9992 (n = 8) Y = 67228x - 43
		0.005 (*334 → 314 m/z*)	0.005	68 – 92 (81)	11 (5)	0.01 – 1.0 ng/mL (0.001 – 0.10 mg/kg). r = 0.9998 (n = 8) Y = 54125 - 189
			0.05	67 – 73 (70)	3.3 (5)	
	High water group: Potato (tubers)	0.005 (*334 → 294 m/z*)	0.005	87 – 97 (91)	4.1 (5)	0.01 – 1.0 ng/mL (0.001 – 0.01 mg/kg). r = 0.9973 (n = 8) Y = 326104x - 2262
			0.05	75 – 94 (82)	8.8 (5)	
		0.005 (*334 → 314 m/z*)	0.005	84 – 96 (89)	5.8 (5)	0.01 – 1.0 ng/mL (0.001 – 0.05 mg/kg).

			0.05	76 – 92 (82)	7.8 (5)	r = 0.9981 (n = 8) Y = 249591x -1353
	High acid group: Grapes	0.005 (*334 → 294 m/z*)	0.005	77 – 92 (84)	6.4 (5)	0.01 – 1.0 ng/mL (0.001 – 0.01 mg/kg). r = 0.9981 (n = 8) Y = 418021x + 413
			0.05	76 – 83 (80)	4.1 (5)	
		0.005 (*334 → 314 m/z*)	0.005	78 – 92 (84)	6.0 (5)	0.01 – 1.0 ng/mL (0.001 – 0.10 mg/kg). r = 0.997 (n = 8) Y = 310975x + 340
			0.05	78 – 83 (81)	2.5 (5)	
	High oil group:	0.005 (*334 → 294 m/z*)	0.005	74 – 77 (75)	1.6 (5)	0.01 – 1.0 ng/mL (0.001 – 0.01 mg/kg).

	Soybeans (seeds)		0.05	64 – 80 (75)	8.8 (5)	r = 0.9997 (n=8) Y = 585707x - 173
		0.005 (*334 → 314 m/z*)	0.005	72 – 80 (77)	4.1 (5)	0.01 – 1.0 ng/mL (0.001 – 0.10 mg/kg). r = 0.9996 (n=8) Y = 439438x + 572
			0.05	65 – 81 (77)	8.8 (5)	

Table B.5.1.2.5-4:Analytical validation data for the determination of N-des-Me-1'-CH_2OH-S-2840 B in barley (whole plant), wheat (grain), potato (tubers), grapes and soybeans (seeds)

Analyte	Matrix	LOQ (mg/kg)	Recovery fortification level (mg/kg)	Recoveries % range (mean)	Repeatability % RSD (n)	Linearity
N-des-Me-1'-CH_2OH-S-2840 B	Barley (whole plant)	0.005 (*334 → 294 m/z*)	0.005	78 – 101 (92)	13 (5)	0.01 – 1.0 ng/mL (0.001 – 0.10 mg/kg). r = 0.9931 (n = 7) Y = 490882x - 2709
			0.05	69 – 81 (76)	7.9 (5)	

		0.005 (*334 → 314 m/z*)	0.005	80 – 109 (97)	15 (5)	0.01 – 1.0 ng/mL (0.001 – 0.1 mg/kg). r = 0.993 (n = 7) Y = 522183x - 3459
			0.05	63 – 85 (77)	12 (5)	
	Wheat (grain)	0.005 (*334 → 294 m/z*)	0.005	60 – 88 (77)	17 (5)	0.01 – 1.0 ng/mL (0.001 – 0.10 mg/kg). r = 0.9991 (n = 8) Y = 52044x -9
			0.05	74 – 80 (77)	2.9 (5)	
		0.005 (*334 → 314 m/z*)	0.005	63 – 83 (74)	13 (5)	0.01 – 1.0 ng/mL (0.001 – 0.10 mg/kg). r = 0.9993 (n = 8) Y = 57438x + 68
			0.05	72 – 79 (75)	3.6 (5)	
	Potato (tubers)	0.005 (*334 → 294 m/z*)	0.005	85 – 101 (90)	7.3 (5)	0.01 – 1.0 ng/mL (0.001 – 0.10 mg/kg). r = 0.9982 (n = 8) Y = 234618x - 1330
			0.05	75 – 93 (84)	7.8 (5)	

		0.005 (*334 → 314 m/z*)	0.005	82 – 95 (88)	5.5 (5)	0.01 – 1.0 ng/mL (0.001 – 0.10 mg/kg). r = 0.9975 (n = 8) Y = 253576x - 1283
			0.05	78 – 96 (85)	8.5 (5)	
	Grapes	0.005 (*334 → 294 m/z*)	0.005	70 – 95 (85)	11 (5)	0.01 – 1.0 ng/mL (0.001 – 0.10 mg/kg). r = 0.9973 (n = 8) Y = 294873x + 1517
			0.05	86 – 94 (89)	3.7 (5)	
		0.005 (*334 → 314 m/z*)	0.005	74 – 87 (81)	8.1 (5)	0.01 – 1.0 ng/mL (0.001 – 0.10 mg/kg). r = 0.9972 (n = 8) Y = 325017x + 1512
			0.05	82 – 90 (86)	3.4 (5)	
	Soybeans (seeds)	0.005 (*334 → 294 m/z*)	0.005	77 – 86 (81)	4.8 (5)	0.01 – 1.0 ng/mL (0.001 – 0.10 mg/kg). r = 0.9986

			0.05	72 – 88 (82)	7.4 (5)	(n = 8) Y = 382516x - 385
		0.005 (*334 → 314 m/z*)	0.005	75 – 86 (81)	5.2 (5)	0.01 – 1.0 ng/mL (0.001 – 0.10 mg/kg). r = 0.9984 (n = 8) Y = 417173x - 243
			0.05	76 – 85 (82)	4.3 (5)	

Specificity

Specificity was demonstrated by retention time match with a reference standard. Analysis of unfortified control samples and reagent blanks demonstrated no significant interference (> 30% of the LOQ) at the retention time of interest.

The ion transitions monitored are appropriate.

LC-MS/MS with primary and confirmatory ion transitions is considered a highly specific technique. Therefore, additional methods to confirm the identity of the analytes are not considered necessary.

Chromatograms for standards, reagent blank, control samples and samples fortified at the LOQ and at the higher level have been provided for all matrices. No significant interference (>30% of the LOQ) between the peak and any of the plant commodity matrices was observed. Analyte identity was confirmed by retention time match with reference standard.

Matrix Effects

Matrix effects were investigated by comparing peak areas of matrix-matched standards (90 % matrix amount) with solvent standards at identical concentrations.

Matrix effects were determined to be significant (>±20%) in a number of matrices. Therefore, matrix matched standards were used for quantification throughout.

Linearity

Linearity was demonstrated by the analysis of six to eight standards of increasing concentration. For N-des-Me-S-2840, the range of standard concentrations used was 0.02 – 2.0 ng/mL, equivalent to 0.002 – 0.20 mg/kg active substance in the samples. For N-des-Me-CH_2OH-S-2840A and N-des-Me-CH_2OH-S-2840B, the range of standard concentrations used was 0.01 – 0.10 ng/mL, equivalent to 0.001 – 0.10 mg/kg in N-des-Me-CH_2OH-S-2840A and N-des-Me-CH_2OH-S-2840B in the samples. All responses were linear with a correlation coefficient of (r) of ≥ 0.995.

Accuracy

Recovery samples were prepared by spiking blank homogenized samples with N-des-Me-S-2840, N-des-Me-CH_2OH-S-2840A or N-des-Me-CH_2OH-S-2840B standard and analysing them by the method described. The spike concentrations were in the range 0.01 to 0.10 mg/kg. Five samples were prepared at each fortification level. Mean recovery levels were within the range 70 - 107 % and are acceptable.

Precision

Precision was determined from the accuracy recovery data. Five samples were prepared at each fortification level. The %RSD at each fortification level was <17% and are considered acceptable.

Stability of stock solutions

The stock solutions prepared in methanol were stored at 1 °C to 10 °C for at least 64 days in the dark, which was sufficient to cover the length of time they were used in this study. After this time freshly prepared dilutions of the stock solutions were compared to freshly prepared dilutions of freshly prepared stock solutions. One mass transition per analyte was evaluated. Results obtained are summarised in the table below:

Analyte	Solvent of stock solution	Standard conc. of diluted stock solution (ng/mL)	Storage period (Days)	Recovery of Stored Stock Solution (Freshly prepared Stock Solution = 100 %)
N-des-Me-S-2840 (320→280 m/z)	Methanol	20	64	110%
N-des-Me-1'-CH_2OH-S-2840A (336→278 m/z)	Methanol	20	82	105%
N-des-Me-1'-CH_2OH-S-2840B (336→278 m/z)	Methanol	20	82	96%

Stability of stock solutions was adequately shown for N-des-Me-S-2840 and N-des-Me-CH_2OH-S-2840A.

Stability of Solvent Calibration Solutions

The calibration solutions prepared in water/methanol (3:1, v/v) were stored at 1° C to 10° C for 46 days in the dark, which was sufficient to cover the length of time they were used in the study. After this time at least six solvent standard solutions were compared to freshly prepared solvent standard solutions of the same concentration. One mass transition per analyte was evaluated. Results obtained are summarised in the table below.

Analyte	Solvent for calibration solutions	Standard concentration (ng/mL)	Storage period (days)	Mean difference (%) of stored solution compared to a freshly prepared solution
N-des-Me-S-2840 (318 → 278 m/z)	Water/methanol (3:1, v/v)	0.02 – 2.0	46	+ 2.8
N-des-Me-CH_2OH-S-2840A (334 → 278 m/z)	Water/methanol (3:1, v/v)	0.01 – 1.0	46	+ 5.7
N-des-Me-CH_2OH-S-	Water/methanol (3:1, v/v)	0.01 – 1.0	46	+ 12

2840B (334 → 278 m/z)				

Stability of solvent calibration solutions was adequately shown for N-des-Me-S-2840 and N-des-Me-CH_2OH-S-2840A. Analyte N-des-Me-CH_2OH-S-2840B was slightly outside the acceptable range of ± 10% as outlined in SANTE/2020/12830 rev.1. However, data was generated prior to SANTE/2020/12830 rev.1. As the data only indicates the difference between fresh and stored solutions is +12%, which is within the acceptable limits outlined in prior guidance (SANCO/825/00 Rev 8.1) it's deemed to have minimal effect on suitability of the method.

Storage stability of extracts

Storage stability of extracts was determined by fortifying the extracts at the LOQ level (0.01 mg/kg). The sample was stored at 1 °C – 10 °C for at least fifteen days in the dark alongside one control sample. After this time, the final extracts were re-analysed against freshly prepared calibration standards. Only one mass transition per analyte was evaluated.

N-des-Me-S-2840, N-des-Me-CH_2OH-S-2840A and N-des-Me-CH_2OH-S-2840B were analysed for in barley (whole plant), wheat (grain), potato (tubers), grapes and soybean (seeds). The results are shown below.

N-des-Me-S-2840 (318 → 278 m/z)

Matrix	Fortification level (mg/kg)	Recoveries (mean) % [n]	Percent of the initial recovery calculated from unrounded mean recovery values (%)	Days of storage
Barley (whole plant)	0.01	95 – 103 (98) [5]	94	21
Wheat (grain)	0.01	70 – 92 (82) [5]	94	25
Potato (tubers)	0.01	87 – 116 (106) [5]	104	21
Grapes	0.01	82 – 100 (91) [5]	89	28
Soybean (seeds)	0.01	98 – 105 (101) [5]	95	17

N-des-Me-1'-CH_2OH-S-2840A (334 → 278 m/z)

Matrix	Fortification level (mg/kg)	Recoveries (mean) % [n]	Percent of the initial recovery calculated from unrounded mean recovery values (%)	Days of storage
Barley (whole plant)	0.005	94 – 110 (104) [5]	103	16
Wheat (grain)	0.005	62 – 87 (77) [5]	106	26
Potato (tubers)	0.005	87 – 94 (92) [5]	100	22
Grapes	0.005	93 – 101 (97) [5]	115	24
Soybean (seeds)	0.005	88 – 98 (92) [5]	122	15

N-des-Me-CH_2OH-S-2840B (334 → 278 m/z)

Matrix	Fortification level (mg/kg)	Recoveries (mean) % [n]	Percent of the initial recovery calculated from unrounded mean recovery values (%)	Days of storage
Barley (whole plant)	0.005	100 – 116 (106) [5]	115	16
Wheat (grain)	0.005	52 – 89 (76) [5]	99	26
Potato (tubers)	0.005	98 – 103 (100) [5]	111	22
Grapes	0.005	93 – 108 (101) [5]	119	24
Soybean (seeds)	0.005	92 – 104 (97) [5]	120	15

Acceptable recoveries were shown for all tested metabolites and in all crop matrices. As a result of this data, it is concluded that stability of extracts is confirmed for at least 15 days for N-des-Me-S-2840, N-des-Me-CH_2OH-S-2840A and N-des-Me-CH_2OH-S-2840B in barley (whole plant), wheat (grain), potato (tubers), grapes and soybean (seeds).

Extraction efficiency

High water

In the available metabolism study on apples, radioactive residues were extracted twice with acetonitrile/water (1:1, v/v) and once with acetonitrile. The TRR in apple rinse, peel

and pulp amounted to 0.249 and 0.3 mg/kg for the phenyl- and pyrazolyl- label respectively.

For the phenyl label, the apple rinse removed 0.145 mg/kg (58.2 %TRR). Apple peel accounted for 0.093 mg/kg (37.3 %TRR) with 0.084 mg/kg (33.7 %TRR) extracted. Apple pulp accounted for 0.011 mg/kg (4.4 %TRR) with 0.010 mg/kg (4.0 %TRR) extracted.

For the pyrazolyl label, the apple rinse removed 0.192 mg/kg (64.0 %TRR). Apple peel accounted for 0.094 mg/kg (31.3 %TRR) with 0.084 mg/kg (28.0% TRR) extracted. Apple pulp accounted for 0.014 mg/kg (4.7 %TRR) with 0.013 mg/kg (4.3% TRR) extracted.

Sufficient TRR (>89%) was extracted from both peel and pulp in both radiolabels. The extraction procedure used in this study is similar to that used in the metabolism study (acetonitrile/water (1:1 v/v). Therefore, the extraction efficiency has been sufficiently addressed for high water commodities.

Dry/high starch

In the available foliar metabolism study on rice, radioactive residues were extracted twice with acetonitrile/water (1:1, v/v) and once with acetonitrile. Sufficient TRR was extracted from phenyl labelled samples (0.047 mg/kg (95.9 %TRR) in grain and 0.755 mg/kg (81.5 %TRR) in straw), and pyrazolyl labelled samples (0.061 mg/kg (95.3 %TRR) in grain and 0.732 mg/kg (86.0 %TRR) in straw). Sufficient TRR was also extracted from rice hulls (phenyl: 83.0 %TRR (1.395 mg/kg), pyrazolyl: 85.5 %TRR (1.306 mg/kg))

The extraction procedure used in this study is similar to that used in the metabolism study (acetonitrile/water (1:1 v/v). Therefore, the extraction efficiency has been sufficiently addressed for dry/high starch commodities.

High oil.

In the available metabolism study on soybean, radioactive residues were extracted twice with acetonitrile/water (1:1, v/v) and once with acetonitrile.

Pod samples were rinsed prior to solvent extraction with residues of 0.055 mg/kg (7.4 %TRR) and 0.065 mg/kg (5.4 %TRR) found for the phenyl and pyrazolyl label respectively. The TRR extracted from pods via solvent extraction was <70% (phenyl: 58.8 %TRR (0.436 mg/kg), pyrazolyl: 67.9 %TRR (0.815 mg/kg)).

From seed, <70% TRR was extracted for the phenyl label (57.9 %TRR (0.022 mg/kg). Sufficient TRR was extracted for the pyrazolyl label (89.0 %TRR (0.195 mg/kg).

For soybean, <70% TRR was extracted from pods (phenyl and pyrazolyl label) and seeds (phenyl label). Further attempts were made in the study to extract residues from the remaining unextracted material where remaining radioactivity was >0.01 mg/kg. In these additional extracts, while analytes were identified, none were quantified above 0.001 mg/kg, and as such the solvents are sufficient to extract the relevant analytes.

The extraction procedure used in this study is similar to that used in the metabolism study (2 x acetonitrile/water (1:1 v/v) followed by acetonitrile). Therefore, the extraction efficiency has been sufficiently addressed for high oil commodities

High acid

There is no extraction efficiency data for high acid commodities. Nevertheless, bridging between high water commodities and high acid commodities is acceptable for slightly acidic matrices, e.g. apple, tomato, grapes. As extraction efficiency is validated in apples, it can be bridged to high acid commodities.

Conclusion

Validation data was generated prior to the introduction of SANTE/2020/12830 rev.1. The method can be considered sufficiently validated to the requirements as outlined in SANCO/3029/99 rev.4. The method meets the requirements outlined under section 4.2 of SANTE/2020/12830 rev.1 “minimum validation requirements for the assessment of existing methods for risk assessment”. Therefore, the method can be considered sufficiently validated in accordance with SANTE/2020/12830 rev.1.

The method is acceptable in the areas of specificity, linearity, accuracy, and precision. Extraction efficiency is considered sufficiently addressed. The LOQ of the method is 0.01 mg/kg (N-Des-Me-S-2840) and 0.005 mg/kg (N-des-Me-CH_2OH-S-2840A and N-des-Me-CH_2OH-S-2840B).

Matrix effect was investigated as part of the study and found with significant for a number of samples. Matrix matched standards were used for quantification for all analytes. Stability of stock and calibration solutions and final extracts was investigated and while slight deviations are noted, have minimal impact on the validity of the method.

Reference:	KCA 4.1.2/20
Report Title:	Validation of an Analytical Method for the Determination of S-2399 and its Metabolites in Cereal (Whole Plant and Grain), Potato (Tubers), Grapes and Soybean (Seeds) – Amendment No. 1 to Final Report
Author(s) & Year:	[redacted] 2017
Document No, Authority registration No	Study No.: S16-03371 (SUM-1601V); Report No.: TPA-0057

Guideline(s):	SANCO/3029/99 rev. 4
Deviations:	No
GLP or GEP:	Yes
Acceptability:	Yes
Study relied upon:	Yes

The following HPLC-MS/MS method was validated for grapes, potato (tuber), soybean (seeds), and wheat (grain and whole plant) for inpyrfluxam, 3'-OH-S-2840, DFPA-$CONH_2$, N-des-Me-DFPA, DFPA, 1'-COOH-S-2840A and B and 1'-CH_2OH-S-2840A and B and used in studies to support the residue data requirements.

Samples for analysis of DFPA, 1'-COOH-S-2840A and B and 1'-CH_2OH-S-2840A and B residues are subjected to acid hydrolysis. Therefore, the quantified residue levels will include both free and conjugated forms.

Principle of the method

Samples of grapes, potato (tuber), soybean (seeds) and wheat (whole plant and grain) were homogenised before analysis by HPLC-MS/MS.

Samples (20 g) of grapes, potato (tuber), soybean (seeds) and wheat (whole plant and grain) are weighed into 250 mL bottles and 50 mL (60 mL for wheat (grain)) of acetonitrile/water (1:1 v/v) is added. The samples are homogenised using a high-speed homogeniser (8000 rpm) for 2 minutes and then mechanically shaken for 30 minutes. The extracts are filtered through filter paper and Celite (40 g suspended in methanol (80 mL)) in a Buchner funnel. The extraction/filtration process is repeated twice more and the extracts combined and made up to 200 mL with acetonitrile/water (1:1, v/v).

Different procedures for the clean-up of sample extracts were necessary and different HPLC conditions were used. These are described below.

Inpyrfluxam, 3'-OH-S-2840 and DFPA-$CONH_2$

An aliquot (1.0 mL) of the combined extract is transferred into a test tube and evaporated to dryness under nitrogen at 40ºC and reconstituted in 10 mL of water/methanol (3:1 v/v). The resulting sample concentration is 0.01 g sample/mL.

N-des-Me-DFPA

An aliquot (2 mL) of the combined extract is hydrolysed with 5 mL of 6M hydrochloric acid for 6 hours at 100°C and diluted to 15 mL with water. The extract is cleaned up with a Chem Elut cartridge, eluted with ethyl acetate (4 x 25 mL) into a round bottomed flask and evaporated to dryness under vacuum at 40°C. The sample is reconstituted in 5 mL of water/methanol (3:1, v/v) with sonication before being made up to 10 mL with water/methanol (3:1, v/v). The resulting sample concentration is 0.02 g sample/mL.

DFPA, 1'-COOH-S-2840A and B and 1'-CH_2OH-S-2840A and B

An aliquot (2 mL) of the combined extract is hydrolysed with 2 mL of 4M hydrochloric acid for 4 hours at 100°C, cooled to room temperature and cleaned up using an Oasis HLB cartridge (conditioned with 5 mL of methanol followed by 10 mL of water). The extract is loaded onto the cartridge and the analytes are eluted with 10 mL of methanol. The sample is evaporated to 5 mL under nitrogen at 40°C. The sample is diluted with water to 20 mL prior to analysis. The resulting sample concentration is 0.01 g sample/mL.

All samples are analysed by high performance liquid chromatography with tandem mass specific detection (HPLC-MS/MS) in both positive and negative ion modes. The analytical parameters are shown below:

HPLC conditions (Inpyrfluxam, 3'-OH-S-2840, DFPA-$CONH_2$)

Column Accucore Phenyl-Hexyl, 2.6 µm, 4.6 mm x 50 mm

Mobile phase A = Methanol containing 0.1% (v/v) formic acid
B = Water containing 0.1% (v/v) formic acid

Time (minutes)	Flow rate (µL/min)	%A	%B
0.0	1000	20.0	80.0
5.5	1000	90.0	10.0
6.0	1000	90.0	10.0
6.1	1000	20.0	80.0
7.5	1000	20.0	80.0

Column temperature 40.0 °C

Injection volume 10.0 µL

Retention time ~4.5 minutes (Inpyrfluxam)
~4.2 minutes (3'-OH-S-2840)
~0.9 minutes (DFPA-$CONH_2$)

MS conditions

Instrument SCIEX TripleQuad 6500 System, SCIEX (Triple quadrupole mass spectrometer)

Ionisation Type Electrospray ionisation (ESI, Turbulon Spray)

Polarity Positive/negative ion switching mode

Scan Type MRM
Capillary voltage 5000 V/ - 4500 V

Mass transition monitored (*m/z*)	Inpyrfluxam	3'-OH-S-2840	DFPA-$CONH_2$
	334 → 294* (pos)	348 → 175 (neg)	176 → 136* (pos)
	334 → 238 (pos)	348 → 131* (neg)	176 → 156 (pos)

Ion spray turbo heater (TEM) 550 °C

* Proposed for quantification

HPLC conditions (N-des-Me-DFPA)

Column Accucore Phenyl-Hexyl, 2.6 µm, 4.6 mm x 50 mm
Mobile phase A = Acetonitrile containing 0.1% (v/v) acetic acid
B = Water containing 0.1% (v/v) acetic acid

Time (minutes)	Flow rate (µL/min)	%A	%B
0.0	1000	10.0	90.0
2.0	1000	90.0	10.0
3.0	1000	90.0	10.0
3.1	1000	10.0	90.0
4.5	1000	10.0	90.0

Column temperature 40.0 °C
Injection volume 30.0 µL
Retention time ~1.0 minutes

MS conditions

Instrument SCIEX TripleQuad 6500 System, SCIEX (Triple quadrupole mass spectrometer)
Ionisation Type Electrospray ionisation (ESI, Turbulon Spray)
Polarity Negative ion mode
Scan Type MRM
Capillary voltage - 4500 V
Mass transition monitored (*m/z*) 161 → 141* (neg)
161 → 66 (neg)
Ion spray turbo heater (TEM) 550 °C

* Proposed for quantification

HPLC conditions (DFPA, 1'-COOH-S-2840A and B, 1'-CH_2OH-S-2840A and B)

Column Accucore Phenyl-Hexyl, 2.6 µm, 4.6 mm x 50 mm

Mobile phase A = Methanol containing 0.05% (v/v) acetic acid
B = Water containing 0.05% (v/v) acetic acid

Time (minutes)	Flow rate (µL/min)	%A	%B
0.0	1000	20.0	80.0
4.5	1000	60.0	40.0
4.6	1000	90.0	10.0
5.0	1000	90.0	10.0
5.1	1000	20.0	80.0
6.5	1000	20.0	80.0

Column temperature 60.0 °C

Injection volume 30.0 µL

Retention time ~1.1 minutes (DFPA)
~3.8 minutes (1'-COOH-S-2840A)
~4.0 minutes (1'-COOH-S-2840B)
~3.6 minutes (1'-CH_2OH-S-2840A)
~3.9 minutes (1'-CH_2OH-S-2840B)

MS conditions

Instrument SCIEX TripleQuad 6500 System, SCIEX (Triple quadrupole mass spectrometer)

Ionisation Type Electrospray ionisation (ESI, Turbulon Spray)

Polarity Positive/negative ion switching mode

Scan Type MRM

Capillary voltage 5500 V/ - 4500 V

Mass transition monitored (*m/z*)

DFPA	1'-COOH-S-2840A and B	1'-CH_2OH-S-2840A and B
177 → 137[1] (pos)	364 → 278[1] (pos)	350 → 292[1] (pos)
175 → 91[2] (neg)	364 → 318[3] (pos)	350 → 312[5] (pos)
	362 → 318[4] (neg)	350 → 262[6] (pos)

Ion spray turbo heater (TEM) 550 °C

[1]Proposed for quantification
[2]Confirmatory for grapes and wheat (grain)
[3]Confirmatory for grapes, soybean and wheat (whole plant and grain)
[4]Confirmatory potato (tuber)
[5]Confirmatory for grapes, potato (tuber) and wheat (grain)

[6]Confirmatory for soybean (seeds) and wheat (whole plant)

HPLC conditions (DFPA)

Column Develosil RP aqueous C30 140A, 3.0 µm, 3.0 mm x 150 mm

Mobile phase A = Methanol containing 0.05% (v/v) acetic acid
B = Water containing 0.05% (v/v) acetic acid

Time (minutes)	Flow rate (µL/min)	%A	%B
0.0	1000	20.0	80.0
3.0	1000	50.0	50.0
3.1	1000	90.0	10.0
4.5	1000	90.0	10.0
4.6	1000	20.0	80.0
6.0	1000	20.0	80.0

Column temperature 60.0 °C
Injection volume 30.0 µL
Retention time ~2.0 minutes

MS conditions

Instrument SCIEX TripleQuad 6500 System, SCIEX (Triple quadrupole mass spectrometer)
Ionisation Type Electrospray ionisation (ESI, Turbulon Spray)
Polarity Positive ion mode
Scan Type MRM
Capillary voltage 5500 V
Mass transition monitored (*m/z*) 177 → 137* (pos)
Ion spray turbo heater (TEM) 550 °C

* Proposed to be used for confirmation in potato (tuber), soybean and wheat (whole plant)

Table B.5.1.2.5-5: Analytical validation data for the determination of inpyrfluxam in grapes, potato (tubers), soybean (seeds), wheat (whole plant) and wheat (grain)

Analyte	Matrix	LOQ (mg/kg)	Recovery fortification level (mg/kg)	Recoveries % range (mean)	Repeatability % RSD (n)	Linearity
Inpyrfluxam	Grapes	0.01 (*334 → 294 m/z*)	0.01	98 – 108 (103)	4.5 (5)	0.02 – 2.0 ng/mL (0.002 – 0.20 mg/kg) r = 0.9994 (n = 8) y = 421028.8320 x + 2635.4777
			0.1	97 – 105 (101)	3.1 (5)	
		0.01 (*334 → 238 m/z*)	0.01	93 – 110 (101)	6.8 (5)	0.02 – 2.0 ng/mL (0.002 – 0.20 mg/kg) r = 0.9994 (n = 8) y = 551777.9149 x + 4598.2464
			0.1	96 – 109 (102)	5.0 (5)	
	Potato (tuber)	0.01 (*334 → 294 m/z*)	0.01	90 – 113 (102)	9.0 (5)	0.02 – 2.0 ng/mL (0.002 – 0.20 mg/kg) r = 0.9982 (n = 8) y = 331050.2246 x + 2461.9202
			0.1	90 – 97 (93)	3.4 (5)	
		0.01 (*334 → 238 m/z*)	0.01	92 – 111 (104)	7.2 (5)	0.02 – 2.0 ng/mL (0.002 – 0.20 mg/kg) r = 0.9988 (n = 8) y = 425834.7904 x + 3158.4516
			0.1	88 – 99 (93)	4.4 (5)	

	Soybean (seeds)	0.01 (*334 → 294 m/z*)	0.01	93 – 104 (101)	4.5 (5)	0.02 – 2.0 ng/mL (0.002 – 0.20 mg/kg) r = 0.9992 (n = 8) y = 406777.2978 x + 1656.1375
			0.1	92 – 100 (96)	3.8 (5)	
		0.01 (*334 → 238 m/z*)	0.01	91 – 105 (98)	6.3 (5)	0.02 – 2.0 ng/mL (0.002 – 0.20 mg/kg) r = 0.9990 (n = 8) y = 530120.6761 x + 2873.5215
			0.1	91 – 101 (96)	4.4 (5)	
	Wheat (whole plants)	0.01 (*334 → 294 m/z*)	0.01	103 – 112 (108)	4.0 (5)	0.02 – 2.0 ng/mL (0.002 – 0.20 mg/kg) r = 0.9998 (n = 8) y = 260736.8096 x -621.9531
			0.1	96 – 98 (97)	0.9 (5)	
		0.01 (*334 → 238 m/z*)	0.01	99 – 110 (104)	3.9 (5)	0.02 – 2.0 ng/mL (0.002 – 0.20 mg/kg) r = 0.9999 (n = 8) y = 341510.1306 x + 351.7047
			0.1	91 – 99 (96)	3.2 (5)	
	Wheat (grain)	0.01 (*334 → 294 m/z*)	0.01	90 – 108 (97)	7.2 (5)	0.02 – 2.0 ng/mL (0.002 – 0.20 mg/kg) r = 0.9993 (n = 8) y = 380768.8985 x + 1492.7106
			0.1	88 – 102 (91)	6.6 (5)	

		0.01 (*334 → 238 m/z*)	0.01	93 – 104 (97)	5.0 (5)	0.02 – 2.0 ng/mL (0.002 – 0.20 mg/kg) r = 0.9997 (n = 8) y = 497517.9129 x + 2948.0227
			0.1	85 – 101 (91)	6.7 (5)	

Table B.5.1.2.5-6: Analytical validation data for the determination of DFPA-CONH$_2$ in grapes, potato (tubers), soybean (seeds), wheat (whole plant) and wheat (grain)

Analyte	Matrix	LOQ (mg/kg)	Recovery fortification level (mg/kg)	Recoveries % range (mean)	Repeatability % RSD (n)	Linearity
DFPA-CONH$_2$	Grapes	0.01 *(176 → 136 m/z)*	0.01	104 – 116 (109)	4.3 (5)	0.02 – 2.0 ng/mL (0.002 – 0.20 mg/kg) r = 0.9981 (n = 8) y = 525386.8833x + 4698.5627
			0.1	96 – 115 (106)	6.5 (5)	
		0.01 *(176 → 156 m/z)*	0.01	101 – 113 (108)	4.1 (5)	0.02 – 2.0 ng/mL (0.002 – 0.20 mg/kg) r = 0.9986 (n = 8) y = 431693.9216x + 3838.9772
			0.1	96 – 118 (105)	8.1 (5)	
	Potato (tuber)	0.01 *(176 → 136 m/z)*	0.01	100 – 115 (107)	5.4 (5)	0.02 – 2.0 ng/mL (0.002 – 0.20 mg/kg) r = 0.9991 (n = 8) y = 331893.0028x + 2739.0594
			0.1	92 – 105 (98)	5.4 (5)	

		0.01 *(176 → 156 m/z)*	0.01	100 – 113 (106)	5.4 (5)	0.02 – 2.0 ng/mL (0.002 – 0.20 mg/kg) r = 0.9987 (n = 8) y = 281235.0441x + 2061.0408
			0.1	92 – 106 (97)	6.4 (5)	
	Soybean (seeds)	0.01 *(176 → 136 m/z)*	0.01	95 – 108 (100)	5.3 (5)	0.02 – 2.0 ng/mL (0.002 – 0.20 mg/kg) r = 0.9984 (n = 8) y = 300860.3655x + 2189.7434
			0.1	93 – 101 (98)	3.3 (5)	
		0.01 *(176 → 156 m/z)*	0.01	95 – 104 (99)	3.3 (5)	0.02 – 2.0 ng/mL (0.002 – 0.20 mg/kg) r = 0.9975 (n = 8) y = 245736.7753x + 1749.3166
			0.1	95 – 103 (100)	4.0 (5)	
	Wheat (whole plants)	0.01 *(176 → 136 m/z)*	0.01	95 – 107 (101)	4.2 (5)	0.02 – 2.0 ng/mL (0.002 – 0.20 mg/kg) r = 0.9984 (n = 8) y = 278963.5572x - 88.1544
			0.1	89 – 96 (93)	2.9 (5)	
		0.01 *(176 → 156 m/z)*	0.01	91 – 104 (100)	5.4 (5)	0.02 – 2.0 ng/mL (0.002 – 0.20 mg/kg) r = 0.9996 (n = 8) y = 225505.7081x + 370.7575
			0.1	90 – 95 (92)	2.4 (5)	
	Wheat (grain)	0.01 *(176 → 136 m/z)*	0.01	107 – 114 (110)	2.6 (5)	0.02 – 2.0 ng/mL (0.002 – 0.20 mg/kg)

			0.1	99 – 103 (101)	1.6 (5)	r = 0.9994 (n = 8) y = 485036.8542x + 2007.8937
		0.01 *(176 → 156 m/z)*	0.01	104 – 116 (110)	4.6 (5)	0.02 – 2.0 ng/mL (0.002 – 0.20 mg/kg) r = 0.9993 (n = 8) y = 417037.1285x + 287.7198
			0.1	100 – 104 (102)	1.5 (5)	

Table B.5.1.2.5-7: Analytical validation data for the determination of 3'-OH-S-2840 in grapes, potato (tubers), soybean (seeds), wheat (whole plant) and wheat (grain)

Analyte	Matrix	LOQ (mg/kg)	Recovery fortification level (mg/kg)	Recoveries % range (mean)	Repeatability % RSD (n)	Linearity
3'-OH-S-2840	Grapes	0.01 *(348 → 175 m/z)*	0.01	103 – 118 (109)	5.3 (5)	0.02 – 2.0 ng/mL (0.002 – 0.20 mg/kg) r = 0.9996 (n = 8) y = 251544.3793x - 1173.7504
			0.1	97 – 105 (102)	3.2 (5)	
		0.01 *(348 → 131 m/z)*	0.01	101 – 121 (109)	7.4 (5)	0.02 – 2.0 ng/mL (0.002 – 0.20 mg/kg) r = 0.9952 (n = 8) y = 180926.9087x - 748.6784
			0.1	95 – 108 (100)	5.2 (5)	
	Potato (tuber)	0.01 *(348 → 175 m/z)*	0.01	87 – 114 (102)	10 (5)	0.02 – 2.0 ng/mL (0.002 – 0.20 mg/kg) r = 0.9999 (n = 8) y = 209881.8266x + 773.6424
			0.1	90 – 95 (92)	2.0 (5)	

		0.01 *(348 → 131 m/z)*	0.01	98 – 111 (103)	5.4 (5)	0.02 – 2.0 ng/mL (0.002 – 0.20 mg/kg) r = 0.9990 (n = 8) y = 155937.5707x + 360.8146
			0.1	89 – 96 (93)	3.4 (5)	
	Soybean (seeds)	0.01 *(348 → 175 m/z)*	0.01	96 – 108 (101)	4.9 (5)	0.02 – 2.0 ng/mL (0.002 – 0.20 mg/kg) r = 0.9996 (n = 8) y = 162180.4998x - 495.6418
			0.1	92 – 97 (94)	2.1 (5)	
		0.01 *(348 → 131 m/z)*	0.01	91 – 105 (97)	5.7 (5)	0.02 – 2.0 ng/mL (0.002 – 0.20 mg/kg) r = 0.9995 (n = 8) y = 116570.5029x + 267.1938
			0.1	92 – 99 (96)	3.2 (5)	
	Wheat (whole plants)	0.01 *(348 → 175 m/z)*	0.01	94 – 119 (104)	9.5 (5)	0.02 – 2.0 ng/mL (0.002 – 0.20 mg/kg) r = 0.9992 (n = 8) y = 200021.3926x + 326.4425
			0.1	90 – 96 (92)	2.7 (5)	
		0.01 *(348 → 131 m/z)*	0.01	93 – 112 (103)	6.7 (5)	0.02 – 2.0 ng/mL (0.002 – 0.20 mg/kg) r = 0.9990 (n = 8) y = 130461.2088x + 31.4589
			0.1	90 – 99 (94)	3.6 (5)	
	Wheat (grain)	0.01 *(348 → 175 m/z)*	0.01	104 – 118 (109)	4.8 (5)	0.02 – 2.0 ng/mL (0.002 – 0.20 mg/kg)

			0.1	98 – 107 (102)	3.9 (5)	r = 0.9993 (n = 8) y = 395161.4042x + 2177.7101
		0.01 *(348 → 131 m/z)*	0.01	105 – 119 (110)	4.8 (5)	0.02 – 2.0 ng/mL (0.002 – 0.20 mg/kg) r = 0.9998 (n = 8) y = 302592.4440x + 1678.2886
			0.1	105 – 119 (110)	4.4 (5)	

Table B.5.1.2.5-8: Analytical validation data for the determination of N-des-Me-DFPA in grapes, potato (tubers), soybean (seeds), wheat (whole plant) and wheat (grain)

Analyte	Matrix	LOQ (mg/kg)	Recovery fortification level (mg/kg)	Recoveries % range (mean)	Repeatability % RSD (n)	Linearity
N-des-Me-DFPA	Grapes	0.01 *(161 → 141 m/z)*	0.01	92 – 96 (94)	3.5 (5)	0.04 – 4.0 ng/mL (0.002 – 0.20 mg/kg) r = 1.0000 (n = 8) y = 56445.2501x-483.1045
			0.1	76 – 86 (80)	5.5 (5)	
		0.01 *(161 → 66 m/z)*	0.01	75 – 104 (83)	14 (5)	0.04 – 4.0 ng/mL (0.002 – 0.20 mg/kg) r = 0.9997 (n = 8) y = 4832.9117x - 54.2155
			0.1	76 – 87 (81)	5.9 (5)	
	Potato (tuber)	0.01 *(161 → 141 m/z)*	0.01	58 – 86 (70)	15 (5)	0.04 – 2.4 ng/mL (0.002 – 0.12 mg/kg)

			0.1	60 – 84 (77)	13 (5)	r = 0.9965 (n = 7) y = 118681.6570x + 308.7323
		0.01 *(161 → 66 m/z)*	0.01	56 – 89 (70)	17 (5)	0.04 – 2.4 ng/mL (0.002 – 0.12 mg/kg) r = 0.9951 (n = 7) y = 10402.5199x + 84.2183
			0.1	60 – 83 (77)	13 (5)	
	Soybean (seeds)	0.01 *(161 → 141 m/z)*	0.01	86 – 97 (92)	4.9 (5)	0.04 – 4.0 ng/mL (0.002 – 0.20 mg/kg) r = 0.9997 (n = 8) y = 55889.9859x -328.0572
			0.1	84 – 91 (88)	3.0 (5)	
		0.01 *(161 → 66 m/z)*	0.01	93 – 106 (101)	5.2 (5)	0.04 – 4.0 ng/mL (0.002 – 0.20 mg/kg) r = 0.9986 (n = 8) y = 4768.0243x - 34.7208
			0.1	83 – 89 (87)	2.9 (5)	
	Wheat (whole plants)	0.01 *(161 → 141 m/z)*	0.01	72 – 78 (75)	2.9 (5)	0.04– 4.0 ng/mL (0.002 – 0.20 mg/kg) r = 0.9999 (n = 8) y = 106431.5026x - 2197.1796
			0.1	68 – 76 (71)	6.6 (5)	
		0.01 *(161 → 66 m/z)*	0.01	75 – 88 (83)	6.0 (5)	0.04 – 4.0 ng/mL (0.002 – 0.20 mg/kg) r = 0.9995 (n = 8) y = 9449.6263x - 103.7764
			0.1	62 – 79 (71)	9.3 (5)	

	Wheat (grain)	0.01 *(161 → 141 m/z)*	0.01	87 – 93 (90)	2.6 (5)	0.04 – 4.0 ng/mL (0.002 – 0.20 mg/kg) r = 0.9999 (n = 8) y = 118901.4022x - 195.0273
			0.1	79 – 93 (82)	2.1 (5)	
		0.01 *(161 → 66 m/z)*	0.01	86 – 94 (92)	3.8 (5)	0.04 – 4.0 ng/mL (0.002 – 0.20 mg/kg) r = 0.9996 (n = 8) y = 10434.7526x + 41.2010
			0.1	81 – 85 (83)	2.2 (5)	

Table B.5.1.2.5-9: Analytical validation data for the determination of DFPA in grapes, potato (tubers), soybean (seeds), wheat (whole plant) and wheat (grain)

Analyte	Matrix	LOQ (mg/kg)	Recovery fortification level (mg/kg)	Recoveries % range (mean)	Repeatability % RSD (n)	Linearity
DFPA	Grapes	0.01 *(177 → 137 m/z)*	0.01	97 – 113 (104)	5.6 (5)	0.02 – 2.0 ng/mL (0.002 – 0.20 mg/kg) r = 0.9987 (n = 8) y = 259900.3737x + 2415.6382
			0.1	99 – 104 (102)	2.2 (5)	
		0.01 *(175 → 91 m/z)*	0.01	88 – 122 (105)	12 (5)	0.02 – 2.0 ng/mL (0.002 – 0.20 mg/kg) r = 0.9995 (n = 8) y = 68833.0762x -90.4620
			0.1	93 – 110 (102)	6.6 (5)	
	Potato (tuber)	0.01 *(177 → 137 m/z)*	0.01	108 – 114 (110)	2.2 (5)	0.02 – 2.0 ng/mL (0.002 – 0.20 mg/kg)

			0.1	101 – 118 (109)	6.5 (5)	r = 0.9991 (n = 8) y = 342137.0290x + 401.9078
		0.01 *(177 → 137 m/z)*	0.01	99 – 105 (103)	2.3 (5)	0.02 – 2.0 ng/mL (0.002 – 0.20 mg/kg) r = 0.9998 (n = 7) y = 274403.1335x + 1215.7642
			0.1	103 – 117 (110)	4.9 (5)	
	Soybean (seeds)	0.01 *(177 → 137 m/z)*	0.01	84 – 112 (102)	11 (5)	0.02 – 2.0 ng/mL (0.002 – 0.20 mg/kg) r = 0.9996 (n = 8) y = 319319.8601x -215.2114
			0.1	81 – 84 (82)	1.4 (5)	
		0.01 *(177 → 137 m/z)*	0.01	82 – 108 (97)	10 (5)	0.02 – 2.0 ng/mL (0.002 – 0.20 mg/kg) r = 0.9989 (n = 8) y = 346495.3889x -1626.4527
			0.1	78 – 84 (81)	3.7 (5)	
	Wheat (whole plants)	0.01 *(177 → 137 m/z)*	0.01	103 – 124 (115)	7.5 (5)	0.02 – 2.0 ng/mL (0.002 – 0.20 mg/kg) r = 0.9995 (n = 8) y = 406244.6433x -1150.0427
			0.1	97 – 108 (104)	4.3 (5)	
		0.01 *(177 → 137 m/z)*	0.01	98 – 109 (106)	4.2 (5)	0.02 – 2.0 ng/mL (0.002 – 0.20 mg/kg) r = 0.9997 (n = 8) y = 349212.6573x + 1751.4785
			0.1	97 – 113 (104)	5.6 (5)	

	Wheat (grain)	0.01 *(177 → 137 m/z)*	0.01	87 – 115 (96)	11 (5)	0.02 – 2.0 ng/mL (0.002 – 0.20 mg/kg) r = 0.9990 (n = 8) y = 224526.4836x + 1380.0910
			0.1	78 – 91 (84)	6.7 (5)	
		0.01 *(175 → 91 m/z)*	0.01	93 – 118 (106)	11 (5)	0.02 – 2.0 ng/mL (0.002 – 0.20 mg/kg) r = 0.9957 (n = 8) y = 24476.5916x + 254.3351
			0.1	95 – 107 (100)	5.7 (5)	

Table B.5.1.2.5-10: Analytical validation data for the determination of 1'-COOH-S-2840A in grapes, potato (tubers), soybean (seeds), wheat (whole plant) and wheat (grain)

Analyte	Matrix	LOQ (mg/kg)	Recovery fortification level (mg/kg)	Recoveries % range (mean)	Repeatability % RSD (n)	Linearity
1'-COOH-S-2840A	Grapes	0.005 *(364 → 278 m/z)*	0.005	69 -101 (82)	14 (5)	0.01 – 1.0 ng/mL (0.001 – 0.10 mg/kg) r = 0.9997 (n = 8) y = 628921.8005x + 372.9045
			0.05	74 – 88 (82)	7.5 (5)	
		0.005 *(364 → 318 m/z)*	0.005	70 – 101 (81)	15 (5)	0.01 – 1.0 ng/mL (0.001 – 0.10 mg/kg) r = 0.9998 (n = 8) y = 464583.2905x + 341.4198
			0.05	75 – 89 (82)	8.2 (5)	

	Potato (tuber)	0.005 *(364 → 278 m/z)*	0.005	91 – 114 (98)	9.4 (5)	0.01 – 1.0 ng/mL (0.001 – 0.10 mg/kg) r = 0.9995 (n = 8) y = 454424.3546x - 50.7174
			0.05	87 – 99 (90)	6.1 (5)	
		0.005 *(362 → 318 m/z)*	0.005	94 – 117 (103)	8.4 (5)	0.01 – 1.0 ng/mL (0.001 – 0.10 mg/kg) r = 0.9997 (n = 8) y = 350056.7040x + 183.2819
			0.05	87 – 97 (91)	5.1 (5)	
	Soybean (seeds)	0.005 *(364 → 278 m/z)*	0.005	73 – 93 (85)	11 (5)	0.01 – 1.0 ng/mL (0.001 – 0.10 mg/kg) r = 0.9998 (n = 8) y = 792825.8768x + 1032.0503
			0.05	80 – 83 (82)	2.0 (5)	
		0.005 *(364 → 318 m/z)*	0.005	66 – 100 (83)	15 (5)	0.01 – 1.0 ng/mL (0.001 – 0.10 mg/kg) r = 0.9995 (n = 8) y = 528257.4651x - 3374.6657
			0.05	79 – 86 (84)	3.2 (5)	
	Wheat (whole plants)	0.005 *(364 → 278 m/z)*	0.005	97 – 106 (102)	3.7 (5)	0.01 – 1.0 ng/mL (0.001 – 0.10 mg/kg) r = 0.9997 (n = 8) y = 773370.4275x + 266.9958
			0.05	93 – 99 (95)	2.4 (5)	
		0.005 *(364 → 318 m/z)*	0.005	92 – 121 (107)	12 (5)	0.01 – 1.0 ng/mL (0.001 – 0.10 mg/kg)

			0.05	94 – 99 (97)	2.4 (5)	r = 0.9996 (n = 8) y = 556981.7272x - 631.4152
	Wheat (grain)	0.005 *(364 → 278 m/z)*	0.005	86 – 101 (93)	6.1 (5)	0.01 – 1.0 ng/mL (0.001 – 0.10 mg/kg) r = 1.0000 (n = 8) y = 654889.6431x - 500.6557
			0.05	82 – 96 (88)	6.3 (5)	
		0.005 *(364 → 318 m/z)*	0.005	83 – 107 (92)	10 (5)	0.01 – 1.0 ng/mL (0.001 – 0.10 mg/kg) r = 0.9998 (n = 8) y = 453199.4240x - 1281.3175
			0.05	81 – 97 (87)	7.5 (5)	

Table B.5.1.2.5-11: Analytical validation data for the determination of 1'-COOH-S-2840B in grapes, potato (tubers), soybean (seeds), wheat (whole plant) and wheat (grain)

Analyte	Matrix	LOQ (mg/kg)	Recovery fortification level (mg/kg)	Recoveries % range (mean)	Repeatability % RSD (n)	Linearity
1'-COOH-S-2840B	Grapes	0.005 *(364 → 278 m/z)*	0.005	63 – 94 (77)	14 (5)	0.01 – 1.0 ng/mL (0.001 – 0.10 mg/kg) r = 0.9995 (n = 8) y = 400903x + 631.1838
			0.05	79 – 90 (83)	5.7 (5)	
		0.005 *(364 → 318 m/z)*	0.005	67 – 95 (80)	13 (5)	0.01 – 1.0 ng/mL (0.001 – 0.10 mg/kg)

			0.05	78 – 89 (84)	5.6 (5)	r = 0.9993 (n = 8) y = 994362.5081x + 711.3802
	Potato (tuber)	0.005 *(364 → 278 m/z)*	0.005	88 – 98 (94)	4.2 (5)	0.01 – 1.0 ng/mL (0.001 – 0.10 mg/kg) r = 0.9997 (n = 8) y = 997949.8783x + 4149.8323
			0.05	86 – 96 (90)	4.5 (5)	
		0.005 *(362 → 318 m/z)*	0.005	87 – 102 (94)	6.2 (5)	0.01 – 1.0 ng/mL (0.001 – 0.10 mg/kg) r = 0.9998 (n = 8) y = 394559.2238x - 807.7959
			0.05	84 – 98 (91)	5.5 (5)	
	Soybean (seeds)	0.005 *(364 → 278 m/z)*	0.005	74 – 84 (81)	5.2 (5)	0.01 – 1.0 ng/mL (0.001 – 0.10 mg/kg) r = 0.9998 (n = 8) y = 575042.9566x + 1710.7631
			0.05	81 – 85 (83)	2.7 (5)	
		0.005 *(364 → 318 m/z)*	0.005	68 – 87 (79)	10 (5)	0.01 – 1.0 ng/mL (0.001 – 0.10 mg/kg) r = 0.9997 (n = 8) y = 1297359.5255x + 8299.2003
			0.05	82 – 85 (83)	2.2 (5)	
	Wheat (whole plants)	0.005 *(364 → 278 m/z)*	0.005	100 – 110 (104)	3.7 (5)	0.01 – 1.0 ng/mL (0.001 – 0.10 mg/kg) r = 0.9999 (n = 8) y = 600339.6757x + 2094.8653
			0.05	94 – 96 (95)	0.7 (5)	

		0.005 *(364 → 318 m/z)*	0.005	91 – 110 (102)	7.4 (5)	0.01 – 1.0 ng/mL (0.001 – 0.10 mg/kg) r = 0.9996 (n = 8) y = 1453144.0913x + 7834.9661
			0.05	94 – 96 (95)	1.2 (5)	
	Wheat (grain)	0.005 *(364 → 278 m/z)*	0.005	85 – 106 (93)	8.6 (5)	0.01 – 1.0 ng/mL (0.001 – 0.10 mg/kg) r = 0.9997 (n = 8) y = 549768.5214x + 288.3498
			0.05	81 – 93 (86)	5.7 (5)	
		0.005 *(364 → 318 m/z)*	0.005	90 – 105 (95)	6.1 (5)	0.01 – 1.0 ng/mL (0.001 – 0.10 mg/kg) r = 0.9997 (n = 8) y = 1154147.8967x + 541.8852
			0.05	82 – 94 (86)	5.6 (5)	

Table B.5.1.2.5-12: Analytical validation data for the determination of 1'-CH_2OH-S-2840A In grapes, potato (tubers), soybean (seeds), wheat (whole plant) and wheat (grain)

Analyte	Matrix	LOQ (mg/kg)	Recovery fortification level (mg/kg)	Recoveries % range (mean)	Repeatability % RSD (n)	Linearity
1'-CH_2OH-S-2840A	Grapes	0.005 *(350 → 292 m/z)*	0.005	69 – 94 (85)	11 (5)	0.01 – 1.0 ng/mL (0.001 – 0.10 mg/kg) r = 0.9996 (n = 8) y = 923709.6232x + 210.7631
			0.5	78 – 91 (86)	6.3 (5)	
		0.005 *(350 → 312 m/z)*	0.005	82 – 104 (92)	9.9 (5)	0.01 – 1.0 ng/mL (0.001 – 0.10 mg/kg)

			0.05	83 – 92 (87)	5.1 (5)	r = 0.9995 (n = 8) y = 352830x - 50.6120
	Potato (tuber)	0.005 *(350 → 292 m/z)*	0.005	88 – 115 (101)	9.8 (5)	0.01 – 1.0 ng/mL (0.001 – 0.10 mg/kg) r = 0.9993 (n = 8) y = 923277.4521x + 1961.4574
			0.05	92 – 102 (96)	4.4 (5)	
		0.005 *(350 → 312 m/z)*	0.005	91 – 107 (99)	6.1 (5)	0.01 – 1.0 ng/mL (0.001 – 0.10 mg/kg) r = 0.9991 (n = 8) y = 350800.9188x + 1099.9589
			0.05	91 – 100 (96)	3.4 (5)	
	Soybean (seeds)	0.005 *(350 → 292 m/z)*	0.005	87 – 92 (90)	2.7 (5)	0.01 – 1.0 ng/mL (0.001 – 0.10 mg/kg) r = 0.9998 (n = 8) y = 141686.6536x - 413.2084
			0.05	80 – 83 (82)	1.9 (5)	
		0.005 *(350 → 262 m/z)*	0.005	79 – 89 (85)	6.4 (5)	0.01 – 1.0 ng/mL (0.001 – 0.10 mg/kg) r = 0.9995 (n = 8) y = 283320.0315x - 526.7150
			0.05	79 – 83 (82)	2.0 (5)	
	Wheat (whole plants)	0.005 *(350 → 292 m/z)*	0.005	99 – 111 (105)	5.0 (5)	0.01 – 1.0 ng/mL (0.001 – 0.10 mg/kg) r = 0.9995 (n = 8) y = 1309950.2897x + 1172.0020
			0.05	94 – 101 (97)	3.1 (5)	

		0.005 *(350 → 262 m/z)*	0.005	97 – 117 (106)	7.0 (5)	0.01 – 1.0 ng/mL (0.001 – 0.10 mg/kg) r = 0.9993 (n = 8) y = 260652.9535x + 804.3454
			0.05	95 – 99 (97)	1.7 (5)	
	Wheat (grain)	0.005 *(350 → 292 m/z)*	0.005	92 – 112 (102)	7.3 (5)	0.01 – 1.0 ng/mL (0.001 – 0.10 mg/kg) r = 0.9998 (n = 8) y = 1198998.2516x + 1904.9290
			0.05	85 – 96 (89)	5.1 (5)	
		0.005 *(350 → 312 m/z)*	0.005	102 – 112 (106)	4.5 (5)	0.01 – 1.0 ng/mL (0.001 – 0.10 mg/kg) r = 0.9995 (n = 8) y = 448735.6440x - 125.6428
			0.05	86 – 92 (88)	2.9 (5)	

Table B.5.1.2.5-13: Analytical validation data for the determination of 1'-CH_2OH-S-2840B in grapes, potato (tubers), soybean (seeds), wheat (whole plant) and wheat (grain)

Analyte	Matrix	LOQ (mg/kg)	Recovery fortification level (mg/kg)	Recoveries % range (mean)	Repeatability % RSD (n)	Linearity
1'-CH_2OH-S-2840B	Grapes	0.005 *(350 → 292 m/z)*	0.005	79 – 109 (94)	12 (5)	0.01 – 1.0 ng/mL (0.001 – 0.10 mg/kg) r = 0.9995 (n = 8) y = 907049x + 678.7643
			0.05	83 – 101 (92)	7.1 (5)	
		0.005 *(350 → 312 m/z)*	0.005	83 – 109 (93)	11 (5)	0.01 – 1.0 ng/mL (0.001 – 0.10 mg/kg)

			0.05	87 – 103 (94)	6.3 (5)	r = 0.9994 (n = 8) y = 384626.1442x + 621.5906
	Potato (tuber)	0.005 *(350 → 292 m/z)*	0.005	88 – 111 (103)	9.1 (5)	0.01 – 1.0 ng/mL (0.001 – 0.10 mg/kg) r = 0.9994 (n = 8) y = 915914.1554x + 582.2020
			0.05	90 – 102 (95)	4.6 (5)	
		0.005 *(350 → 312 m/z)*	0.005	97 – 108 (102)	4.6 (5)	0.01 – 1.0 ng/mL (0.001 – 0.10 mg/kg) r = 0.9988 (n = 8) y = 387026.9121x - 1213.3472
			0.05	93 – 104 (97)	4.7 (5)	
	Soybean (seeds)	0.005 *(350 → 292 m/z)*	0.005	96 – 106 (101)	3.6 (5)	0.01 – 1.0 ng/mL (0.001 – 0.10 mg/kg) r = 0.9999 (n = 8) y = 1432476.2590x + 21.5854
			0.05	81 – 85 (83)	1.8 (5)	
		0.005 *(350 → 262 m/z)*	0.005	96 – 113 (106)	6.6 (5)	0.01 – 1.0 ng/mL (0.001 – 0.10 mg/kg) r = 0.9998 (n = 8) y = 296265.0074x - 265.2242
			0.05	82 – 85 (84)	1.6 (5)	
	Wheat (whole plants)	0.005 *(350 → 292 m/z)*	0.005	89 – 115 (105)	9.5 (5)	0.01 – 1.0 ng/mL (0.001 – 0.10 mg/kg) r = 0.9999

			0.05	91 – 99 (96)	3.6 (5)	(n = 8) y = 1459242.3463x + 2052.5815
		0.005 *(350 → 262 m/z)*	0.005	97 – 111 (105)	5.6 (5)	0.01 – 1.0 ng/mL (0.001 – 0.10 mg/kg) r = 0.9998 (n = 8) y = 294484.8984x + 291.3478
			0.05	96 – 100 (97)	2.0 (5)	
	Wheat (grain)	0.005 *(350 → 292 m/z)*	0.005	102 – 111 (106)	4.0 (5)	0.01 – 1.0 ng/mL (0.001 – 0.10 mg/kg) r = 0.9998 (n = 8) y = 1337880.6267x - 2240.9236
			0.05	86 – 98 (90)	5.5 (5)	
		0.005 *(350 → 312 m/z)*	0.005	94 – 115 (102)	7.7 (5)	0.01 – 1.0 ng/mL (0.001 – 0.10 mg/kg) r = 0.9998 (n = 8) y = 555709.4175x - 699.5783
			0.05	82 – 99 (89)	7.7 (5)	

Specificity

LC-MS/MS with primary and confirmatory ion transitions is considered a highly specific technique. Therefore, additional methods to confirm the identity of the analytes are not considered necessary. Chromatograms for standards, reagent blank, control samples and samples fortified at the LOQ have been provided for all matrices. No significant interference (>30% of the LOQ) between the analyte peaks and any of the plant commodity matrices was observed. Analyte identity was confirmed by retention time match with reference standard.

Matrix Effects

Matrix effects were investigated by comparing peak areas of matrix-matched standards (90 % matrix amount) with solvent standards at identical concentrations.

Matrix effects were determined to be significant (>±20%) in a number of matrices. Therefore, matrix matched standards were used for quantification throughout.

Linearity

Linearity was demonstrated by the analysis of at least seven standards of increasing concentration. For inpyrfluxam, 3'-OH-S-2840, DFPA-$CONH_2$ and DFPA the range of standard concentrations used was 0.02 – 2.0 ng/mL, equivalent to 0.002 – 0.20 mg/kg analyte in the samples. For 1'-CH_2OH-S-2840A, 1'-CH_2OH-S-2840B, 1'-COOH-S-2840A and 1'-COOH-S-2840B the range of standard concentrations used was 0.01 – 1.0 ng/mL, equivalent to 0.001 – 0.10 mg/kg analyte in the samples.

For N-des-Me-DFPA, all except potato (tubers) the range of standard concentrations was 0.04 – 4.0 ng/mL, equivalent to 0.002 – 0.2 mg/kg. For potato (tubers), the range was 0.04 – 2.4 ng/mL, equivalent to 0.002 – 0.12 mg/kg.

The responses were linear with correlation coefficients of (r) of ≥ 0.99.

Accuracy

Recovery samples were prepared by spiking control samples with known concentrations of analytes and analysing them following the method described above.

For inpyrfluxam, 3'-OH-S-2840, DFPA-$CONH_2$, N-des-Me-DFPA and DFPA five recovery determinations were performed at 0.01 mg/kg and at 0.1 mg/kg. For 1'-COOH-S-2840A and B, 1'-CH_2OH-S-2840A and B five recovery determinations were performed at 0.005 mg/kg and at 0.05 mg/kg. Analysis was performed by extraction and single injection.

Mean recovery levels were within the acceptable range (60 – 120% at 0.005 mg/kg and 0.01 mg/kg; 70 – 120% at 0.05 mg/kg and 0.1 mg/kg).

Precision

Precision was determined from the accuracy recovery data. Five samples were prepared at each fortification level. The calculated %RSD for all matrices at each level was acceptable (≤30% at 0.005 mg/kg and 0.01 mg/kg; ≤ 20% at 0.5 mg/kg and 0.1 mg/kg).

Stability of stock solutions

The stock solutions prepared in methanol were stored between 1 °C and 10 °C in the dark for 104 days (inpyrfluxam) or 170 days (3'-OH-S-2840, DFPA-$CONH_2$, N-des-Me-DFPA, DFPA, 1'-COOH-S-2840A and B, 1'-CH_2OH-S-2840A and B). After this time, the stock solutions were compared against freshly prepared stock solutions by single injection. One mass transition per analyte was evaluated.

Stock solutions were stable for the storage periods investigated (<10% difference from initial analysis), and the duration covered is sufficient to support the length of storage used

within the study.

Stability of Solvent Calibration solution

The calibration solutions prepared in water/methanol (3:1, v/v) were stored between 1 °C and 10 °C in the dark for 27 days (inpyrfluxam, 3'-OH-S-2840, DFPA-$CONH_2$, N-des-Me-DFPA) or 43 days (DFPA, 1'-COOH-S-2840A and B, 1'-CH_2OH-S-2840A and B). This storage period was sufficient to cover the length of time they were stored for in this study. After this time, solvent standard solutions were compared to freshly prepared solvent standard solutions of the same concentration by single injection. One mass transition per analyte was evaluated. Results obtained are summarised in the table below.

Analyte	Solvent for calibration solutions	Standard conc. (ng/mL)	Storage period (Days)	Difference (%) of stored solution compared to a freshly prepared solution
Inpyrfluxam 334 → 294 *m/z*	Water / Methanol (3:1, v/v)	0.10	27	- 8.8
		1.0	27	- 11
3'-OH-S-2840 348 → 131 *m/z*	Water / Methanol (3:1, v/v)	0.10	27	- 9.3
		1.0	27	- 10
DFPA-$CONH_2$ 176 → 136 *m/z*	Water / Methanol (3:1, v/v)	0.10	27	- 10
		1.0	27	- 9.8
N-des-Me-DFPA 161 → 141 *m/z*	Water / Methanol (3:1, v/v)	0.10	27	- 17
		1.0	27	- 13
DFPA 177 → 137 m/z	Water / Methanol (3:1, v/v)	0.10	43	5.8
		1.0	43	3.0
1'-COOH-S-2840A 362 → 318 m/z	Water / Methanol (3:1, v/v)	0.050	43	- 4.2
		0.50	43	2.5
1'-COOH-S-2840B 362 → 318 m/z	Water / Methanol (3:1, v/v)	0.050	43	17
		0.50	43	6.8
1'-CH_2OH-S-2840A 350 → 292 m/z	Water / Methanol (3:1, v/v)	0.050	43	- 7.0
		0.50	43	4.8

1'-CH_2OH-S-2840B 350 → 292 m/z	Water / Methanol (3:1, v/v)	0.050	43	5.8
		0.50	43	4.5

Stability of solvent calibration solutions was adequately shown for 3'-OH-S-2840, DFPA-$CONH_2$, DFPA, 1'-COOH-S-2840A, 1'-CH_2OH-S-2840A and 1'-CH_2OH-S-2840B. The stability of inpyrfluxam, N-des-Me-DFPA, and 1'-COOH-S-2840B are shown to be outside the acceptable range of ±10% as outlined in SANTE/2020/12830. However, data was generated prior to SANTE/2020/12830 rev.1. As the data only indicates the difference between fresh and stored solutions is at maximum -17%, which is within the acceptable limits outlined in prior guidance (SANCO/825/00 Rev 8.1). it's deemed to have minimal effect on the suitability the method.

Storage stability of extracts.

Storage stability of extracts was determined by fortifying the extracts at the LOQ level (0.01 mg/kg). The sample was stored at 1 °C – 10 °C for at least 12 days in the dark alongside one control sample. After this time, the final extracts were re-analysed against freshly prepared calibration standards. Only one mass transition per analyte was evaluated.

Grapes

Analyte	Fortification level (mg/kg)	Mean recovery 1st Injection (n = 5) (%)	Mean recovery 2nd Injection (n = 5) (%)	Days of storage (1st to 2nd Injection)	Difference (%) of recoveries after storage to recoveries before storage
Inpyrfluxam (334→294 *m/z*)	0.01	103	107	22	3.6
3'-OH-S-2840 (348→175 *m/z*)	0.01	109	100	22	-9.4
DFPA-$CONH_2$ (176→136 *m/z*)	0.01	109	116	22	7.0
N-des-Me-DFPA (161→141 *m/z*)	0.01	94	86	20	-7.6

DFPA (177→137 *m/z*)	0.01	104	99	28	-4.6
1'-COOH-S-2840A (364→278 *m/z*)	0.005	82	81	28	-1.8
1'-COOH-S-2840B (364→278 *m/z*)	0.005	77	81	28	3.6
1'-CH_2OH-S-2840A (350→292 *m/z*)	0.005	85	91	28	6.2
1'-CH_2OH-S-2840B (350→292 *m/z*)	0.005	94	87	28	-7.0

Potato (tuber)

Analyte	Fortification level (mg/kg)	Mean recovery 1st Injection (n = 5) (%)	Mean recovery 2nd Injection (n = 5) (%)	Days of storage (1st to 2nd Injection)	Difference (%) of recoveries after storage to recoveries before storage
Inpyrfluxam (334→294 *m/z*)	0.01	102	91	26	-11
3'-OH-S-2840 (348→175 *m/z*)	0.01	102	97	26	-4.8
DFPA-$CONH_2$ (176→136 *m/z*)	0.01	107	87	26	-19
N-des-Me-DFPA (161→141 *m/z*)	0.01	70	75	26	5.8

DFPA (177→137 *m/z*)	0.01	110	98	15	-12
1'-COOH-S-2840A (364→318 *m/z*)	0.005	98	88	15	-10
1'-COOH-S-2840B (364→318 *m/z*)	0.005	94	93	15	-1.2
1'-CH_2OH-S-2840A (350→292 *m/z*)	0.005	101	99	15	-2.2
1'-CH_2OH-S-2840B (350→292 *m/z*)	0.005	103	105	15	2.8

Soybean seed

Analyte	Fortification level (mg/kg)	Mean recovery 1st Injection (n = 5) (%)	Mean recovery 2nd Injection (n = 5) (%)	Days of storage (1st to 2nd Injection)	Difference (%) of recoveries after storage to recoveries before storage
Inpyrfluxam (334→294 *m/z*)	0.01	101	102	15	0.6
3'-OH-S-2840 (348→175 *m/z*)	0.01	101	101	15	-0.4
DFPA-$CONH_2$ (176→136 *m/z*)	0.01	100	94	15	-5.6
N-des-Me-DFPA (161→141 *m/z*)	0.01	92	78	14	-14

DFPA (177→137 *m/z*)	0.01	102	102	18	-0.4
1'-COOH-S-2840A (364→278 *m/z*)	0.005	85	92	18	6.8
1'-COOH-S-2840B (364→278 *m/z*)	0.005	81	94	18	12
1'-CH_2OH-S-2840A (350→292 *m/z*)	0.005	90	92	18	2.0
1'-CH_2OH-S-2840B (350→292 *m/z*)	0.005	101	101	18	0.2

Wheat (whole plant)

Analyte	Fortification level (mg/kg)	Mean recovery 1st Injection (n = 5) (%)	Mean recovery 2nd Injection (n = 5) (%)	Days of storage (1st to 2nd Injection)	Difference (%) of recoveries after storage to recoveries before storage
Inpyrfluxam (334→294 *m/z*)	0.01	108	104	16	-3.8
3'-OH-S-2840 (348→175 *m/z*)	0.01	104	105	16	1.0
DFPA-$CONH_2$ (176→136 *m/z*)	0.01	101	96	16	-4.8
N-des-Me-DFPA (161→141 *m/z*)	0.01	81	75	12	6.0

DFPA (177→137 *m/z*)	0.01	115	127	17	12
1'-COOH-S-2840A (364→278 *m/z*)	0.005	102	105	17	3.0
1'-COOH-S-2840B (364→278 *m/z*)	0.005	104	102	17	-2.6
1'-CH_2OH-S-2840A (350→292 *m/z*)	0.005	105	106	17	1.0
1'-CH_2OH-S-2840B (350→292 *m/z*)	0.005	105	115	17	11

Wheat (grain)

Analyte	Fortification level (mg/kg)	Mean recovery 1st Injection (n = 5) (%)	Mean recovery 2nd Injection (n = 5) (%)	Days of storage (1st to 2nd Injection)	Difference (%) of recoveries after storage to recoveries before storage
Inpyrfluxam (334→294 *m/z*)	0.01	97	94	23	-3.2
3'-OH-S-2840 (348→175 *m/z*)	0.01	109	90	23	-20
DFPA-$CONH_2$ (176→136 *m/z*)	0.01	110	96	23	-14
N-des-Me-DFPA (161→141 *m/z*)	0.01	90	102	22	12

DFPA (177→137 *m/z*)	0.01	96	98	18	1.8
1'-COOH-S-2840A (364→278 *m/z*)	0.005	93	93	18	0
1'-COOH-S-2840B (364→278 *m/z*)	0.005	93	97	18	3.6
1'-CH_2OH-S-2840A (350→292 *m/z*)	0.005	102	94	18	-7.8
1'-CH_2OH-S-2840B (350→292 *m/z*)	0.005	106	103	18	-2.8

Acceptable recoveries were shown for all tested metabolites and in all crop matrices. As a result of this data, it is concluded that stability of extracts is confirmed for at least 15 days for inpyrfluxam, 3'-OH-S-2840, DFPA-$CONH_2$, N-des-Me-DFPA, DFPA, 1'-COOH-S-2840A, 1'-COOH-S-2840B, 1'-CH_2OH-S-2840A, and 1'-CH_2OH-S-2840B in grapes, potato (tubers), wheat (whole plant), wheat (grain) and soybean (seeds).

It is noted that the mean recovery of DFPA was 127% after storage, however this was +12% from the first sampling point (115%). Therefore, despite 120% recoveries at the later sampling point, this could be attributed to the method rather than the stability of the analyte in the extract. Stability can be considered acceptable.,

Extraction efficiency

Extraction efficiency has been sufficiently addressed for high water, dry/high starch, high oil and high acid commodities under KCA 4.1.2/19. The extraction procedure of the current method is similar to the extraction procedure used and evaluated for Study KCA 4.1.2/19. Therefore, the same conclusions can apply to the current study; extraction efficiency has been sufficiently addressed for high water, dry/high starch, high oil and high acid commodities.

See KCA 4.1.2/19 for full consideration of extraction efficiency.

Conclusion

Validation data was generated prior to the introduction of SANTE/2020/12830 rev.1. The method can be considered sufficiently validated to the requirements as outlined in SANCO/3029/99 rev.4. The method meets the requirements outlined under section 4.2 of SANTE/2020/12830 rev.1 "minimum validation requirements for the assessment of

existing methods for risk assessment". Therefore, the method can be considered sufficiently validated in accordance with SANTE/2020/12830 rev.1.

The method is acceptable in the areas of specificity, linearity, accuracy, and precision. Extraction efficiency is considered sufficiently addressed. The LOQ of the method is 0.01 mg/kg (inpyrfluxam, 3'-OH-S-2840, DFPA-CONH2, DFPA and N-des-Me-DFPA) and 0.005 mg/kg (1'-CH2OH-S-2840A, 1'-CH2OH-S-2840B, 1'-COOH-S-2840A and 1'-COOH-S-2840B).

Matrix effect was investigated as part of the study and found with significant for a number of samples. Matrix matched standards were used for quantification for all analytes. Stability of stock and calibration solutions and final extracts was investigated; slight deviations are noted but have minimal impact on the study.

Reference:	KCA 4.1.2/21
Report Title:	S-2399: Validation of Valent's Method RM-50C-1, Determination of Residues of S-2399, 3′-OHS-2840, 1′-CH_2OH-S-2840-A,1′-CH_2OH-S-2840-B, DFPA-$CONH_2$, 1′-COOH-S-2840-A, and 1′-COOH-S-2840-B in Crops (including Conjugated Forms of the Metabolites Converted to their Aglycones)
Author(s) & Year:	[redacted] (2017)
Document No, Authority registration No	Study No.: VP-39140; Report No.: 201700135
Guideline(s):	Residue Analytical Method Series 860 – Residue Chemistry Guidelines OCSPP 860.1340
Deviations:	No
GLP or GEP:	Yes
Acceptability:	Yes
Study relied upon:	Yes

The following HPLC-MS/MS method was validated for grapes, potato (tuber), soybean (seeds), barley (whole plant) and wheat (grain) for inpyrfluxam, 3′-OHS-2840, 1′-CH_2OH-S-2840-A,1′-CH_2OH-S-2840-B, DFPA-$CONH_2$, 1′-COOH-S-2840-A, and 1′-COOH-S-2840-B and used in studies to support the residue data requirements.

Final Volume A was analysed for inpyrfluxam, 3′-OH-S-2840, 1′-CH_2OH-S-2840A, 1′-CH_2OH-S-2840B, DFPA-$CONH_2$.

Final Volume B was analysed for 1'-COOH-S-2840A and B and 1'-CH_2OH-S-2840A and B after acid hydrolysis. Therefore, the quantified residue levels will include both free and conjugated forms.

Analytes 1′-CH_2OH-S-2840A and 1′-CH_2OH-S-2840B were analysed in both Final Volume A (free), and Final Volume B. Final volume B contains an acid hydrolysis step therefore, the residues represent the sum of free and conjugated forms of the analyte.

Principle of the method

Weigh 10.0 g (± 0.1 g) of homogenised sample into a 50 mL polypropylene centrifuge tube (2.5 ± 0.1 g for livestock feed matrices (corn forage or stover)). Add 40 mL of acetonitrile/water (1:1, v/v) to the centrifuge tube containing the sample and shake on reciprocating shaker for 10 minutes. Soak samples overnight at room temperature or place in a refrigerator for longer storage periods.

Shake the sample for 30 minutes on reciprocating shaker then centrifuge the sample for approximately 5 minutes at 2000 rpm or as needed to separate the solids from the extraction solvent. Pour the sample extract through a funnel containing a GFA glass filter into a stoppable 100 mL graduated cylinder.

Add 25 mL of acetonitrile/water (1:1, v/v) to the centrifuge tube containing the sample. Sample may be hand shaken or struck against counter to break up pellet if tightly on bottom prior to shaking. Shake on a reciprocating shaker for 30 minutes. Centrifuge the sample for approximately 5 minutes and pour the sample extract through the funnel, containing a GFA glass filter, into the 100 mL graduated cylinder containing the first extract. Repeat this step once more.

Add 1 mL of 0.5 M sodium acetate solution to the graduated cylinder, stopper and mix. Make up to 100 mL using acetonitrile/water (1:1, v/v) and mix. This extract can be stored in a refrigerator or freezer before the following step.

A: Hexane/Ethyl Acetate Partition

Place 10 mL (20 mL for animal feed matrices (corn forage/stover)) aliquot of the extraction solution into a 250 mL round bottom flask and evaporate the acetonitrile in a water bath set at ≤40 °C. Transfer the sample to a 50 mL centrifuge tube. Rinse the round bottom flask with a small amount of HPLC water and add to the centrifuge tube. Bring the centrifuge tube to 10 mL with HPLC water.

Add 20 mL of hexane/ethyl acetate (4:1, v/v) to the round bottom flask and sonicate briefly. Add solvent to the centrifuge tube and shake for one minute and allow to separate. Pipette the top solvent layer into a stoppable 100 mL graduated cylinder. Extract the aqueous phase in the centrifuge tube two additional times with 20 mL hexane/ethyl acetate (4:1, v/v) adding each solvent top to the 100 mL graduated cylinder containing the first extraction.

Using hexane/ethyl acetate (4:1), make the graduated cylinder up to 70 mL and mix. Set aside or place in refrigerator for storage until completion of the next step.

B: Strata-X Column Cleanup A

Transfer the aqueous sample from Step A to a 250 mL round bottom flask and evaporate any remaining solvent residue off using a rotary evaporator and water bath set to ≤40 °C. Attach a Strata-X Phase Extraction Tube to a vacuum manifold. Condition the column using gravity with 5mL of methanol followed by 10 mL of deionized water.

Transfer the aqueous sample to the column and allow to drain through the column by gravity. Rinse the round bottom flask with 10mL of methanol/water (1:9, v/v) and transfer to the column allowing to drain through the column by gravity. Discard these eluents.

Place a 15 mL graduated centrifuge tube under the column and elute the residues with 12 mL of acetonitrile/water (3:1, v/v). Remove the centrifuge tube and make up to 14 mL acetonitrile/water (3:1, v/v). Cap the centrifuge tube and mix.

C: Final Volume A

Place 35 mL of hexane/ethyl acetate extract from Step A into a 250 mL round bottom flask. Add 7 mL of acetonitrile/water eluent from Step B. Evaporate the sample to dryness using a rotary-evaporator and water bath set to ≤40 °C. Ethyl acetate (~10 mL) may be added to sample to azeotrope the water.

Reconstitute the residues in 2.5 mL of methanol/HPLC water (1:1. v/v), sonicate then transfer the extract to a vial for storage. Store in a freezer until LC-MS/MS analysis. Filter a portion of the final volume extract through a syringe equipped with a PTFE 0.22 µm filter into an autosampler vial.

D: Hydrolysis

Transfer 7 mL of acetonitrile/water from Step B into a 100 mL round bottom flask. Evaporate the acetonitrile off using a rotary-evaporator and water bath set to ≤40 °C. Pipette the water to a vial. Add two 1 mL portions of deionised water to rinse the round bottom flask and add to the vial. Make the vial up to 5 mL with deionised water.

Add 1 mL of concentrated HCL (12N) to the vial, cap, and place in a preheated block in a bath set at 95 °C for 1 hour.

Remove the vial from the heating block and allow to cool for about 10-15 minutes. Add 500 µL of 50% NaOH solution and 2mL of 2 M sodium acetate solution. Recap the vial and mix.

E: Strata-X Column Cleanup B

Attach a Strata-X Solid Phase Extraction Tube to the vacuum manifold. Condition the column using gravity with 5 mL of methanol followed by 10 mL of deionized water.

Transfer the aqueous sample to the column. Rinse the vial with two 1 mL portions of deionised water and add each rinse to the column. Allow to flow through the column by gravity. Add 20 mL of deionised water to the column and allow to flow through. Add 10 mL of methanol/water (1:9, v/v) to the column and allow to flow through the column. Discard these eluents.

Remove the column from the vacuum manifold and place the column in the neck of a 100 mL round bottom flask. Elute the residues from the column by adding 15 mL of acetonitrile, allowing to flow through the column into the round bottom flask by gravity.

F: Final Volume B

Evaporate the sample to dryness using a rotary evaporator and water bath set to ≤40 °C. Ethyl acetate (~5 mL) may be added to the sample to azeotrope the water.

Reconstitute the residues in 2.5 mL of methanol/water (1:1, v/v), or internal standard Final Volume Solution B (if using optional internal standards). Sonicate the sample before transferring the extract to a vial for storage. Store in a freezer until LC/MS/MS analysis. Transfer a portion of the final volume extract to an autosampler vial.

HPLC conditions (inpyrfluxam, DFPA-$CONH_2$, 1'-COOH-S-2840A and B, 1'-CH_2OH-S-2840A and B, 3'-OH-S-2840)

Column Eclipse XDB-C8, 5 µm, 4.6 mm x 150 mm

Mobile phase A = 5mM ammonium acetate in HPLC water
B = 5mM ammonium acetate in methanol

Time (minutes)	Flow rate (µL/min)	%A	%B
0.0	400	50.0	50.0
1.0	400	50.0	50.0
6.0	400	10.0	90.0
14.0	400	10.0	90.0
14.5	400	50.0	50.0
21.0	400	50.0	50.0

Column temperature 40.0 °C
Injection volume 25.0 µL
Retention time ~4.65 minutes (DFPA-$CONH_2$)

~6.31 minutes (1'-COOH-S-2840A)
~7.3 minutes (1'-COOH-S-2840B)
~10.07 minutes (1'-CH_2OH-S-2840A)
~10.53 minutes (1'-CH_2OH-S-2840B)
~12.15 minutes (3'-OH-S-2840)
~12.7 minutes (Inpyrfluxam)

MS conditions (DFPA-$CONH_2$)

Instrument	Applied Biosystems API 4000 mass spectrometer
Ionisation Type	Electrospray ionisation (ESI)
Polarity	Positive ion mode
Scan Type	MRM
Ion spray voltage	4500 V
Mass transition monitored (*m/z*)	176 → 136* 176 → 156
Temperature (TEM)	550 °C

* Proposed for quantification

MS conditions (1'-COOH-S-2840A and B)

Instrument	Applied Biosystems API 4000 mass spectrometer
Ionisation Type	Electrospray ionisation (ESI)
Polarity	Negative ion mode
Scan Type	MRM
Ion spray voltage	- 4000 V
Mass transition monitored (*m/z*)	362 → 318* 362 → 131
Temperature (TEM)	500 °C

* Proposed for quantification

MS conditions (1'-CH_2OH-S-2840A and B)

Instrument	Applied Biosystems API 4000 mass spectrometer
Ionisation Type	Electrospray ionisation (ESI)
Polarity	Positive ion mode
Scan Type	MRM
Capillary voltage	4500 V
Mass transition monitored (*m/z*)	350 → 292* 350 → 159
Temperature (TEM)	300 °C

* Proposed for quantification

MS conditions (3'-OH-S-2840)

Instrument	Applied Biosystems API 4000 mass spectrometer
Ionisation Type	Electrospray ionisation (ESI)
Polarity	Negative ion mode
Scan Type	MRM
Capillary voltage	- 4500 V
Mass transition monitored (*m/z*)	348 → 130.9* 348 → 175
Temperature (TEM)	350 °C

* Proposed for quantification

MS conditions (Inpyrfluxam)

Instrument	Applied Biosystems API 4000 mass spectrometer
Ionisation Type	Electrospray ionisation (ESI)
Polarity	Positive ion mode
Scan Type	MRM
Capillary voltage	3500 V
Mass transition monitored (*m/z*)	334 → 258* 334 → 294
Temperature (TEM)	400 °C

* Proposed for quantification

Table B.5.1.2.5-14: Analytical validation data for the determination of inpyrfluxam in corn grain, soyabean seed, apples and corn stover.

Analyte	Matrix	LOQ (mg/kg)	Recovery fortification level (mg/kg)	Recoveries % range (mean)	Repeatability % RSD (n)	Linearity
Inpyrfluxam	Corn Grain	0.01 *(334 → 258.0 m/z)*	0.01	76.9 – 84.6 (81.7)	3.53 (5)	1 – 25 µg/L R^2 = 0.99980 (N = 5) Y = -3.51E-23x^2 + 4.32E+00x + -9.55E-02
			0.1	81.2 – 90.7 (86.0)	4.94 (5)	

	Soyabean Seed	0.01 *(334 → 258.0 m/z)*	0.01	73.9 – 80.7 (78.1)	3.69 (5)	1 – 25 µg/L R^2 = 0.99998 (N = 5) Y = -7.10E-02x^2 + 4.58E+00x + -1.43E-01
			0.1	74.9 – 81.8 (78.0)	3.86 (5)	
	Apples	0.01 *(334 → 258.0 m/z)*	0.01	79.9 – 96.5 (87.3)	7.26 (5)	1 – 25 µg/L R^2 = 0.99979 (N = 5) Y = -2.59E-03x^2 + 4.29E+00x + 6.48E-02
			0.1	85.0 – 90.6 (88.0)	2.35 (5)	
	Corn Stover	0.02 *(334 → 258.0 m/z)*	0.02	75.6 – 80.9 (78.6)	2.88 (5)	1 – 25 µg/L R^2 = 0.99995 (N = 5) Y = -3.90E-02x^2 + 5.18E+00x + -1.28E-01
			0.2	70.1 – 73.7 (72.1)	2.18 (5)	

Table B.5.1.2.5-15: Analytical validation data for the determination of 3'-OH-S-2840 in corn grain, soyabean seed, apples and corn stover.

Analyte	Matrix	LOQ (mg/kg)	Recovery fortification level (mg/kg)	Recoveries % range (mean)	Repeatability % RSD (n)	Linearity
3'-OH-S-2840	Corn Grain	0.01 *(348 → 130.9 m/z)*	0.01	70.5 – 78.4 (75.6)	4.08 (5)	1 – 25 µg/L R^2 = 0.99991 (N = 5) Y = 7.55E-02x^2 + 4.23E+00x + -1.79E-01
			0.1	81.2 – 90.7 (86.7)	4.14 (5)	
	Soyabean Seed	0.01	0.01	73.4 – 88.8 (82.2)	6.87 (5)	1 – 25 µg/L R^2 = 0.99990

		(348 → 130.9 m/z)	0.1	86.2 – 93.6 (90.8)	3.8 (5)	(N = 5) Y = 5.54E-02x^2 + 4.34E+00x + -1.64E-01
	Apples	0.01 *(348 → 130.9 m/z)*	0.01	79.5 – 91.9 (88.0)	5.87 (5)	1 – 25 µg/L R^2 = 0.99964 (N = 5) Y = 8.25E-02x^2 + 3.70E-00x + 3.75E-02
			0.1	82.8 – 89.9 (87.6)	3.34 (5)	
	Corn Stover	0.02 *(348 → 130.9 m/z)*	0.02	71.7 – 91.2 (82.1)	10.41 (5)	1 – 25 µg/L R^2 = 0.99993 (N = 5) Y = -1.90E-02x^2 + 3.27E+00x + -3.84E-02
			0.2	73.1 – 77.1 (75.3)	1.98 (5)	

Table B.5.1.2.5-16: Analytical validation data for the determination of 1'-CH_2OH-S-2840A (Free) in corn grain, soyabean seed, apples and corn stover.

Analyte	Matrix	LOQ (mg/kg)	Recovery fortification level (mg/kg)	Recoveries % range (mean)	Repeatability % RSD (n)	Linearity
1'-CH_2OH-S-2840A (Free)	Corn Grain	0.01 *(350 → 292 m/z)*	0.01	73.8 – 85.1 (82.2)	5.77 (5)	1 – 25 µg/L R^2 = 0.99987 (N = 5) Y = -4.06E-02x^2 + 4.15E+00x + -1.20E-01
			0.1	81.8 – 91.7 (85.2)	4.44 (5)	
	Soyabean Seed	0.01 *(350 → 292 m/z)*	0.01	76.5 – 86.7 (82.5)	4.71 (5)	1 – 25 µg/L R^2 = 0.99948 (N = 5) Y = 8.30E-04x^2 + 4.07E+00x + -4.00E-02
			0.1	85.2 – 102.6 (95.2)	7.45 (5)	
	Apples	0.01 *(350 → 292 m/z)*	0.01	74.3 – 82.6 (79.1)	4.64 (5)	1 – 25 µg/L R^2 = 0.99982 (N = 5)
			0.1	78.7 – 88.6 (84.3)	5.84 (5)	

						Y = 2.64E-02x^2 + 3.86+00x + -3.39E-02
	Corn Stover	0.02 *(350 → 292 m/z)*	0.02	71.3 – 92.3 (79.7)	9.64 (5)	1 – 25 µg/L R^2 = 0.99988 (N = 5) Y = -2.52E-02x^2 + 4.70E+00x + -3.82E-02
			0.2	77.4 – 82.0 (79.5)	2.12 (5)	

Table B.5.1.2.5-17: Analytical validation data for the determination of 1'-CH_2OH-S-2840B (Free) in corn grain, soyabean seed, apples and corn stover.

Analyte	Matrix	LOQ (mg/kg)	Recovery fortification level (mg/kg)	Recoveries % range (mean)	Repeatability % RSD (n)	Linearity
1'-CH_2OH-S-2840B (Free)	Corn Grain	0.01 *(350 → 292 m/z)*	0.01	74.2 – 84.3 (80.6)	4.64 (5)	1 – 25 µg/L R^2 = 1.0 (N = 5) Y = -4.78E-02x^2 + 4.10E+00x + -1.61E-01
			0.1	82.2 – 87.2 (84.6)	2.43 (5)	
	Soyabean Seed	0.01 *(350 → 292 m/z)*	0.01	73.7 – 82.3 (79.0)	4.37 (5)	1 – 25 µg/L R^2 = 0.99978 (N = 5) Y = -1.90E-03x^2 + 3.91E+00x + -6.36E-02
			0.1	81.4 – 84.6 (82.8)	1.62 (5)	
	Apples	0.01 *(350 → 292 m/z)*	0.01	71.2 – 88.4 (79.6)	10.18 (5)	1 – 25 µg/L R^2 = 0.99979 (N = 5) Y = -5.02E-03x^2 + 3.77E+00x + 1.31E-02
			0.1	81.0 – 91.4 (85.2)	4.5 (5)	
	Corn Stover	0.02 *(350 → 292 m/z)*	0.02	73.0 – 89.2 (79.6)	7.95 (5)	1 – 25 µg/L R^2 = 0.99970 (N = 5) Y = -4.05E-03x^2 +
			0.2	76.0 – 78.8 (77.9)	1.44 (5)	

						4.44E+00x + -1.94E-02

Table B.5.1.2.5-18: Analytical validation data for the determination of DFPA-$CONH_2$ in corn grain, soyabean seed, apples and corn stover.

Analyte	Matrix	LOQ (mg/kg)	Recovery fortification level (mg/kg)	Recoveries % range (mean)	Repeatability % RSD (n)	Linearity
DFPA-$CONH_2$	Corn Grain	0.01 *(176 → 136 m/z)*	0.01	81.7 – 91.1 (84.0)	4.76 (5)	1 – 25 µg/L R^2 = 0.99931 (N = 5) Y = -3.40E-01x^2 + 1.09E+01x + -2.88E-01
			0.1	80.1 – 87.5 (84.1)	5.25 (5)	
	Soyabean Seed	0.01 *(176 → 136 m/z)*	0.01	70.1 – 90.0 (76.2)	10.4 (5)	1 – 25 µg/L R^2 = 0.99996 (N = 5) Y = 2.82E-02x^2 + 1.01E+01x + -1.56E-02
			0.1	74.8 – 85.6 (79.8)	5.31 (5)	
	Apples	0.01 *(176 → 136 m/z)*	0.01	81.5 – 100.0 (87.6)	9.2 (5)	1 – 25 µg/L R^2 = 0.99957 (N = 5) Y = 8.57E-03x^2 + 4.52E+00x + 1.18E-01
			0.1	76.7 – 92.5 (85.1)	7.59 (5)	
	Corn Stover	0.02 *(176 → 136 m/z)*	0.02	81.2 – 95.7 (86.7)	6.36 (5)	1 – 25 µg/L R^2 = 0.99966 (N = 5) Y = -2.33E-01x^2 + 1.13E+01x + -7.35E-02
			0.2	82,6 – 92.1 (86.2)	4.13 (5)	

Table B.5.1.2.5-19: Analytical validation data for the determination of 1'-COOH-S-2840A in corn grain, soyabean seed, apples and corn stover.

Analyte	Matrix	LOQ (mg/kg)	Recovery fortification level (mg/kg)	Recoveries % range (mean)	Repeatability % RSD (n)	Linearity
1'-COOH-S-2840A	Corn Grain	0.01 *(362 → 318 m/z)*	0.01	77.7 – 102.5 (94.1)	10.22 (5)	1 – 25 µg/L R^2 = 0.99986 (N = 5) Y = 3.37E-03x^2 + 4.53E+00x + 2.34E-03
			0.1	87.6 – 103.6 (93.9)	6.59 (5)	
	Soyabean Seed	0.01 *(362 → 318 m/z)*	0.01	77.1 – 103.9 (93.8)	11.11 (5)	1 – 25 µg/L R^2 = 0.99982 (N = 5) Y = 5.47E-02x^2 + 4.56E+00x + -4.82E-02
			0.1	72.7 – 99.2 (89.3)	11.23 (5)	
	Apples	0.01 *(362 → 318 m/z)*	0.01	84.3 – 89.1 (87.0)	2.62 (5)	1 – 25 µg/L R^2 = 0.99993 (N = 5) Y = 2.12E-02x^2 + 4.51E+00x + 4.54E-02
			0.1	85.3 – 91.2 (88.4)	3.15 (5)	
	Corn Stover	0.02	0.02	93.8 – 107.3 (99.4)	8.57 (5)	1 – 25 µg/L

		(362 → 318 m/z)	0.2	90.7 – 100.6 (93.2)	3.48 (5)	R^2 = 0.99993 (N = 5) Y = -6.06E-03x^2 + 4.66E+00x + -2.51E-02

Table B.5.1.2.5-20: Analytical validation data for the determination of 1'-COOH-S-2840B in corn grain, soyabean seed, apples and corn stover.

Analyte	Matrix	LOQ (mg/kg)	Recovery fortification level (mg/kg)	Recoveries % range (mean)	Repeatability % RSD (n)	Linearity
1'-COOH-S-2840B	Corn Grain	0.01 *(362 → 318 m/z)*	0.01	80.3 – 106.5 (96.8)	10.31 (5)	1 – 25 µg/L R^2 = 0.99997 (N = 5) Y = 6.51E-02x^2 + 6.33E+00x + -7.90E-02
			0.1	87.5 – 100.3 (92.8)	5.85 (5)	
	Soyabean Seed	0.01 *(362 → 318 m/z)*	0.01	91.7 – 106.4 (100.0)	7.46 (5)	1 – 25 µg/L R^2 = 0.99920 (N = 5) Y = -6.93E-02x^2 + 6.74E+00x + -6.53E-02
			0.1	76.3 – 99.7 (90.5)	9.52 (5)	
	Apples	0.01 *(362 → 318 m/z)*	0.01	82.5 – 90.8 (86.4)	3.96 (5)	1 – 25 µg/L R^2 = 0.99995 (N = 5) Y = 5.68E-02x^2 + 6.25E+00x + 5.21E-02
			0.1	82.0 – 90.2 (86.1)	3.75 (5)	
	Corn Stover	0.02 *(362 → 318 m/z)*	0.02	93.8 – 107.3 (102.1)	6.11 (5)	1 – 25 µg/L R^2 = 0.99984 (N = 5) Y = -1.24E-01x^2 +
			0.2	90.7 – 100.6 (93.6)	4.55 (5)	

						6.88E+00x + -8.12E-02

Table B.5.1.2.5-21: Analytical validation data for the determination of 1'-CH_2OH-S-2840A in corn grain, soyabean seed, apples and corn stover.

Analyte	Matrix	LOQ (mg/kg)	Recovery fortification level (mg/kg)	Recoveries % range (mean)	Repeatability % RSD (n)	Linearity
1'-CH_2OH-S-2840A	Corn Grain	0.01 *(350 → 292 m/z)*	0.01	85.0 – 104.4 (96.6)	9.09 (5)	1 – 25 µg/L R^2 = 0.99999 (N = 5) Y = 8.58E-03x^2 + 3.57E+00x + -4.63E-02
			0.1	92.0 – 101.6 (94.7)	4.18 (5)	
	Soyabean Seed	0.01 *(350 → 292 m/z)*	0.01	88.4 – 106.5 (102.0)	7.55 (5)	1 – 25 µg/L R^2 = 0.99993 (N = 5) Y = 5.66E-04x^2 + 3.45E+00x + 168E-02
			0.1	72.4 – 97.3 (89.5)	11.36 (5)	
	Apples	0.01 *(350 → 292 m/z)*	0.01	83.1 – 101.1 (93.3)	8.07 (5)	1 – 25 µg/L R^2 = 0.99990 (N = 5) Y = 2.21E-02x^2 + 3.11E+00x + 5.12E-02
			0.1	82.9 – 92.2 (86.3)	4.27 (5)	
	Corn Stover	0.02 *(350 → 292 m/z)*	0.02	99.4 – 117.6 (107.8)	8.04 (5)	1 – 25 µg/L R^2 = 0.99950 (N = 5) Y = 1.98E-02x^2 + 3.38E+00x + 9.18E-02
			0.2	92.9 – 101.6 (95.8)	3.66 (5)	

Table B.5.1.2.5-22: Analytical validation data for the determination of 1'-CH_2OH-S-2840B in corn grain, soyabean seed, apples and corn stover.

Analyte	Matrix	LOQ (mg/kg)	Recovery fortification level (mg/kg)	Recoveries % range (mean)	Repeatability % RSD (n)	Linearity
1'-CH_2OH-S-2840B	Corn Grain	0.01 *(350 → 292 m/z)*	0.01	82.0 – 105.5 (96.8)	3.66 (5)	1 – 25 µg/L R^2 = 0.99996 (N = 5) Y = -1.63E-02x^2 + 3.92E+00x + -4.47E-03
			0.1	82.7 – 98.2 (89.6)	6.32 (5)	
	Soyabean Seed	0.01 *(350 → 292 m/z)*	0.01	78.1 – 102.7 (93.7)	10.15 (5)	1 – 25 µg/L R^2 = 0.99989 (N = 5) Y = 1.34E-02x^2 + 3.77E+00x + -1.70E-02
			0.1	73.9 – 92.5 (87.1)	8.61 (5)	
	Apples	0.01 *(350 → 292 m/z)*	0.01	84.5 – 95.1 (91.3)	5.06 (5)	1 – 25 µg/L R^2 = 0.99999 (N = 5) Y = 4.26E-02x^2 + 3.45E+00x + -7.10E-03
			0.1	86.4 – 97.4 (90.5)	4.58 (5)	
	Corn Stover	0.02 *(350 → 292 m/z)*	0.02	88.9 – 110.6 (102.2)	8.89 (5)	1 – 25 µg/L R^2 = 0.99973 (N = 5) Y = -1.16E-02x^2 + 3.88E+00x + -3.70E-02
			0.2	87.4 – 98.5 (91.5)	4.72 (5)	

Specificity:

Specificity was demonstrated by retention time match with a reference standard. Analysis of unfortified control samples and reagent blanks demonstrated no significant interference (> 30% of the LOQ) at the retention time of interest.

Matrix Effects:

Solvent based standards were used for quantification. Matrix effects were not investigated as part of the study. Samples were fortified with deuterated internal standards prior to analysis and the peak area of analyte and internal standard determined within the samples

using separate mass transitions, and the peak ratio calculated and used for quantification. The use of an IL-IS in the calibration and quantification corrects for potential matrix effect, therefore no further consideration is required.

Linearity:

Linearity was demonstrated using a quadratic fit model. Five samples were prepared over the concentration range of 1 – 25 ug/L. Calibration standards were fortified with analyte and internal standard and the peak area for each determined separately, and the peak ratio calculated between the two values and used for quantification. The calculated coefficient of determination was ≥0.999 and considered acceptable.

As per the method plan, any sample with a detector response greater than the largest calibration standard response must be appropriately diluted using methanol/water (1:1, v/v) or internal standard solution so that the analyte response falls within the calibration standard range.

Accuracy:

Recovery samples were prepared by spiking plant matrices with active substance and metabolite standards and analysing them by the method described. The spike concentrations were in the range 0.01 to 0.1 mg/kg for corn grain, soyabean seed and apples, and 0.02 to 0.2 mg/kg for corn stover. Five samples were prepared at each fortification level. Mean recovery levels were within the range 72.1 - 107.8% and are acceptable.

Precision:

Precision was determined from the accuracy recovery data. Five samples were prepared at each fortification level, however the % RSD at each fortification level was < 20%.

Stock stability

The stability of stock solutions has not been addressed as part of the current study.

Stability of calibration solution

The stability of calibration solutions has not been addressed as part of the current study.

Extract stability

Extract stability was not investigated as part of the current study. Isotopic labelled internal standards were used for validation. When employed to quantify residues in support of residue studies, extract stability should be confirmed via acceptable concurrent recoveries and/or the use of isotopic labelled internal standards.

Extraction efficiency:

Extraction efficiency has been sufficiently addressed for high water, dry/high starch and high oil commodities under KCA 4.1.2/19. The extraction procedure of the current method is similar to the extraction procedure used and evaluated for Study KCA 4.1.2/19. Therefore, the same conclusions can apply to the current study; extraction efficiency has been sufficiently addressed for high water, dry/high starch and high oil commodities.

See KCA 4.1.2/19 for full consideration of extraction efficiency.

Conclusion

Validation data was generated prior to the introduction of SANTE/2020/12830 rev.1. The method can be considered sufficiently validated to the requirements as outlined in SANCO/3029/99 rev.4. The method meets the requirements outlined under section 4.2 of SANTE/2020/12830 rev.1 “minimum validation requirements for the assessment of existing methods for risk assessment”. Therefore, the method can be considered sufficiently validated in accordance with SANTE/2020/12830 rev.1.

The method is acceptable in the areas of specificity, linearity, accuracy, and precision. Extraction efficiency is considered sufficiently addressed. The LOQ of the method is 0.01 mg/kg for all analytes with the exception of corn stover, which has the LOQ of 0.02 mg/kg.

Stock stability, calibration stability and matrix stability were not addressed as part of the validation data. However, this is deemed to have minimal impact on the validity of the study.

Matrix effects were not investigated as part of the study and solvent based solutions were used. IL-IS have been used for validation. IL-IS must be used, as suggested by the study, until matrix effects are sufficiently demonstrated.

Reference:	KCA 4.1.2/28
Report Title:	Magnitude of S-2399 and Metabolites 1’-CH_2OH-S-2840 A&B (including conjugate) and 1’-COOH-S-2840 A&B Residues in Laying Hen Tissues and Eggs from a 28-Day Feeding Study
Author(s) & Year:	[redacted], 2017
Document No, Authority registration No	Study No.: 213-057-09 (2815W) Report No.: TPR-0015
Guideline(s):	OPPTS 860.1480, PMRA DIR-98-02

Deviations:	No
GLP or GEP:	Yes
Acceptability:	Yes
Study relied upon:	Yes

The following HPLC-MS/MS method was validated for poultry matrices (egg, fat, liver, meat) for inpyrfluxam, 1′-CH_2OH-S-2840-A, 1′-CH_2OH-S-2840-B, 1′-COOH-S-2840-A, and 1′-COOH-S-2840-B and used in studies to support the residue data requirements.

Samples for analysis of 1'-CH_2OH-S-2840A and B residues are subjected to acid hydrolysis. Therefore, the quantified residue levels will include both free and conjugated forms.

Principle of the method

Egg (egg whites and yolk)

Inpyrfluxam, 1'-COOH-S-2840A and 1'-COOH-S-2840B

Weigh 5g of eggs into a 50 mL centrifuge bottle, add 25 mL of hexane:acetone (1:1, v/v) and three stainless steel grinding balls.

Grind for 2 minutes at 1500 RPM then centrifuge at 2800 rpm for 10 minutes. Transfer the sample to a 100 mL cylinder and filter through a funnel plugged with glass wool. Repeat the extraction process twice more with 25 mL of acetone.

Make up to 100 mL with acetone. Transfer the mixture to an amber bottle and mix well (initial extract).

Inpyrfluxam, 1'-COOH-S-2840A and B Clean-Up

Transfer 2 mL of the initial extract to a 125 mL bottle containing 50 mL hexane then add 20 mL acetonitrile. Shake for 5 minutes then transfer to a separatory funnel and allow the phases to separate. Collect the acetonitrile layer in a 125 mL flat bottom flask. Return the hexane layer to the 125 mL bottle and repeat the previous step collecting a total of 40 mL of acetonitrile in the same 125 mL flat bottom flask. Discard the hexane layer.

Evaporate the acetonitrile solution to dryness at 35°C then reconstitute with 10 mL methanol/water (1:1, v/v). Finally, filter the solution through a 0.45 µm nylon filter. The sample is diluted as necessary prior to analysis.

Hydrolysis of 1'-CH_2OH-S-2840A and 1'-CH_2-OH-S-2840B

Transfer 4 mL of initial extract to a 50 mL flat bottom flask. Evaporate at 35 °C to near dryness, then reconstitute with 2 mL acetonitrile:water (1:1, v/v). Sonicate to dissolve. Add 1.0 mL of 1M HCL and heat in an oil bath, under flux, for 4 hours at 100 °C. Cool to room temperature, then add 5 mL of water. Sonicate to dissolve.

Prepare an SPE cartridge with 5 mL of methanol followed by 10 mL of water. Load the sample onto the pre-conditioned cartridge. Rinse the flask with 2 x 5 mL of methanol:water (1:1, v/v) and transfer to the cartridge. Add 10 mL of methanol to the SPE cartridge and collect the eluent in a 15 mL tube, vortex to mix. Transfer the methanol elute to a graduated cylinder and make up the volume to 20 mL with water, transfer to an amber bottle and mix well. Transfer aliquots to autosampler vials for analysis.

Fat

Inpyrfluxam, 1'-COOH-S-2840A and 1'-COOH-S-2840B

Weigh 10 g of fat into a 50 mL centrifuge bottle, add 25 mL of hexane:acetone (4:1, v/v) and three stainless steel grinding balls.

Grind for 2 minutes at 1500 RPM then centrifuge at 2800 rpm for 10 minutes. Transfer the sample to a 100 mL cylinder and filter through a funnel plugged with glass wool. Repeat the extraction process twice more with 25 mL of acetone

Make up to 100 mL with acetone. Transfer the mixture to an amber bottle and mix well (initial extract).

Inpyrfluxam, 1'-COOH-S-2840A and B Clean-Up

Transfer 2 mL of the initial extract to a 125 mL bottle containing 50 mL hexane then add 20 mL acetonitrile. Shake for 5 minutes then transfer to a separatory funnel and allow the phases to separate. Collect the acetonitrile layer in a 125 mL flat bottom flask. Return the hexane layer to the 125 mL bottle and repeat the previous step collecting a total of 40 mL of acetonitrile in the same 125 mL flat bottom flask. Discard the hexane layer.

Evaporate the acetonitrile solution to dryness at 35°C then reconstitute with 20 mL methanol:water (1:1, v/v). Finally, filter the solution through a 0.45 µm nylon filter. The sample is diluted as necessary prior to analysis.

Hydrolysis of 1'-CH_2OH-S-2840A and 1'-CH_2-OH-S-2840B

Transfer 4 mL of initial extract to a 50 mL flat bottom flask. Evaporate at 35 °C to near dryness, then reconstitute with 2 mL acetonitrile:water (1:1, v/v). Sonicate to dissolve. Add 1.0 mL of 1M HCL and heat in an oil bath, under flux, for 4 hours at 100 °C. Cool to room temperature, then add 5 mL of water. Sonicate to dissolve.

Prepare an SPE cartridge with 5 mL of methanol followed by 10 mL of water. Load the sample onto the pre-conditioned cartridge. Rinse the flask with 2 x 5 mL of methanol:water (1:1, v/v) and transfer to the cartridge. Add 10 mL of methanol to the SPE cartridge and collect the eluent in a 15 mL tube. Transfer the methanol elute to a graduated cylinder and make up the volume to 20 mL with water, transfer to an amber bottle and mix well. Transfer aliquots to autosampler vials for analysis.

Liver

Inpyrfluxam, 1'-COOH-S-2840A and 1'-COOH-S-2840B

Weigh 5g of tissue into a 50 mL centrifuge bottle, add 25 mL of acetonitrile:water (1:1, v/v) and three stainless steel grinding balls.

Grind for 2 minutes at 1500 RPM then centrifuge at 2800 rpm for 10 minutes. Transfer the sample to a 100 mL cylinder and filter through a funnel plugged with glass wool. Repeat the extraction process again with 25 mL of acetonitrile:water (1:1, v/v), then a third time with 25 mL of acetonitrile.

Make up to 100 mL with acetonitrile:water (1:1, v/v). Transfer the mixture to an amber bottle and mix well (initial extract).

Inpyrfluxam, 1'-COOH-S-2840A and B Clean-Up

Transfer 100 µL of the initial extract into 400 µL of methanol:water (1:1, v/v). Vortex to mix. Filter through a 0.45 µm nylon filter. Dilute as necessary prior to analysis.

Hydrolysis of 1'-CH_2OH-S-2840A and 1'-CH_2-OH-S-2840B

Transfer 4 mL of initial extract to a 50 mL flat bottom flask. Add 1.0 mL of 1M HCL and heat in an oil bath, under flux, for 4 hours at 100 °C. Cool to room temperature then add 5 mL of water. Sonicate to dissolve.

Prepare an SPE cartridge with 5 mL of methanol followed by 10 mL of water. Load the sample onto the pre-conditioned cartridge. Rinse the flask with 2 x 5 mL of methanol:water (1:1, v/v) and transfer to the cartridge. Add 10 mL of methanol to the SPE cartridge and collect the eluent in a 15 mL tube. Transfer the methanol elute to a graduated cylinder and make up the volume to 20 mL with water, transfer to an amber bottle and mix well. Transfer aliquots to autosampler vials for analysis.

Muscle

Inpyrfluxam, 1'-COOH-S-2840A and 1'-COOH-S-2840B

Weigh 10 g of tissue into a 50 mL centrifuge bottle, add 25 mL of acetonitrile:water (1:1, v/v) and three stainless steel grinding balls.

Grind for 2 minutes at 1500 RPM then centrifuge at 2800 rpm for 10 minutes. Transfer the sample to a 100 mL cylinder and filter through a funnel plugged with glass wool. Repeat the extraction process again with 25 mL of acetonitrile:water (1:1, v/v), then a third time with 25 mL of acetonitrile.

Make up to 100 mL with acetonitrile:water (1:1, v/v). Transfer the mixture to an amber bottle and mix well (initial extract).

Inpyrfluxam, 1'-COOH-S-2840A and B Clean-Up

Transfer 1 mL of the initial extract into a 10 mL flask and dilute to mark with methanol:water (1:1, v/v). Vortex to mix. Filter through a 0.45 µm nylon centrifuge filter. Dilute as necessary before analysis.

Hydrolysis of 1'-CH_2OH-S-2840A and 1'-CH_2-OH-S-2840B

Transfer 2 mL of initial extract to a 50 mL flat bottom flask. Add 1.0 mL of 1M HCL and heat in an oil bath, under flux, for 4 hours at 100 °C. Cool to room temperature then add 5 mL of water. Sonicate to dissolve.

Prepare an SPE cartridge with 5 mL of methanol followed by 10 mL of water. Load the sample onto the pre-conditioned cartridge. Rinse the flask with 2 x 5 mL of methanol:water (1:1, v/v) and transfer to the cartridge. Add 10 mL of methanol to the SPE cartridge and collect the eluent in a 15 mL tube. Transfer the methanol elute to a graduated cylinder and make up the volume to 20 mL with water, transfer to an amber bottle and mix well. Transfer aliquots to autosampler vials for analysis.

HPLC conditions

Column ACE 3 C18-PFP, 3.0 µm, 2.1 mm x 150 mm

Mobile phase A = 0.1% acetic acid in HPLC grade water
B = 0.1% acetic acid in HPLC grade methanol

Time (minutes)	Flow rate (µL/min)	%A	%B
0.0	400	95.0	5.0
0.1	400	95.0	5.0
10.0	400	5.0	95.0
11.0	400	5.0	95.0
11.1	400	95.0	5.0
16.0	400	95.0	5.0

Column temperature 40.0 °C

Injection volume 15.0 µL

Retention time ~10.5 minutes (Inpyrfluxam)
~8.9 minutes (1'-COOH-S-2840A)
~9.0 minutes (1'-COOH-S-2840B)
~8.6 minutes (1'-CH_2OH-S-2840A)

~8.9 minutes (1'-CH_2OH-S-2840B)

MS conditions (inpyrfluxam, 1'-CH_2OH-S-2840A and B)

Instrument	Applied Biosystems MDS/SCIEX API 5500	
Ionisation Type	Electrospray ionisation (ESI)	
Polarity	Positive ion mode	
Scan Type	MRM	
Ionspray voltage	5000V	
Mass transition monitored (*m/z*)	Inpyrfluxam	1'-CH_2OH-S-2840A and B
	334 → 238*	350 → 292*
	334 → 258	350 → 312
Nebulizer temperature (TEM)	600 °C	

* Proposed for quantification

MS conditions (1'-COOH-S-2840A and B)

Instrument	Applied Biosystems MDS/SCIEX API 5500
Ionisation Type	Electrospray ionisation (ESI)
Polarity	Negative ion mode
Scan Type	MRM
Ionspray voltage	- 4500 V
Mass transition monitored (*m/z*)	362 → 318* 362 → 131
Nebulizer temperature (TEM)	600 °C

* Proposed for quantification

Table B.5.1.2.5-23: Analytical validation data for the determination of inpyrfluxam in poultry matrices

Analyte	Matrix	LOQ (mg/kg)	Recovery fortification level (mg/kg)	Recoveries % range (mean)	Repeatability % RSD (n)	Linearity
Inpyrfluxam	Egg	0.01 *(334.2 → 238.2 m/z)*	0.01	79 – 96 (87)	7.7 (5)	0.02 – 8.0 ng/ml (0.002 – 0.8 mg/kg). r= 0.9999 (n = 9) y = 953000x + 3760
			0.5	93 – 98 (95)	2.7 (5)	0.02 – 8.0 ng/ml (0.002 – 0.8 mg/kg). r = 1.0 (n = 9) y = 77600x + 2590
		0.01 *(334.2 → 258.2 m/z)*	0.01	81 – 93 (87)	5.9 (5)	Linearity preformed using solvent based calibration standards. Representative linearity given above.
			0.5	92 – 97 (94)	2.9 (5)	
	Muscle	0.01 *(334.2 → 238.2 m/z)*	0.01	85 – 89 (86)	1.9 (5)	
			0.5	96 – 108 (102)	4.5 (5)	
		0.01 *(334.2 → 258.2 m/z)*	0.01	86 – 92 (89)	2.4 (5)	
			0.5	96 – 106 (102)	4.3 (5)	
	Liver	0.01 *(334.2 → 238.2 m/z)*	0.01	76 – 80 (77)	2.1	
			0.5	70 – 83 (75)	7.4	
		0.01	0.01	75 – 81 (78)	3.6	

		(334.2 → 258.2 m/z)	0.5	69 – 82 (74)	7.3	
	Fat	0.01 *(334.2 → 238.2 m/z)*	0.01	81 – 95 (87)	6.5 (5)	
			0.5	89 – 105 (98)	6.0 (5)	
		0.01 *(334.2 → 258.2 m/z)*	0.01	76 – 99 (85)	10.1 (5)	
			0.5	93 – 105 (100)	4.4 (5)	

Table B.5.1.2.5-24: Analytical validation data for the determination of 1'-COOH-S-2840A in poultry matrices

Analyte	Matrix	LOQ (mg/kg)	Recovery fortification level (mg/kg)	Recoveries % range (mean)	Repeatability % RSD (n)	Linearity
1'-COOH-S-2840A	Egg	0.005 *(362.2 → 318.2 m/z)*	0.005	63 – 77 (68)	8.8 (5)	0.01 – 4.0 ng/ml (0.001 – 0.4 mg/kg). r= 0.9989 (n = 9) y = 476000x + 1600
			0.25	78 – 80 (79)	1.1 (5)	
		0.005 *(362.2 → 131.0 m/z)*	0.005	63 – 77 (69)	9.3 (5)	0.01 – 4.0 ng/ml (0.001 – 0.4 mg/kg). r= 0.9988 (n = 9) y = 56400x + 287
			0.25	78 – 82 (80)	2.1 (5)	
	Muscle	0.005 *(362.2 → 318.2 m/z)*	0.005	72 – 80 (76)	3.9 (5)	Linearity preformed using solvent based
			0.25	82 – 92 (88)	4.3 (5)	

		0.005 *(362.2 → 131.0 m/z)*	0.005	59 – 78 (69)	10.7 (5)	calibration standards. Representative linearity given above.
			0.25	84 – 92 (89)	3.7 (5)	
	Liver	0.005 *(362.2 → 318.2 m/z)*	0.005	87 – 96 (91)	4.0	
			0.25	80 – 94 (85)	7.0	
		0.005 *(362.2 → 131.0 m/z)*	0.005	78 – 108 (94)	12.8	
			0.25	80 - 95 (86)	7.4	
	Fat	0.005 *(362.2 → 318.2 m/z)*	0.005	62 – 84 (71)	13.0 (5)	
			0.25	70 – 86 (80)	8.0	
		0.005 *(362.2 → 131.0 m/z)*	0.005	55 – 89 (75)	17.5 (5)	
			0.25	72 – 88 (81)	7.7 (5)	

Table B.5.1.2.5-25: Analytical validation data for the determination of 1'-COOH-S-2840B poultry matrices

Analyte	Matrix	LOQ (mg/kg)	Recovery fortification level (mg/kg)	Recoveries % range (mean)	Repeatability % RSD (n)	Linearity
1'-COOH-S-2840B	Egg	0.005 *(362.2 → 318.2 m/z)*	0.005	73 – 83 (78)	4.9 (5)	0.01 – 4.0 ng/ml (0.001 – 0.4 mg/kg). r= 0.9997 (n = 9) y = 433000x + 1630
			0.25	87 – 92 (90)	2.1 (5)	
		0.005	0.005	62 – 98 (86)	16.5 (5)	0.01 – 4.0 ng/ml (0.001 – 0.4 mg/kg).
			0.25	87 – 91 (89)	1.9 (5)	

		(362.2 → 131.0 m/z)				r= 0.9999 (n = 9) y = 439000x + 937
	Muscle	0.005 *(362.2 → 318.2 m/z)*	0.005	83 – 94 (88)	4.5 (5)	Linearity preformed using solvent based calibration standards. Representative linearity given above.
			0.25	97 – 107 (104)	3.8 (5)	
		0.005 *(362.2 → 131.0 m/z)*	0.005	80 – 102 (90)	9.7 (5)	
			0.25	96 – 109 (103)	4.8 (5)	
	Liver	0.005 *(362.2 → 318.2 m/z)*	0.005	86 – 93 (90)	3.1 (5)	
			0.25	79 – 95 (86)	7.7 (5)	
		0.005 *(362.2 → 131.0 m/z)*	0.005	92 – 99 (95)	3.1 (5)	
			0.25	82 – 94 (87)	5.5 (5)	
	Fat	0.005 *(362.2 → 318.2 m/z)*	0.005	80 – 107 (95)	10.8 (5)	
			0.25	83 – 100 (96)	7.7 (5)	
		0.005 *(362.2 → 131.0 m/z)*	0.005	66 – 95 (83)	13.8 (5)	
			0.25	81 – 97 (92)	7.0 (5)	

Table B.5.1.2.5-26: Analytical validation data for the determination of, 1'-CH_2OH-S-2840A in poultry matrices

Analyte	Matrix	LOQ (mg/kg)	Recovery fortification level (mg/kg)	Recoveries % range (mean)	Repeatability % RSD (n)	Linearity
1'-CH_2OH-	Egg	0.005	0.005	70 – 83 (77)	7.6 (5)	

S-2840A		*(350.2 → 292.2 m/z)*	0.25	67 – 88 (78)	9.5 (5)	0.01 – 4.0 ng/ml (0.001 – 0.4 mg/kg). r= 0.9992 (n = 9) y = 1050000x + 973
		0.005 *(350.2 → 312.2 m/z)*	0.005	67 – 79 (73)	6.2 (5)	0.01 – 4.0 ng/ml (0.001 – 0.4 mg/kg). r= 0.9997 (n = 9) y = 432000x + 1160
			0.25	67 – 87 (77)	9.3 (5)	
	Muscle	0.005 *(350.2 → 292.2 m/z)*	0.005	73 – 77 (76)	2.3 (5)	Linearity preformed using solvent based calibration standards. Representative linearity given above.
			0.25	87 – 92 (89)	2.8 (5)	
		0.005 *(350.2 → 312.2 m/z)*	0.005	73 – 80 (75)	3.7 (5)	
			0.25	86 – 93 (90)	3.2 (5)	
	Liver	0.005 *(350.2 → 292.2 m/z)*	0.005	72 – 100 (81)	13.8 (5)	
			0.25	26 – 80* 65 – 80 (73)	8.7 (4)	
		0.005 *(350.2 → 312.2 m/z)*	0.005	68 – 105 (85)	16.3 (5)	
			0.25	28 – 78* 66 – 78 (73)	7.4 (4)	
	Fat	0.005 *(350.2 → 292.2 m/z)*	0.005	70 – 81 (74)	6.4 (5)	
			0.25	70 – 87 (80)	8.7 (5)	

		0.005 *(350.2 → 312.2 m/z)*	0.005	73 – 84 (80)	5.1 (5)	
			0.25	70 – 86 (80)	8.6 (5)	

* Outlier removed from data set by Dixon Q-test

Table B.5.1.2.5-27: Analytical validation data for the determination of 1'-CH_2OH-S-2840B in poultry matrices

Analyte	Matrix	LOQ (mg/kg)	Recovery fortification level (mg/kg)	Recoveries % range (mean)	Repeatability % RSD (n)	Linearity
1'-CH_2OH-S-2840B	Egg	0.005 *(350.2 → 292.2 m/z)*	0.005	72 – 78 (75)	3.6 (5)	0.01 – 4.0 ng/ml (0.001 – 0.4 mg/kg). r= 1.000 (n = 9) y = 961000x + 1430
			0.25	70 – 89 (79)	8.7 (5)	
		0.005 *(350.2 → 312.2 m/z)*	0.005	69 – 80 (75)	6.6 (5)	0.01 – 4.0 ng/ml (0.001 – 0.4 mg/kg). r= 0.9999 (n = 9) y = 439000x + 937
			0.25	73 – 87 (79)	6.6 (5)	
	Muscle	0.005 *(350.2 → 292.2 m/z)*	0.005	74 – 81 (77)	3.5 (5)	Linearity preformed using solvent based calibration standards. Representative linearity given above.
			0.25	88 – 92 (90)	2.3 (5)	
		0.005 *(350.2 → 312.2 m/z)*	0.005	71 – 79 (76)	4.0 (5)	
			0.25	86 – 92 (90)	2.9 (5)	
	Liver	0.005	0.005	72 – 104 (85)	16.0 (5)	

		(350.2 → 292.2 m/z)	0.25	25 – 77* 63 – 77 (72)	8.4 (4)	
		0.005 *(350.2 → 312.2 m/z)*	0.005	70 – 106 (85)	16.0 (5)	
			0.25	25 – 78* 66 – 78 (72)	7.6 (4)	
	Fat	0.005 *(350.2 → 292.2 m/z)*	0.005	73 – 83 (79)	5.1 (5)	
			0.25	71 – 89 (82)	8.5 (5)	
		0.005 *(350.2 → 312.2 m/z)*	0.005	66 – 75 (72)	5.1 (5)	
			0.25	70 – 90 (82)	9.2 (5)	

* Outlier removed from data set by Dixon Q-test

Specificity:
Specificity was demonstrated by retention time match with a reference standard. Analysis of unfortified control samples and reagent blanks demonstrated no significant interference (> 30% of the LOQ) at the retention time of interest.

Two mass transitions were investigated and are considered acceptable.

Matrix Effects:

Matrix effects were not investigated, and matrix matched standards were not used; therefore, a consideration of matrix effects is required. In this case, a consideration of accuracy data determined by spiking of blank samples in conjunction with linearity data using calibration standards is sufficient to demonstrate that significant matrix effects are not observed, therefore the use of solvent-based standards is acceptable.

Linearity:

Linearity was demonstrated by the analysis of nine standards of increasing concentration. For parent inpyrfluxam the range of standard concentrations used was 0.02 – 8.0 ng/mL, equivalent to 0.002 – 0.8 mg/kg. For the metabolites, standard concentration ranged from 0.01 – 4.0 ng/mL, equivalent to 0.001 – 0.4 mg/kg. The responses were linear with correlation coefficient (r) of ≥0.9988.

Accuracy:

Recovery samples were prepared by spiking each matrix with parent inpyrfluxam standard, and each metabolite standard separately, and analysing them by the method described. The spike concentrations for inpyrfluxam were in the range 0.01 to 0.5 mg/kg. The spike

concentration for the metabolites was 0.005 – 0.25 mg/kg. Five samples were prepared at each fortification level.

For fortification levels ≤ 0.01 mg/kg (0.005 mg/kg), mean recoveries ranged from 68% to 95%, therefore within the acceptable range of 60 – 120%. For fortification levels >0.01 - ≤0.1 mg/kg (0.01 mg/kg) the mean recoveries ranged from 77 – 89%, therefore between the range of 70 – 120% and are acceptable.

For fortification levels between >0.1 – 1.0 (0.25, 0.5 mg/kg), the mean recoveries ranged between 72 – 104% and are considered acceptable.

For metabolites 1'-CH_2OH-S-2840A and 1'-CH_2OH-S-2840B in liver at the fortification level of 0.25 mg/kg, the lower recovery of 28% and 25%, respectively, was identified as an outlier via Grubbs test and not considered further. Therefore, only four samples were used for accuracy and precision for liver at the higher fortification level.

Precision:

Precision was determined from the accuracy recovery data. Five samples were prepared at each fortification level, and the %RSD reported were acceptable. As indicated above, for liver at the higher fortification only four samples were used for accuracy and precision as one value was identified as an outlier. One outlier of the overall dataset is acceptable as outlined in SANTE/2020/12830, Rev.1.

Stock Stability

~~Stock stability has not been addressed as part of the study. Waiting on confirmation.~~
The stability of methanol stock solutions of 1'-COOH-S-2840 was determined by comparing the response of a freshly prepared dilution of a stock solution stored for 8 days at 1 to 10 °C in the dark, against a freshly prepared stock solution by five-fold injection. The difference between the response of the two samples was negligible (-0.8%). The methanol stock solutions of 1'-COOH-S-2840 have been demonstrated to be stable for 8 days when stored at 1 to 10 °C in the dark.

The stability of 1′-CH_2OH-S-2840 in stock solutions has not been addressed.

Calibration stability:

~~Calibration stability has not been addressed as part of the study. Waiting on confirmation.~~
The stability of water/methanol (3/1, v/v) standard solutions of 1'-COOH-S-2840 was determined by comparing the response standard solution stored for 8 days at 1 to 10 °C in the dark, against a freshly prepared standard solution by five-fold injection. The difference between the response of the two samples was negligible (1.0%). The water/methanol (3/1, v/v) standard solutions of 1'-COOH-S-2840 have been demonstrated to be stable for 8 days when stored at 1 to 10 °C in the dark. It is noted that matrix-matched calibration standards were used. The final extract data above shows the analyte is stable for 8 days

at 1 to 10 °C in the dark and therefore this is suitable to support the stability of analyte in calibration solutions.

The stability of 1′-CH_2OH-S-2840 in standard solutions has not been addressed.

Extract Stability

Stability of residues in sample extracts has been satisfactorily addressed in the feeding study, as procedural recovery samples were extracted and stored for the same length of time as the test sample extracts. The recoveries were within the acceptable range of 70 – 120%.

Extraction efficiency:

Egg

In the current study, egg was extracted once with hexane:acetone (1:1, v/v), followed by two more extractions using acetone. In the poultry metabolism study, radioactive residues were extracted twice with acetonitrile/water (1:1, v/v), then once by acetonitrile.

Based on the available data, the extraction efficiency of hexane: acetone (1:1, v/v) has not been demonstrated.

Muscle

For muscle the extraction procedure used in the poultry metabolism study was a three-step extraction using acetonitrile:water (1:1, v/v) twice, followed by acetonitrile. Muscle was analysed in sub-samples (breast and thigh). The total TRR extracted was 91.67% and 92.31% from breast and thigh muscle, respectively, for the pyrazolyl- label, and 91.3% and 80% was extracted from breast and thigh muscle for the phenyl- label. The same extraction procedure was used in the current study therefore, extraction efficiency has been sufficiently addressed.

Liver

For liver the extraction procedure used in the poultry metabolism study was a three-step extraction using acetonitrile:water (1:1, v/v) twice, followed by acetonitrile. In total 94.32% and 91.37% TRR was extracted for the pyrazolyl- and phenyl- label, respectively. The same extraction procedure was used in the current study therefore, extraction efficiency has been sufficiently addressed.

Fat

For fat the extraction procedure used in the poultry metabolism study was a three-step extraction using hexane:acetone (4:1, v/v) once, followed by acetone twice. Fat was analysed in sub-samples (subcutaneous and abdominal fat). The total TRR extracted was 98.44% and 99.02% from abdominal and subcutaneous fat, respectively, for the pyrazolyl-label. For the phenyl- label 96.81% and 97.53% was extracted from abdominal and

subcutaneous fat, respectively. The same extraction procedure was used in the current study therefore, extraction efficiency has been sufficiently addressed.

Conclusion

Validation data was generated prior to the introduction of SANTE/2020/12830 Rev.1. The method can be considered sufficiently validated to the requirements as outlined in SANCO/3029/99 Rev.4 for risk assessment. The method meets the requirements outlined under Section 4.2 of SANTE/2020/12830 Rev.1 “minimum validation requirements for the assessment of existing methods for risk assessment”. Therefore, the method can be considered sufficiently validated in accordance with SANTE/2020/12830 for risk assessment purposes for 1′-CH_2OH-S-2840B and inpyrfluxam.

The method is acceptable in the areas of specificity, linearity, accuracy, and precision. The LOQ of the method is 0.01 mg/kg (inpyrfluxam) and 0.005 mg/kg for 1′-CH_2OH-S-2840A, 1′-CH_2OH-S-2840B, 1′-COOH-S-2840A and 1′-COOH-S-2840B.

Stability of stock and calibration solutions has not been addressed for 1′-CH_2OH-S-2840B and inpyrfluxam as part of the current study. Extraction efficiency has not been addressed for eggs. See Vol 1 (Section 2.5.1) for further consideration. Extraction efficiency is considered sufficiently addressed for other matrices.

The method is sufficiently validated in accordance with SANTE/2020/12830 Rev.1 for the determination of 1′-COOH-S-2840A and 1′-COOH-S-2840B in animal matrices.

Reference:	KCA 4.1.2/29
Report Title:	Magnitude of S-2399 and Metabolites 1’-CH_2OH-S-2840 A&B (including conjugate) and 1’-COOH-S-2840 A&B Residues in Bovine Tissues and Milk from a 28-Day Feeding Study
Author(s) & Year:	[redacted], 2016
Document No, Authority registration No	Study No.: 213-056-10 (2814W) Report No.: TPR-0013
Guideline(s):	OPPTS 860.1480, PMRA DIR-98-02, Section 8 OECD Guidelines for the testing of chemicals: Residues in Livestock 505 (January 8, 2007) MAFF in Japan (12-Nousan-No. 8147, 3-2-1, 2000)
Deviations:	No
GLP or GEP:	Yes
Acceptability:	Yes
Study relied upon:	Yes

The following HPLC-MS/MS method was validated for ruminant matrices (milk, liver, kidney muscle and fat) for the determination of inpyrfluxam, 1′-CH_2OH-S-2840-A,1′-CH_2OH-S-2840-B, 1′-COOH-S-2840-A, and 1′-COOH-S-2840-B and used in studies to support the residue data requirements.

Samples for analysis of 1'-CH_2OH-S-2840A and B residues are subjected to acid hydrolysis. Therefore, the quantified residue levels will include both free and conjugated forms.

Principle of the method

Milk (including skim milk)

Inpyrfluxam, 1'-COOH-S-2840A and 1'-COOH-S-2840B

Weigh 10 g of milk into a 50 mL centrifuge bottle, add 25 mL of acetone and three stainless steel grinding balls.

Grind for 2 minutes at 1500 RPM then centrifuge at 2800 rpm for 10 minutes. Transfer the sample to a 100 mL cylinder and filter through a funnel plugged with glass wool. Repeat the extraction process again with 25 mL of acetone, then a third time with 25 mL of acetone:water (1:1, v/v).

Make up to 100 mL with acetone. Transfer the mixture to an amber bottle and mix well (initial extract).

Inpyrfluxam, 1'-COOH-S-2840A and B Clean-Up

Transfer 50 µL of the initial extract into 450 µL of methanol:water (1:1, v/v). Vortex to mix. Filter through a 0.45 µm nylon centrifuge filter. Dilute as necessary before analysis.

Hydrolysis of 1'-CH_2OH-S-2840A and 1'-CH_2-OH-S-2840B

Transfer 2 mL of initial extract to a 50 mL flat bottom flask then add 1 mL of 1 M HCL. Heat under reflux for 4 hours at 100°C in oil bath. Cool to room temperature, then add 5 mL of water. Sonicate to dissolve.

Prepare an SPE cartridge with 5 mL of methanol followed by 10 mL of water. Load the sample onto the pre-conditioned cartridge. Rinse the flask with 2 x 5 mL of methanol:water (1:1, v/v) and transfer to the cartridge. Add 10 mL of methanol to the SPE cartridge and collect the eluent in a 15 mL tube. Transfer the methanol elute to a graduated cylinder and make up the volume to 20 mL with water, transfer to an amber bottle and mix well. Transfer aliquots to autosampler vials for analysis.

Cream

Inpyrfluxam, 1'-COOH-S-2840A and 1'-COOH-S-2840B

Weigh 5 g of cream into a 50 mL centrifuge bottle, add 25 mL of acetone and three stainless steel grinding balls.

Grind for 2 minutes at 1500 RPM then centrifuge at 2800 rpm for 10 minutes. Transfer the sample to a 100 mL cylinder and filter through a funnel plugged with glass wool. Repeat the extraction process again with 25 mL of acetone, then a third time with 25 mL of acetone:water (1:1, v/v).

Make up to 100 mL with acetone. Transfer the mixture to an amber bottle and mix well (initial extract).

Inpyrfluxam, 1'-COOH-S-2840A and B Clean-Up

Transfer 100 µL of the initial extract into 400 µL of methanol:water (1:1, v/v). Vortex to mix. Filter through a 0.45 µm nylon centrifuge filter. Dilute as necessary before analysis.

Hydrolysis of 1'-CH_2OH-S-2840A and 1'-CH_2-OH-S-2840B

Transfer 2 mL of initial extract to a 50 mL flat bottom flask then add 1 mL of 1 M HCL. Heat under reflux for 4 hours at 100°C in oil bath. Cool to room temperature, then add 5 mL of water. Sonicate to dissolve.

Prepare an SPE cartridge with 5 mL of methanol followed by 10 mL of water. Load the sample onto the pre-conditioned cartridge. Rinse the flask with 2 x 5 mL of methanol:water (1:1, v/v) and transfer to the cartridge. Add 5 mL of methanol to the SPE cartridge and collect the eluent in a 15 mL tube. Transfer the methanol elute to a graduated cylinder and make up the volume to 10 mL with water, transfer to an amber bottle and mix well. Transfer aliquots to autosampler vials for analysis.

Liver, Kidney and Muscle

Inpyrfluxam, 1'-COOH-S-2840A and 1'-COOH-S-2840B

Weigh 10 g of tissue into a 50 mL centrifuge bottle, add 25 mL of acetonitrile:water (1:1, v/v) and three stainless steel grinding balls.

Grind for 2 minutes at 1500 RPM then centrifuge at 2800 rpm for 10 minutes. Transfer the sample to a 100 mL cylinder and filter through a funnel plugged with glass wool. Repeat the extraction process again with 25 mL of acetonitrile:water (1:1, v/v), then a third time with 25 mL of acetonitrile.

Make up to 100 mL with acetonitrile:water (1:1, v/v). Transfer the mixture to an amber bottle and mix well (initial extract).

Inpyrfluxam, 1'-COOH-S-2840A and B Clean-Up

Transfer 50 µL of the initial extract into 450 µL of methanol:water (1:1, v/v). Vortex to mix. Filter through a 0.45 µm nylon centrifuge filter. Dilute as necessary before analysis.

Hydrolysis of 1'-CH_2OH-S-2840A and 1'-CH_2-OH-S-2840B

Transfer 2 mL of initial extract to a 50 mL flat bottom flask then add 1 mL of 1 M HCL. Heat under reflux for 4 hours at 100°C in oil bath. Cool to room temperature, then add 5 mL of water. Sonicate to dissolve.

Prepare an SPE cartridge with 5 mL of methanol followed by 10 mL of water. Load the sample onto the pre-conditioned cartridge. Rinse the flask with 2 x 5 mL of methanol:water (1:1, v/v) and transfer to the cartridge. Add 10 mL of methanol to the SPE cartridge and collect the eluent in a 15 mL tube. Transfer the methanol elute to a graduated cylinder and make up the volume to 20 mL with water, transfer to an amber bottle and mix well. Transfer aliquots to autosampler vials for analysis.

Fat

Inpyrfluxam, 1'-COOH-S-2840A and 1'-COOH-S-2840B

Weigh 10 g of fat into a 50 mL centrifuge bottle, add 25 mL of hexane:acetone (4:1, v/v) and three stainless steel grinding balls.

Grind for 2 minutes at 1500 RPM then centrifuge at 2800 rpm for 10 minutes. Transfer the sample to a 100 mL cylinder and filter through a funnel plugged with glass wool. Repeat the extraction process twice more with 25 mL of acetone.

Make up to 100 mL with acetone. Transfer the mixture to an amber bottle and mix well (initial extract).

Inpyrfluxam, 1'-COOH-S-2840A and B Clean-Up

Transfer 2 mL of the initial extract to a 125 mL bottle containing 50 mL hexane then add 20 mL acetonitrile. Shake for 5 minutes then transfer to a separatory funnel and allow the phases to separate. Collect the acetonitrile layer in a 125 mL flat bottom flask. Return the hexane layer to the 125 mL bottle and repeat the previous step collecting a total of 40 mL of acetonitrile in the same 125 mL flat bottom flask. Discard the hexane layer.

Evaporate the acetonitrile solution to dryness at 35°C then reconstitute with 20 mL methanol:water (1:1, v/v). Finally, filter the solution through a 0.45 µm nylon filter. The sample is diluted as necessary prior to analysis.

Hydrolysis of 1'-CH_2OH-S-2840A and 1'-CH_2-OH-S-2840B

Transfer 2 mL of initial extract to a 50 mL flat bottom flask. Evaporate at 35 °C to near dryness, then reconstitute with 2 mL acetonitrile:water (1:1, v/v). Sonicate to dissolve. Add

1.0 mL of 1M HCL and heat in an oil bath, under flux, for 4 hours at 100 °C. Cool to room temperature, then add 5 mL of water. Sonicate to dissolve.

Prepare an SPE cartridge with 5 mL of methanol followed by 10 mL of water. Load the sample onto the pre-conditioned cartridge. Rinse the flask with 2 x 5 mL of methanol:water (1:1, v/v) and transfer to the cartridge. Add 10 mL of methanol to the SPE cartridge and collect the eluent in a 15 mL tube. Transfer the methanol elute to a graduated cylinder and make up the volume to 20 mL with water, transfer to an amber bottle and mix well. Transfer aliquots to autosampler vials for analysis.

HPLC conditions

Column ACE 3 C18-PFP, 3.0 µm, 2.1 mm x 100 mm

Mobile phase A = 0.1% acetic acid in HPLC grade water
B = 0.1% acetic acid in HPLC grade methanol

Time (minutes)	Flow rate (µL/min)	%A	%B
0.0	400	95.0	5.0
0.1	400	95.0	5.0
10.0	400	5.0	95.0
11.0	400	5.0	95.0
11.1	400	95.0	5.0
16.0	400	95.0	5.0

Column temperature 40.0 °C

Injection volume 15.0 µL

Retention time ~10.8 minutes (Inpyrfluxam)
~8.9 minutes (1'-COOH-S-2840A)
~9.0 minutes (1'-COOH-S-2840B)
~8.6 minutes (1'-CH_2OH-S-2840A)
~8.9 minutes (1'-CH_2OH-S-2840B)

MS conditions (inpyrfluxam, 1'-CH_2OH-S-2840A and B)

Instrument Applied Biosystems MDS/SCIEX API 5500

Ionisation Type Electrospray ionisation (ESI)

Polarity Positive ion mode

Scan Type MRM

Ionspray voltage 5000V

Mass transition monitored (*m/z*)

Inpyrfluxam	1'-CH_2OH-S-2840A and B
334 → 238*	350 → 292*
334 → 258	350 → 312

Nebulizer temperature (TEM) 600 °C

* Proposed for quantification

MS conditions (1'-COOH-S-2840A and B)

Instrument	Applied Biosystems MDS/SCIEX API 5500
Ionisation Type	Electrospray ionisation (ESI)
Polarity	Negative ion mode
Scan Type	MRM
Ionspray voltage	- 4500 V
Mass transition monitored (*m/z*)	362 → 318* 362 → 131
Nebulizer temperature (TEM)	600 °C

* Proposed for quantification

Table B.5.1.2.5-28: Analytical validation data for the determination of inpyrfluxam in ruminant matrices

Analyte	Matrix	LOQ (mg/kg)	Recovery fortification level (mg/kg)	Recoveries % range (mean)	Repeatability % RSD (n)	Linearity
Inpyrfluxam	Milk	0.01 *(334.2 → 238.2 m/z)*	0.01	76 – 80 (77)	2.2 (5)	0.02 – 8.0 ng/ml (0.002 – 0.8 mg/kg). r = 0.9999 (n = 9) y = 766000x + 2490
			0.5	77 – 85 (83)	3.9 (5)	
		0.01 *(334.2 → 258.2 m/z)*	0.01	74 – 80 (78)	3.2 (5)	0.02 – 8.0 ng/ml (0.002 – 0.8 mg/kg). r = 0.9998 (n = 9) y = 628000x + 1540
			0.5	77 – 85 (82)	4.0 (5)	
	Muscle	0.01 *(334.2 → 238.2 m/z)*	0.01	71 – 111 (85)	18.1 (5)	Linearity preformed using solvent based calibration standards. Representative linearity given above.
			0.5	101 – 105 (102)	1.5 (5)	
		0.01 *(334.2 → 258.2 m/z)*	0.01	63 – 115 (84)	22.5 (5)	
			0.5	99 – 103 (100)	1.7 (5)	
	Liver	0.01 *(334.2 → 238.2 m/z)*	0.01	78 – 85 (80)	3.7 (5)	
			0.5	84 – 86 (85)	1.0 (5)	
		0.01 *(334.2 → 258.2 m/z)*	0.01	76 – 88 (80)	6.0 (5)	
			0.5	83 – 88 (85)	2.1 (5)	
	Kidney	0.01	0.01	81 – 85 (82)	2.1 (5)	

		(334.2 → 238.2 m/z)	0.5	83 – 94 (88)	4.5 (5)	
		0.01 *(334.2 → 258.2 m/z)*	0.01	81 – 89 (83)	4.0 (5)	
			0.5	81 – 90 (86)	3.8 (5)	
	Fat	0.01 *(334.2 → 238.2 m/z)*	0.01	100 – 114 (105)	5.3 (5)	
			0.5	100 – 108 (104)	2.9 (5)	
		0.01 *(334.2 → 258.2 m/z)*	0.01	93 – 108 (98)	6.4 (5)	
			0.5	100 – 112 (104)	4.6 (5)	

Table B.5.1.2.5-29: Analytical validation data for the determination of 1'-COOH-S-2840A in ruminant matrices

Analyte	Matrix	LOQ (mg/kg)	Recovery fortification level (mg/kg)	Recoveries % range (mean)	Repeatability % RSD (n)	Linearity
1'-COOH-S-2840A	Milk	0.005 *(362.2 → 318.2 m/z)*	0.005	75 – 80 (77)	2.5 (5)	0.01 – 4.0 ng/ml (0.001 – 0.4 mg/kg) r = 0.9997 (n = 9) y = 693000x + 2790
			0.25	84 – 92 (89)	3.7 (5)	
		0.005 *(362.2 → 131.0 m/z)*	0.005	67 – 78 (71)	6.4 (5)	0.01 – 4.0 ng/ml (0.001 – 0.4 mg/kg) r = 0.9996 (n = 9) y = 81800x + 551
			0.25	86 – 91 (89)	2.6 (5)	
	Muscle	0.005	0.005	77 – 85 (80)	4.4 (5)	Linearity preformed using

		(362.2 → 318.2 m/z)	0.25	105 – 110 (108)	1.7 (5)	solvent based calibration standards. Representative linearity given above.
		0.005 *(362.2 → 131.0 m/z)*	0.005	64 – 83 (74)	11.7 (5)	
			0.25	103 – 109 (106)	2.2 (5)	
	Liver	0.005 *(362.2 → 318.2 m/z)*	0.005	87 – 93 (90)	3.1 (5)	
			0.25	100 – 102 (101)	0.7 (5)	
		0.005 *(362.2 → 131.0 m/z)*	0.005	76 – 101 (90)	12.5 (5)	
			0.25	100 – 104 (102)	1.6 (5)	
	Kidney	0.005 *(362.2 → 318.2 m/z)*	0.005	81 – 86 (84)	2.5 (5)	
			0.25	92 – 103 (97)	4.2 (5)	
		0.005 *(362.2 → 131.0 m/z)*	0.005	72 – 94 (84)	10.1 (5)	
			0.25	94 – 100 (97)	2.2 (5)	
	Fat	0.005 *(362.2 → 318.2 m/z)*	0.005	99 – 110 (103)	4.0 (5)	
			0.25	100 – 110 (103)	3.8 (5)	
		0.005 *(362.2 → 131.0 m/z)*	0.005	73 – 113 (91)	17.7 (5)	
			0.25	98 – 107 (101)	3.5 (5)	

Table B.5.1.2.5-30: Analytical validation data for the determination of 1'-COOH-S-2840B in ruminant matrices

Analyte	Matrix	LOQ (mg/kg)	Recovery fortification level (mg/kg)	Recoveries % range (mean)	Repeatability % RSD (n)	Linearity

1'-COOH-S-2840B	Milk	0.005 *(362.2 → 318.2 m/z)*	0.005	76 – 85 (81)	4.2 (5)	0.01 – 4.0 ng/ml (0.001 – 0.4 mg/kg) r = 1.0 (n = 9) y = 675000x + 1490
			0.25	82 – 92 (89)	4.8 (5)	
		0.005 *(362.2 → 131.0 m/z)*	0.005	80 – 102 (87)	10.1 (5)	0.01 – 4.0 ng/ml (0.001 – 0.4 mg/kg) r = 0.9998 (n = 9) y = 81800x + -56.5
			0.25	83 – 92 (88)	4.1 (5)	
	Muscle	0.005 *(362.2 → 318.2 m/z)*	0.005	82 – 88 (85)	3.3 (5)	Linearity preformed using solvent based calibration standards. Representative linearity given above.
			0.25	107 – 110 (108)	1.1 (5)	
		0.005 *(362.2 → 131.0 m/z)*	0.005	79 – 104 (91)	12.3 (5)	
			0.25	108 – 112 (109)	1.5 (5)	
	Liver	0.005 *(362.2 → 318.2 m/z)*	0.005	85 – 105 (93)	8.7 (5)	
			0.25	99 – 102 (101)	1.1 (5)	
		0.005 *(362.2 → 131.0 m/z)*	0.005	87 – 91 (89)	2.0 (5)	
			0.25	101 – 103 (102)	0.7 (5)	
	Kidney	0.005 *(362.2 → 318.2 m/z)*	0.005	80 – 92 (86)	6.2 (5)	
			0.25	93 – 100 (97)	2.7 (5)	
		0.005	0.005	82 – 96 (90)	5.7 (5)	

		(362.2 → 131.0 m/z)	0.25	93 – 101 (96)	3.1 (5)	
	Fat	0.005 *(362.2 → 318.2 m/z)*	0.005	93 – 105 (100)	5.5 (5)	
			0.25	99 – 105 (101)	2.5 (5)	
		0.005 *(362.2 → 131.0 m/z)*	0.005	80 – 115 (91)	15.2 (5)	
			0.25	99 – 110 (103)	4.2 (5)	

Table B.5.1.2.5-31: Analytical validation data for the determination of, 1'-CH_2OH-S-2840A in ruminant matrices

Analyte	Matrix	LOQ (mg/kg)	Recovery fortification level (mg/kg)	Recoveries % range (mean)	Repeatability % RSD (n)	Linearity
1'-CH_2OH-S-2840A	Milk	0.005 *(350.2 → 292.2 m/z)*	0.005	73 – 86 (80)	6.4 (5)	0.01 – 4.0 ng/ml (0.001 – 0.4 mg/kg) r = 0.9999 (n = 9) y = 664000x + 230
			0.25	85 – 92 (90)	3.4 (5)	
		0.005 *(350.2 → 312.2 m/z)*	0.005	70 – 83 (78)	6.1 (5)	0.01 – 4.0 ng/ml (0.001 – 0.4 mg/kg) r = 0.9999 (n = 9) y = 274000x + 336
			0.25	85 – 92 (90)	3.2 (5)	
	Muscle	0.005 *(350.2 → 292.2 m/z)*	0.005	85 – 97 (91)	5.4 (5)	Linearity preformed using solvent based
			0.25	98 – 102 (100)	1.6 (5)	

		0.005 *(350.2 → 312.2 m/z)*	0.005	85 – 100 (93)	6.7 (5)	calibration standards. Representative linearity given above.
			0.25	97 – 103 (100)	2.3 (5)	
	Liver	0.005 *(350.2 → 292.2 m/z)*	0.005	79 – 88 (84)	4.2 (5)	
			0.25	90 – 96 (93)	2.8 (5)	
		0.005 *(350.2 → 312.2 m/z)*	0.005	78 – 89 (84)	5.8 (5)	
			0.25	91 – 97 (94)	2.6 (5)	
	Kidney	0.005 *(350.2 → 292.2 m/z)*	0.005	79 – 84 (82)	2.5 (5)	
			0.25	88 – 98 (93)	4.3 (5)	
		0.005 *(350.2 → 312.2 m/z)*	0.005	79 – 93 (86)	6.7 (5)	
			0.25	88 – 98 (93)	4.3 (5)	
	Fat	0.005 *(350.2 → 292.2 m/z)*	0.005	71 – 81 (77)	5.3 (5)	
			0.25	82 – 103 (92)	8.3 (5)	
		0.005 *(350.2 → 312.2 m/z)*	0.005	66 – 79 (75)	6.9 (5)	
			0.25	82 – 103 (91)	8.3 (5)	

Table B.5.1.2.5-32: Analytical validation data for the determination of 1'-CH_2OH-S-2840B in ruminant matrices

Analyte	Matrix	LOQ (mg/kg)	Recovery fortification level (mg/kg)	Recoveries % range (mean)	Repeatability % RSD (n)	Linearity
1'-CH_2OH-S-2840B	Milk	0.005	0.005	71 – 85 (77)	8.5 (5)	0.01 – 4.0 ng/ml (0.001 – 0.4 mg/kg)
			0.25	83 – 91 (88)	3.5 (5)	

		(350.2 → 292.2 m/z)				r = 0.9999 (n = 9) y = 578000x + 1130
		0.005 *(350.2 → 312.2 m/z)*	0.005	74 – 84 (79)	5.2 (5)	0.01 – 4.0 ng/ml (0.001 – 0.4 mg/kg) r = 0.9998 (n = 9) y = 261000x + 113
			0.25	83 – 91 (89)	3.8 (5)	
	Muscle	0.005 *(350.2 → 292.2 m/z)*	0.005	82 – 96 (88)	6.2 (5)	Linearity preformed using solvent based calibration standards. Representative linearity given above.
			0.25	96 – 100 (97)	1.7 (5)	
		0.005 *(350.2 → 312.2 m/z)*	0.005	80 – 97 (90)	7.6 (5)	
			0.25	96 – 101 (98)	2.0 (5)	
	Liver	0.005 *(350.2 → 292.2 m/z)*	0.005	78 – 83 (80)	2.4 (5)	
			0.25	88 – 94 (91)	2.7 (5)	
		0.005 *(350.2 → 312.2 m/z)*	0.005	76 – 91 (84)	6.9 (5)	
			0.25	89 – 94 (91)	2.4 (5)	
	Kidney	0.005 *(350.2 → 292.2 m/z)*	0.005	75 – 85 (81)	4.6 (5)	
			0.25	84 – 96 (90)	5.0 (5)	
		0.005 *(350.2 → 312.2 m/z)*	0.005	73 – 82 (79)	4.3 (5)	
			0.25	84 – 96 (90)	5.0 (5)	

	Fat	0.005 *(350.2 → 292.2 m/z)*	0.005	70 – 80 (75)	4.8 (5)	
			0.25	82 – 104 (92)	8.7 (5)	
		0.005 *(350.2 → 312.2 m/z)*	0.005	62 – 79 (71)	8.9 (5)	
			0.25	83 – 105 (92)	8.7 (5)	

Specificity:

Specificity was demonstrated by retention time match with a reference standard. Analysis of unfortified control samples and reagent blanks demonstrated no significant interference (> 30% of the LOQ) at the retention time of interest.

Two mass transitions were investigated and are considered acceptable.

Matrix Effects:

The matrix effect was determined from the inpyrfluxam and 1'-COOH-S-2840A and B extracts by determining recovery from spiked control final extracts using the solvent-based calibration curve.

For inpyrfluxam in kidney, liver, muscle and milk, the spiked control extract recoveries were 91%, 90%, 83% and 89%, respectively. These recoveries indicate a matrix effect of no more than 17% for inpyrfluxam in these four matrices.

For the metabolite 1'-COOH-S-2840A, the recoveries were 87%, 91%, 83% and 87% in kidney, liver, muscle and milk respectively. These recoveries indicate a matrix effect of 17% or less for these four matrices.

For the metabolite 1'-COOH-S-2840B, the recoveries were 96%, 100%, 89% and 97% in kidney, liver, muscle and milk respectively. These results indicate a matrix effect of 11% or less for these four matrices.

Matrix effects for the 1'-CH_2OH-S-2840A and B extracts were not determined as these extracts undergo additional cleanup steps and therefore the inpyrfluxam and 1'-COOH-S-2840A and B extracts are considered most likely to exhibit higher matrix effects.

Similarly, the fat matrix effect was not determined as these undergo additional partitioning cleanup steps and greater matrix effect is expected compared to other tissues.

The available chromatograms for reagent blanks and control blanks indicate that some matrix may be expect, but not significant enough to impact the results of the study.

Linearity:

Linearity was demonstrated by the analysis of nine standards of increasing concentration. For parent inpyrfluxam the range of standard concentrations used was 0.02 – 8.0 ng/mL, equivalent to 0.002 – 0.8 mg/kg. For the metabolites, standard concentrations ranged from

0.01 – 4.0 ng/mL, equivalent to 0.001 – 0.4 mg/kg. The responses were linear with correlation coefficient (r) of ≥0.9998.

The method encouraged dilution of the final extracts if necessary to get a response within the linear range.

Accuracy:

Recovery samples were prepared by spiking each matrix with parent inpyrfluxam standard, and each metabolite standard separately, and analysing them by the method described. The spike concentrations for inpyrfluxam were in the range 0.01 to 0.5 mg/kg. The spike concentration for the metabolites was 0.005 – 0.25 mg/kg. Five samples were prepared at each fortification level.

For fortification levels ≤ 0.01 mg/kg (0.005 mg/kg), mean recoveries ranged from 71% to 103%, therefore within the acceptable range of 60 – 120%. For fortification levels >0.01 - ≤0.1 mg/kg (0.01 mg/kg) the mean recoveries ranged from 77 – 105%, therefore between the range of 70 – 120% and are acceptable.

For the fortification levels that were >0.1 mg/kg (0.25 – 0.5 mg/kg) the mean recoveries ranged from 82 – 109%, therefore were within the acceptable range of 70 – 110%.

Precision:

Precision was determined from the accuracy recovery data. Five samples were prepared at each fortification level. The calculated %RSD was within the acceptable limits of 30% (≤0.01 mg/kg), 20% (>0.01 - ≤0.1 mg/kg) and 15% (>0.1 - ≤1.0 mg/kg).

Stock Stability

~~Stock stability has not been addressed as part of the study. Waiting on confirmation.~~
The stability of methanol stock solutions of 1'-COOH-S-2840 was determined by comparing the response of a freshly prepared dilution of a stock solution stored for 8 days at 1 to 10 °C in the dark, against a freshly prepared stock solution by five-fold injection. The difference between the response of the two samples was negligible (-0.8%). The methanol stock solutions of 1'-COOH-S-2840 have been demonstrated to be stable for 8 days when stored at 1 to 10 °C in the dark.

The stability of inpyrfluxam in methanol stock solutions has been addressed in KCA 4.1.2/20 .

The stability of 1′-CH_2OH-S-2840 in stock solutions has not been addressed.

Calibration stability:

~~Calibration stability has not been addressed as part of the study. Waiting on confirmation.~~

The stability of water/methanol (3/1, v/v) standard solutions of 1'-COOH-S-2840 was determined by comparing the response standard solution stored for 8 days at 1 to 10 °C in the dark, against a freshly prepared standard solution by five-fold injection. The difference between the response of the two samples was negligible (1.0%). The water/methanol (3/1, v/v) standard solutions of 1'-COOH-S-2840 have been demonstrated to be stable for 8 days when stored at 1 to 10 °C in the dark. It is noted that matrix-matched calibration standards were used. The final extract data above shows the analyte is stable for 8 days at 1 to 10 °C in the dark and therefore this is suitable to support the stability of analyte in calibration solutions.

The stability of inpyrfluxam in water/methanol (3/1, v/v) standard solutions has been addressed in KCA 4.1.2/20 .

The stability of 1′-CH_2OH-S-2840 in standard solutions has not been addressed.

Extract Stability

Stability of residues in sample extracts has been satisfactorily addressed in the feeding study as procedural recovery samples were extracted and stored for the same length of time as the test sample extracts. The recoveries were within the acceptable range of 70 – 120%.

Extraction efficiency:

Milk

For milk extraction in the ruminant metabolism study, whole milk was centrifuged prior to extraction to separate the skim milk from the milk fat. The skim milk was extracted once with acetone and then once with acetone/water (1:1, v/v). The extracts were combined before analysis. In total 100% TRR was extracted for both the pyrazolyl- and phenyl- label in skim milk. The extraction process of skimmed milk showed good extractability.

In the current study, both cream and whole/skimmed milk were extracted twice with acetone then a final time with acetone:water (1:1, v/v). This is similar to the extract method used in the ruminant metabolism study for skimmed milk. Therefore, extraction efficiency has been sufficiently addressed.

Muscle

For muscle the extraction procedure used in the ruminant metabolism study was a three-step extraction using acetonitrile:water (1:1, v/v) twice, followed by acetonitrile. Muscle was analysed in sub-samples (flank and loin). The total TRR extracted was 100% and 91.67% from flank and loin muscle, respectively, for the pyrazolyl- label, and 95.24% and 93.33% was extracted from flank and loin muscle for the phenyl- label. The same extraction procedure was used in the current study therefore, extraction efficiency has been sufficiently addressed.

Liver

For liver the extraction procedure used in the ruminant metabolism study was a three-step extraction using acetonitrile:water (1:1, v/v) twice, followed by acetonitrile. In total 91.05% and 90.41% TRR was extracted for the pyrazolyl- and phenyl- label, respectively. The same extraction procedure was used in the current study therefore, extraction efficiency has been sufficiently addressed.

Kidney

For kidney the extraction procedure used in the ruminant metabolism study was a three-step extraction using acetonitrile:water (1:1, v/v) twice, followed by acetonitrile. In total 98.15% and 97.65% TRR was extracted for the pyrazolyl- and phenyl- label, respectively. The same extraction procedure was used in the current study therefore, extraction efficiency has been sufficiently addressed.

Fat

For fat the extraction procedure used in the ruminant metabolism study was a three-step extraction using hexane:acetone (4:1, v/v) once, followed by acetone twice. Fat was analysed in sub-samples (omental, subcutaneous and renal fat). The total TRR extracted was 83.33%, 83.33% and 71.43% from omental, subcutaneous and renal fat, respectively, for the pyrazolyl-label. For the phenyl-label 87.50%, 96.55% and 90.24% was extracted from omental, subcutaneous and renal fat, respectively. The same extraction procedure was used in the current study therefore, extraction efficiency has been sufficiently addressed.

Conclusion

Validation data was generated prior to the introduction of SANTE/2020/12830 Rev.1. The method can be considered sufficiently validated to the requirements as outlined in SANCO/3029/99 rev.4 for risk assessment. The method meets the requirements outlined under Section 4.2 of SANTE/2020/12830 Rev.1 “minimum validation requirements for the assessment of existing methods for risk assessment”. Therefore, the method can be considered sufficiently validated in accordance with SANTE/2020/12830 for risk assessment purposes.

The method is acceptable in the areas of specificity, linearity, accuracy, and precision. The LOQ of the method is 0.01 mg/kg (inpyrfluxam) and 0.005 mg/kg for 1′-CH_2OH-S-2840A, 1′-CH_2OH-S-2840B, 1′-COOH-S-2840A and 1′-COOH-S-2840B. Extraction efficiency is considered sufficiently addressed. Stability of stock and calibration solutions has not been addressed as part of the current study.

Reference:	KCA 4.1.2/30
Report Title:	Magnitude of the Residue of S-2399 and its Metabolites in Wheat Processed Fractions in Northern and Southern Europe - 2016
Author(s) & Year:	[redacted] 2018d
Document No, Authority registration No	Study No.: 261-2016 (S16-05040-L1); Report No.: TPR-0081
Guideline(s):	SANCO/3029/99, rev. 4
Deviations:	No
GLP or GEP:	Yes
Acceptability:	Yes
Study relied upon:	Yes

The method is the same as that summarised in report TPA-0057 (KCA 4.1.2/20). Additional validation data has been generated for the determination of inpyrfluxam and its metabolites 3'-OH-S-2840, DFPA-$CONH_2$, N-des-Me-DFPA, DFPA; 1'-COOH-S-2840A and B, 1'-CH_2OH-S-2840A and B in wheat (flour and whole grain bread). Due to their similarity, the validation results generated for wheat (whole plant and grain), or wheat (flour), are considered representative for wheat (bran, shorts, germs, milled by-products, starch, gluten and gluten feed meal).

Samples for analysis of DFPA, 1'-COOH-S-2840A and B and 1'-CH_2OH-S-2840A and B residues are subjected to acid hydrolysis. Therefore, the quantified residue levels will include both free and conjugated forms.

Principle of the method

Samples (20 g for whole grain bread and 5 g for flour) of wheat (whole grain bread or flour) are weighed into 250 mL bottles and 60 mL of acetonitrile/water (1:1 v/v) is added. The samples are homogenised using a high-speed homogeniser (8000 rpm) for 2 minutes then mechanically shaken for 30 minutes. The extracts are filtered through filter paper and Celite (40 g suspended in methanol (80 mL)) in a Buchner funnel. The extraction/filtration process is repeated twice more and the extracts combined and made up to 200 mL with acetonitrile/water (1:1, v/v).

Different procedures for the clean-up of sample extracts were necessary and different HPLC conditions were used. These are described below.

Inpyrfluxam, 3'-OH-S-2840 and DFPA-$CONH_2$

An aliquot (1 mL for whole grain bread and 2 mL for flour) of the combined extract is transferred into a test tube and evaporated to dryness under nitrogen at 40ºC and reconstituted in 10 mL or 5 mL of water/methanol (3:1 v/v) for wheat (whole grain bread) and wheat (flour) respectively. The resulting sample concentration is 0.01 g sample/mL (whole grain bread and flour).

N-des-Me-DFPA

An aliquot (2 mL for whole grain bread and 4 mL for flour) of the combined extract is hydrolysed with 5 mL or 10 mL of 6M hydrochloric acid for 6 hours at 100°C for wheat (whole grain bread) and wheat (flour) respectively. The sample is cooled to room temperature and diluted with 15 mL of water. The extract is transferred to a Chem Elut cartridge, eluted with ethyl acetate (4 x 25 mL) into a round bottomed flask and evaporated to dryness under vacuum at 40°C. The sample is reconstituted in 5 mL or 2.5 mL of water/methanol (3:1, v/v) with sonication for wheat (whole grain bread) and wheat (flour) respectively. The samples are then made up to 10 mL and 5 mL with water/methanol (3:1, v/v) for wheat (whole grain bread) and wheat (flour) respectively. The resulting sample concentration is 0.02 g sample/mL (whole grain bread and flour).

DFPA, 1'-COOH-S-2840A and B and 1'-CH_2OH-S-2840A and B

An aliquot (4 mL) of combined flour extract is evaporated to 2 mL under nitrogen at 40ºC and 2 mL of water is added. A subsequent hydrolysis step is undertaken on the wheat (flour) and wheat (whole grain bread) samples.

An aliquot (2 mL for whole grain bread and 4 mL for flour) of the combined extract is hydrolysed with 2 mL or 4 mL of 4M hydrochloric acid for 4 hours at 100°C for wheat (whole grain bread) and wheat (flour) respectively. The samples are then cooled to room temperature and cleaned up using an Oasis HLB cartridge (conditioned with 5 mL methanol and 10 mL water). The analytes are eluted with 10 mL of methanol and evaporated to 5 mL or 2.5 mL under nitrogen at 40°C for wheat (whole grain bread) and wheat (flour) respectively. The sample is diluted with water to 20 mL or 10 mL prior to analysis for wheat (whole grain bread) and wheat (flour) respectively. The resulting sample concentration is 0.01 g sample/mL (whole grain bread and flour).

If necessary, final extracts are diluted to be within the calibration range of the matrix-matched standard solutions.

HPLC conditions (inpyrfluxam, 3'-OH-S-2840, DFPA-$CONH_2$)

Column	Accucore Phenyl-Hexyl, 2.6 µm, 4.6 mm x 50 mm
Mobile phase	A = Methanol containing 0.1% (v/v) formic acid
	B = Water containing 0.1% (v/v) formic acid

Time (minutes)	Flow rate (µL/min)	%A	%B
0.0	1000	20.0	80.0
5.5	1000	90.0	10.0
6.0	1000	90.0	10.0
6.1	1000	20.0	80.0
7.5	1000	20.0	80.0

Column temperature 40.0 °C
Injection volume 10.0 µL
Retention time ~4.6 minutes (Inpyrfluxam)
~4.3 minutes (3'-OH-S-2840)
~1.0 minutes (DFPA-$CONH_2$)

MS conditions

Instrument SCIEX TripleQuad 6500 System, SCIEX (Triple quadrupole mass spectrometer)
Ionisation Type Electrospray ionisation (ESI, Turbulon Spray)
Polarity Positive/negative ion switching mode
Scan Type MRM
Capillary voltage 5000 V/ - 4500 V

Mass transition monitored (*m/z*)	Inpyrfluxam	3'-OH-S-2840	DFPA-$CONH_2$
	334 → 294* (pos)	348 → 175 (neg)	176 → 136* (pos)
	334 → 238 (pos)	348 → 131* (neg)	176 → 156 (pos)

Ion spray turbo heater (TEM) 550 °C

* Proposed for quantification

HPLC conditions (N-des-Me-DFPA)

Column Accucore Phenyl-Hexyl, 2.6 µm, 4.6 mm x 50 mm
Mobile phase A = Acetonitrile containing 0.1% (v/v) acetic acid
B = Water containing 0.1% (v/v) acetic acid

Time (minutes)	Flow rate (µL/min)	%A	%B
0.0	1000	10.0	90.0
2.0	1000	90.0	10.0
3.0	1000	90.0	10.0
3.1	1000	10.0	90.0
4.5	1000	10.0	90.0

Column temperature 40.0 °C

Injection volume 30.0 µL
Retention time ~1.0 minutes

MS conditions

Instrument SCIEX TripleQuad 6500 System, SCIEX (Triple quadrupole mass spectrometer)
Ionisation Type Electrospray ionisation (ESI, Turbulon Spray)
Polarity Positive/negative ion switching mode
Scan Type MRM
Capillary voltage 5500 V/- 4500 V
Mass transition monitored (*m/z*) 161 → 141* (neg)
161 → 66 (neg)
163 → 68 (pos)
Ion spray turbo heater (TEM) 550 °C

* Proposed for quantification

HPLC conditions (DFPA, 1'-COOH-S-2840A and B, 1'-CH_2OH-S-2840A and B)

Column Accucore Phenyl-Hexyl, 2.6 µm, 4.6 mm x 50 mm
Mobile phase A = Methanol containing 0.05% (v/v) acetic acid
B = Water containing 0.05% (v/v) acetic acid

Time (minutes)	Flow rate (µL/min)	%A	%B
0.0	1000	20.0	80.0
4.5	1000	60.0	40.0
4.6	1000	90.0	10.0
5.0	1000	90.0	10.0
5.1	1000	20.0	80.0
6.5	1000	20.0	80.0

Column temperature 60.0 °C
Injection volume 30.0 µL
Retention time ~1.0 minutes (DFPA)
~3.8 minutes (1'-COOH-S-2840A)
~4.0 minutes (1'-COOH-S-2840B)
~3.7 minutes (1'-CH_2OH-S-2840A)
~3.9 minutes (1'-CH_2OH-S-2840B)

MS conditions

Instrument SCIEX TripleQuad 6500 System, SCIEX (Triple quadrupole mass spectrometer)
Ionisation Type Electrospray ionisation (ESI, Turbulon Spray)

Polarity Positive/negative ion switching mode
Scan Type MRM
Capillary voltage 5500 V/ - 4500 V

Mass transition monitored (*m/z*)	DFPA	1'-COOH-S-2840A and B	1'-CH_2OH-S-2840A and B
	177 → 137* (pos)	364 → 278* (pos)	350 → 292* (pos)
	175 → 91 (neg)	364 → 318 (pos)	350 → 312 (pos)

Ion spray turbo heater (TEM) 550 °C

*Proposed for quantification

HPLC conditions (DFPA – confirmatory method)

Column Develosil RP aqueous C30 140A, 3.0 µm, 3.0 mm x 150 mm
Mobile phase A = Methanol containing 0.05% (v/v) acetic acid
B = Water containing 0.05% (v/v) acetic acid

Time (minutes)	Flow rate (µL/min)	%A	%B
0.0	1000	20.0	80.0
3.0	1000	50.0	50.0
3.1	1000	90.0	10.0
4.5	1000	90.0	10.0
4.6	1000	20.0	80.0
6.0	1000	20.0	80.0

Column temperature 60.0 °C
Injection volume 30.0 µL
Retention time ~2.0 minutes

MS conditions

Instrument SCIEX TripleQuad 6500 System, SCIEX (Triple quadrupole mass spectrometer)
Ionisation Type Electrospray ionisation (ESI, Turbulon Spray)
Polarity Positive ion mode
Scan Type MRM
Capillary voltage 5500 V
Mass transition monitored (*m/z*) 177 → 137* (pos)
Ion spray turbo heater (TEM) 550 °C

* Proposed to be used for confirmation for wheat (flour and whole-grain bread)

Table B.5.1.2.5-33: Analytical validation data for the determination of inpyrfluxam in wheat (flour) and wheat (whole-grain bread)

Analyte	Matrix	LOQ (mg/kg)	Recovery fortification level (mg/kg)	Recoveries % range (mean)	Repeatability % RSD (n)	Linearity
Inpyrfluxam	Wheat (Flour)	0.01 *(334 → 294 m/z)*	0.01	80 – 94 (86)	7.4 (5)	0.02 – 2.0 ng/mL (0.002 – 0.20 mg/kg) r = 0.9978 (n = 8) y = 465534.9172x + 922.2462
			0.1	83 – 89 (87)	2.7 (5)	
		0.01 *(334 → 238 m/z)*	0.01	82 – 90 (86)	4.1 (5)	0.02 – 2.0 ng/mL (0.002 – 0.20 mg/kg) r = 0.9992 (n = 8) y = 615901.1279x + 2122.6602
			0.1	86 – 91 (88)	2.1 (5)	
	Wheat (Whole-grain bread)	0.01 *(334 → 294 m/z)*	0.01	100 – 103 (101)	1.3 (5)	0.02 – 2.0 ng/mL (0.002 – 0.20 mg/kg) r = 0.9988 (n = 8) y = 293003.1198x -285.7271
			0.1	87 – 101 (96)	6.2 (5)	
		0.01 *(334 → 238 m/z)*	0.01	100 – 108 (104)	3.1 (5)	0.02 – 2.0 ng/mL (0.002 – 0.20 mg/kg) r = 0.9979 (n = 8) y = 309864.9937x -181.9397
			0.1	89 – 99 (96)	4.7 (5)	

Table B.5.1.2.5-34: Analytical validation data for the determination of 3'-OH-S-2840 in wheat (flour) and wheat (whole-grain bread)

Analyte	Matrix	LOQ (mg/kg)	Recovery fortification level (mg/kg)	Recoveries % range (mean)	Repeatability % RSD (n)	Linearity
3'-OH-S-2840	Wheat (Flour)	0.01 *(348 → 131 m/z)*	0.01	88 – 107 (97)	8.1 (5)	0.02 – 2.0 ng/mL (0.002 – 0.20 mg/kg) r = 0.9995 (n = 8) y = 91117.2820x + 179.4225
			0.1	89 – 100 (96)	4.9 (5)	
		0.01 *(348 → 175 m/z)*	0.01	92 – 109 (97)	7.3 (5)	0.02 – 2.0 ng/mL (0.002 – 0.20 mg/kg) r = 0.9992 (n = 8) y = 121029.2777x + 508.9453
			0.1	88 – 102 (94)	5.4 (5)	
	Wheat (Whole-grain bread)	0.01 *(348 → 131 m/z)*	0.01	102 -116 (108)	6.1 (5)	0.02 – 2.0 ng/mL (0.002 – 0.20 mg/kg) r = 0.9992 (n = 8) y = 200021.3926x + 326.4425
			0.1	100 – 110 (106)	3.6 (5)	
		0.01 *(348 → 175 m/z)*	0.01	105 – 120 (110)	5.3 (5)	0.02 – 2.0 ng/mL (0.002 – 0.20 mg/kg) r = 0.9993 (n = 8) y = 302601.4947x -162.5527
			0.1	100 – 110 (107)	3.9 (5)	

Table B.5.1.2.5-35: Analytical validation data for the determination of DFPA-$CONH_2$ in wheat (flour) and wheat (whole-grain bread)

Analyte	Matrix	LOQ (mg/kg)	Recovery fortification level (mg/kg)	Recoveries % range (mean)	Repeatability % RSD (n)	Linearity
DFPA-$CONH_2$	Wheat (Flour)	0.01 *(176 → 136 m/z)*	0.01	91 – 105 (98)	5.5 (5)	0.02 – 2.0 ng/mL (0.002 – 0.20 mg/kg) r = 0.9988 (n = 8) y = 131752.3055x + 213.2264
			0.1	92 – 101 (96)	3.6 (5)	
		0.01 *(176 → 156 m/z)*	0.01	88 – 104 (94)	6.8 (5)	0.02 – 2.0 ng/mL (0.002 – 0.20 mg/kg) r = 0.9995 (n = 8) y = 118037.6427x + 368.6439
			0.1	93 – 101 (97)	3.0 (5)	
	Wheat (Whole-grain bread)	0.01 *(176 → 136 m/z)*	0.01	103 – 110 (106)	2.8 (5)	0.02 – 2.0 ng/mL (0.002 – 0.20 mg/kg) r = 0.9995 (n = 8) y = 99475.1277x + 445.1378
			0.1	98 – 107 (103)	4.4 (5)	
		0.01 *(176 → 156 m/z)*	0.01	105 – 116 (109)	3.9 (5)	0.02 – 2.0 ng/mL (0.002 – 0.20 mg/kg) r = 0.9998 (n = 8) y = 102512.2562x + 207.8577
			0.1	97 – 108 (102)	3.9 (5)	

Table B.5.1.2.5-36: Analytical validation data for the determination of N-des-Me-DFPA in wheat (flour) and wheat (whole-grain bread)

Analyte	Matrix	LOQ (mg/kg)	Recovery fortification level (mg/kg)	Recoveries % range (mean)	Repeatability % RSD (n)	Linearity
N-des-Me-DFPA	Wheat (Flour)	0.01 *(161 → 141 m/z)*	0.01	68 – 91 (79)	12 (5)	0.04 – 4.0 ng/mL (0.002 – 0.20 mg/kg) r = 0.9977 (n = 8) y = 12773.5027x -13.1647
			0.1	81 – 91 (87)	4.2 (5)	
		0.01 *(163 → 68 m/z)*	0.01	86 – 101 (92)	6.2 (5)	0.04 – 4.0 ng/mL (0.002 – 0.20 mg/kg) r = 0.9986 (n = 8) y = 18622.5616x + 469.6531
			0.1	88 – 107 (97)	7.3 (5)	
	Wheat (Whole-grain bread)	0.01 *(161 → 141 m/z)*	0.01	86 – 101 (92)	6.2 (5)	0.04 – 2.4 ng/mL (0.002 – 0.12 mg/kg) r = 0.9969 (n = 7) y = 28382.2291x -123.8410
			0.1	88 – 107 (97)	7.3 (5)	
		0.01 *(163 → 68 m/z)*	0.01	89 – 114 (99)	9.5 (5)	0.04– 2.4 ng/mL (0.002 – 0.12 mg/kg) r = 0.9970 (n = 7) y = 12171.2417x + 55.9394
			0.1	72 – 94 (80)	11 (5)	

Table B.5.1.2.5-37: Analytical validation data for the determination of DFPA in wheat (flour) and wheat (whole-grain bread)

Analyte	Matrix	LOQ (mg/kg)	Recovery fortification level (mg/kg)	Recoveries % range (mean)	Repeatability % RSD (n)	Linearity
DFPA	Wheat (Flour)	0.01 *(177 → 137 m/z)*	0.01	75 – 106 (92)	15 (5)	0.02 – 2.0 ng/mL (0.002 – 0.20 mg/kg) r = 0.9996 (n = 8) y = 75879.0085x -207.8217
			0.1	70 – 99 (83)	16 (5)	
		0.01 *(177 → 137 m/z)*	0.01	69 – 113 (90)	24 (5)	0.02 – 2.0 ng/mL (0.002 – 0.20 mg/kg) r = 0.9993 (n = 8) y = 127047.3105x -504.9630
			0.1	70 – 107 (90)	20 (5)	
	Wheat (Whole-grain bread)	0.01 *(177 → 137 m/z)*	0.01	88 – 107 (97)	7.2 (5)	0.02 – 2.0 ng/mL (0.002 – 0.20 mg/kg) r = 0.9986 (n = 8) y = 340492.0667x -2264.9108
			0.1	90 – 95 (93)	2.2 (5)	
		0.01 *(177 → 137 m/z)*	0.01	71 – 125 (103)	19 (5)	0.02 – 2.0 ng/mL (0.002 – 0.20 mg/kg) r = 0.9979 (n = 8) y = 272568.3068x -1321.4157
			0.1	80 – 118 (105)	14 (5)	

Table B.5.1.2.5-38: Analytical validation data for the determination of 1'-CH_2OH-S-2840A in wheat (flour) and wheat (whole-grain bread)

Analyte	Matrix	LOQ (mg/kg)	Recovery fortification level (mg/kg)	Recoveries % range (mean)	Repeatability % RSD (n)	Linearity
1'-CH_2OH-S-2840A	Wheat (Flour)	0.005 *(350 → 292 m/z)*	0.005	73 – 79 (76)	3.0 (5)	0.01 – 1.0 ng/mL (0.001 – 0.10 mg/kg) r = 0.9977 (n = 8) y = 608106.1401x + 521.3859
			0.05	70 – 82 (75)	7.8 (5)	
		0.005 *(350 → 312 m/z)*	0.005	70 – 88 (79)	9.2 (5)	0.01 – 1.0 ng/mL (0.001 – 0.10 mg/kg) r = 0.9976 (n = 8) y = 228274.4009x -579.4506
			0.05	69 – 86 (77)	9.9 (5)	
	Wheat (Whole-grain bread)	0.005 *(350 → 292 m/z)*	0.005	99 – 110 (103)	4.3 (5)	0.01 – 1.0 ng/mL (0.001 – 0.10 mg/kg) r = 0.9980 (n = 8) y = 1112524.2338x -5404.9132
			0.05	88 – 99 (92)	4.5 (5)	
		0.005 *(350 → 312 m/z)*	0.005	103 – 110 (105)	2.6 (5)	0.01 – 1.0 ng/mL (0.001 – 0.10 mg/kg) r = 0.9983 (n = 8) y = 393968.6881x -2134.0159
			0.05	90 – 96 (93)	2.4 (5)	

Table B.5.1.2.5-39: Analytical validation data for the determination of 1'-CH_2OH-S-2840B in wheat (flour) and wheat (whole-grain bread)

Analyte	Matrix	LOQ (mg/kg)	Recovery fortification level (mg/kg)	Recoveries % range (mean)	Repeatability % RSD (n)	Linearity
1'-CH_2OH-S-2840B	Wheat (Flour)	0.005 *(350 → 292 m/z)*	0.005	70 – 84 (76)	6.9 (5)	0.01 – 1.0 ng/mL (0.001 – 0.10 mg/kg) r = 0.9991 (n = 8) y = 641131.8180x - 1582.3948
			0.05	69 – 81 (74)	7.5 (5)	
		0.005 *(350 → 312 m/z)*	0.005	71 – 85 (75)	7.4 (5)	0.01 – 1.0 ng/mL (0.001 – 0.10 mg/kg) r = 0.9988 (n = 8) y = 274523.9809x + 141.4635
			0.05	70 – 80 (74)	70 (5)	
	Wheat (Whole-grain bread)	0.005 *(350 → 292 m/z)*	0.005	96 – 105 (99)	3.8 (5)	0.01 – 1.0 ng/mL (0.001 – 0.10 mg/kg) r = 0.9976 (n = 8) y = 1080235.6769x -5749.4336
			0.05	85 – 93 (90)	3.6 (5)	
		0.005 *(350 → 312 m/z)*	0.005	96 – 109 (103)	4.6 (5)	0.01 – 1.0 ng/mL (0.001 – 0.10 mg/kg) r = 0.9977 (n = 8) y = 440945.3994x -1817.0497
			0.05	84 – 96 (90)	5.5 (5)	

Table B.5.1.2.5-40: Analytical validation data for the determination of 1'-COOH-S-2840A in wheat (flour) and wheat (whole-grain bread)

Analyte	Matrix	LOQ (mg/kg)	Recovery fortification level (mg/kg)	Recoveries % range (mean)	Repeatability % RSD (n)	Linearity
1'-COOH-S-2840A	Wheat (Flour)	0.005 *(364 → 278 m/z)*	0.005	75 – 88 (80)	7.6 (5)	0.01 – 1.0 ng/mL (0.001 – 0.10 mg/kg) r = 0.9983 (n = 8) y = 372683.2596x -197.7579
			0.05	74 – 84 (79)	5.5 (5)	
		0.005 *(364 → 318 m/z)*	0.005	75 – 87 (81)	7.4 (5)	0.01 – 1.0 ng/mL (0.001 – 0.10 mg/kg) r = 0.9984 (n = 8) y = 299213.8915x -1044.6519
			0.05	74 – 85 (79)	5.5 (5)	
	Wheat (Whole-grain bread)	0.005 *(364 → 278 m/z)*	0.005	82 – 105 (92)	10 (5)	0.01 – 1.0 ng/mL (0.001 – 0.10 mg/kg) r = 0.9975 (n = 8) y = 518503.7582x -1909.7531
			0.05	76 – 95 (88)	8.2 (5)	
		0.005 *(364 → 318 m/z)*	0.005	82 – 106 (93)	10 (5)	0.01 – 1.0 ng/mL (0.001 – 0.10 mg/kg) r = 0.9969 (n = 8) y = 434832.7910x -1821.6368
			0.05	78 – 97 (90)	8.4 (5)	

Table B.5.1.2.5-41: Analytical validation data for the determination of 1'-COOH-S-2840B in wheat (flour) and wheat (whole-grain bread)

Analyte	Matrix	LOQ (mg/kg)	Recovery fortification level (mg/kg)	Recoveries % range (mean)	Repeatability % RSD (n)	Linearity
1'-COOH-S-2840B	Wheat (Flour)	0.005 *(364 → 278 m/z)*	0.005	71 – 97 (92)	12 (5)	0.01 – 1.0 ng/mL (0.001 – 0.10 mg/kg) r = 0.9964 (n = 8) y = 234099.1464x -108.9170
			0.05	70 – 86 (78)	9.9 (5)	
		0.005 *(364 → 318 m/z)*	0.005	74 – 89 (79)	7.4 (5)	0.01 – 1.0 ng/mL (0.001 – 0.10 mg/kg) r = 0.9987 (n = 8) y = 645395.2484x -758.3693
			0.05	69 – 86 (77)	9.7 (5)	
	Wheat (Whole-grain bread)	0.005 *(364 → 278 m/z)*	0.005	83 – 97 (89)	7.9 (5)	0.01 – 1.0 ng/mL (0.001 – 0.10 mg/kg) r = 0.9962 (n = 8) y = 266812.8895x -420.8487
			0.05	73 – 90 (85)	8.1 (5)	
		0.005 *(364 → 318 m/z)*	0.005	79 – 100 (88)	9.8 (5)	0.01 – 1.0 ng/mL (0.001 – 0.10 mg/kg) r = 0.9986 (n = 8) y = 811122.0293x -4163.0161
			0.05	71 – 88 (84)	8.8 (5)	

Specificity

LC-MS/MS with primary and confirmatory ion transitions is considered a highly specific technique. Therefore, additional methods to confirm the identity of the analytes are not considered necessary.

Matrix peaks at the retention time of interest were observed for DFPA mass transition 175 → 91 *m/z* in wheat (flour and whole-grain bread). Therefore, two HPLC methods, applying C_{30} or Phenyl-Hexyl as the stationary phase, were used in combination with mass transition 177 → 137 *m/z* for quantitation and confirmation.

For the metabolites 1'-CH_2OH-S-2840A and B and 1'-COOH-S-2840A and B, isomeric peak identity was confirmed by measuring single standard solutions of 1'-CH_2OH-S-2840A and B and 1'-COOH-S-2840A and B. *Cis* and *trans* isomer peaks were separated chromatographically by their different retention times.

Chromatograms for standards, reagent blank, control samples and samples fortified at the LOQ have been provided for all matrices. No significant interference (>30% of the LOQ) between the analyte peaks and any of the plant commodity matrices was observed. Analyte identity was confirmed by retention time match with reference standard.

Matrix Effects

Matrix effects were investigated by comparing peak areas of matrix-matched standards (90 % matrix amount) with solvent standards at identical concentrations.

Matrix effects were determined to be significant (>±20%) in both wheat (flour) and wheat (whole-grain bread) for a number of analytes. Therefore, matrix matched standards were used for quantification throughout.

Linearity

Linearity was demonstrated by the analysis of seven to eight standards of increasing concentration. For inpyrfluxam, 3'-OH-S-2840, DFPA-$CONH_2$ and DFPA the range of standard concentrations used was 0.02 – 2.0 ng/mL, equivalent to 0.002 – 0.20 mg/kg analyte in the samples. For 1'-CH_2OH-S-2840A, 1'-CH_2OH-S-2840B, 1'-COOH-S-2840A and 1'-COOH-S-2840B the range of standard concentrations used was 0.01 – 1.0 ng/mL, equivalent to 0.001 – 0.10 mg/kg analyte in the samples. For N-des-Me-DFPA, for wheat (flour) the range of standard concentrations was 0.04 -4.0 ng/mL, equivalent to 0.002 – 0.2 mg/kg. For wheat (whole-grain bread), the range was 0.04 – 2.4 ng/mL, equivalent to 0.002 – 0.12 mg/kg.

The responses were linear with correlation coefficients (r) of ≥ 0.99.

Accuracy

Recovery samples were prepared by spiking each matrix with the target analyte. The analytes were fortified in two groups:

- Group A: Inpyrfluxam, 3'-OH-S-2840, DFPA-$CONH_2$ and N-des-Me-DFPA
- Group B: DFPA, 1'-CH_2OH-S-2840A and B, and 1'-COOH-S-2840A and B

Two different spiking groups were necessary as DFPA-$CONH_2$ is susceptible to DFPA transformation. Within each group the analytes were fortified jointly and but quantified separately by the method described. The spike concentrations for inpyrfluxam, 3'-OH-S-

2840, DFPA-$CONH_2$, DFPA and N-des-Me-DFPA was 0.01 – 0.1 mg/kg, and the spike concentration for 1'-CH_2OH-S-2840A, 1'-CH_2OH-S-2840B, 1'-COOH-S-2840A and 1'-COOH-S-2840B 0.005 – 0.05 mg/kg. Five samples were prepared at each fortification level. The mean recoveries for all analytes in each matrix are within the acceptable ranges of 60 – 120% (≤0.01 mg/kg) and 70 – 120% (>0.01 - ≤0.1 mg/kg).

Precision

Precision was determined from the accuracy recovery data. Five samples were prepared at each fortification level. The calculated %RSD is within the acceptable limits of ≤30% (≤0.01 mg/kg) and ≤20% (>0.01 - ≤0.1 mg/kg).

Stock stability

The stability of stock solutions has not been addressed as part of the current study.

Stability of calibration solution

The stability of calibration solutions has not been addressed as part of the current study.

Stability of extract

Stability of analytes in extracts were not investigated as matrix matched standards used for quantification were prepared the same day as the work up residue samples. Acceptable procedural recoveries also support the conclusion that stability was sufficient for the purpose of the validation.

Extraction efficiency

Refer to KCA 4.1.2/19

Conclusion

Validation data was generated prior to the introduction of SANTE/2020/12830 Rev.1. The method can be considered sufficiently validated to the requirements as outlined in SANCO/3029/99 rev.4 for risk assessment. The method meets the requirements outlined under Section 4.2 of SANTE/2020/12830 Rev.1 "minimum validation requirements for the assessment of existing methods for risk assessment". Therefore, the method can be considered sufficiently validated in accordance with SANTE/2020/12830 for risk assessment purposes.

The method is acceptable in the areas of specificity, linearity, accuracy, and precision Extraction efficiency is considered sufficiently addressed. The LOQ of the method is 0.01 mg/kg (inpyrfluxam, 3'-OH-S-2840, DFPA-$CONH_2$, DFPA and N-des-Me-DFPA) and 0.005 mg/kg (1'-CH_2OH-S-2840A, 1'-CH_2OH-S-2840B, 1'-COOH-S-2840A and 1'-COOH-S-2840B).

Matrix effects were not investigated in the study, however matrix matched standards were used for quantification. Stability of stock and calibration solutions has not been addressed as part of the current study.

Reference:	KCA 4.1.2/31
Report Title:	Magnitude of residues of S-2399 and its metabolites in barley processed fractions in Northern and Southern Europe – 2016
Author(s) & Year:	[redacted] 2018b
Document No, Authority registration No	Study No.: 262-2016; Report No.: TPR-0082
Guideline(s):	SANCO/3029/99, rev. 4
Deviations:	No
GLP or GEP:	Yes
Acceptability:	Yes
Study relied upon:	Yes

The method is the same as that summarised in report TPA-0057 (KCA 4.1.2/20), and TPR-0081 (KCA 4.1.2/30). Additional validation data has been generated for the determination of inpyrfluxam and its metabolites 3'-OH-S-2840, DFPA-$CONH_2$, N-des-Me-DFPA, DFPA, 1'-COOH-S-2840A and B, 1'-CH_2OH-S-2840A and B in beer.

Samples for analysis of DFPA, 1'-COOH-S-2840A and B and 1'-CH_2OH-S-2840A and B residues are subjected to acid hydrolysis. Therefore, the quantified residue levels will include both free and conjugated forms.

Principle of the method

Samples (20 mL) are weighed into 250 mL bottles. 10 mL of water and 30 mL of acetonitrile are added and shaken by hand for 1 minute. The samples are mechanically shaken for 10 minutes then filtered through cotton into a 100 mL flask. The bottle is rinsed twice with 20 mL of acetonitrile/water (1:1, v/v) and added to the flask. The combined extracts are made up to 200 mL with acetonitrile/water (1:1, v/v).

Different procedures for the clean-up of sample extracts were necessary and different HPLC conditions were used. These are described below.

Inpyrfluxam, 3'-OH-S-2840 and DFPA-$CONH_2$

An aliquot (1 mL) of extract is transferred into a test tube and evaporated to the aqueous remainder under nitrogen at 40ºC. The tube is made up to 10 mL with water/methanol (3:1 v/v). An aliquot is transferred to a HPLC vial for analysis by LC-MS/MS.

N-des-Me-DFPA

An aliquot (2 mL) of the combined extract is hydrolysed with 5 mL of 6M hydrochloric acid for 6 hours at 100°C. The sample is cooled to room temperature and diluted with 15 mL of water. The extract is transferred to a Chem Elut cartridge, eluted with ethyl acetate (4 x 25 mL) into a round bottomed flask and evaporated to dryness under vacuum at 40°C. The sample is reconstituted in 5 mL of water/methanol (3:1, v/v) with sonication. The samples are then made up to 10 mL with water/methanol (3:1, v/v). An aliquot is transferred to a HPLC vial for analysis by LC-MS/MS.

DFPA, 1'-COOH-S-2840A and B and 1'-CH_2OH-S-2840A and B

An aliquot (2 mL) of the combined extract is hydrolysed with 2 mL of 4M hydrochloric acid for 4 hours at 100°C. The samples are cooled to room temperature and cleaned up using an Oasis HLB cartridge (conditioned with 5 mL methanol and 10 mL water). The analytes are eluted with 10 mL of methanol and evaporated to 5 mL under nitrogen at 40°C. The sample is diluted with water to 20 mL prior to analysis.

Analytical parameters are the same as those reported under KCA 4.1.2/30. If necessary, final extracts are diluted to be within the calibration range of the matrix-matched standard solutions.

Table B.5.1.2.5-42: Analytical validation data for the determination of inpyrfluxam in beer

Analyte	Matrix	LOQ (mg/kg)	Recovery fortification level (mg/kg)	Recoveries % range (mean)	Repeatability % RSD (n)	Linearity
Inpyrfluxam	Beer	0.01 *(334 → 294 m/z)*	0.01	80 – 91 (84)	5.6 (5)	0.02 – 2.0 ng/mL r = 0.9991 (n = 8) y = 268114.9608x + 2125.8999
			0.1	96 – 106 (101)	3.7 (5)	
		0.01 *(334 → 238 m/z)*	0.01	75 – 94 (91)	4.4 (5)	0.02 – 2.0 ng/mL r = 0.9969 (n = 8) y = 362345.9837x + 2500.7328
			0.1	95 – 105 (101)	4.1 (5)	

Table B.5.1.2.5-43: Analytical validation data for the determination of 3'-OH-S-2840 in beer

Analyte	Matrix	LOQ (mg/kg)	Recovery fortification level (mg/kg)	Recoveries % range (mean)	Repeatability % RSD (n)	Linearity
3'-OH-S-2840	Beer	0.01 *(348 → 131 m/z)*	0.01	69 – 102 (88)	15 (5)	0.02 – 2.0 ng/mL r = 0.9972 (n = 8) y = 12657.6163x + 120.7732
			0.1	92 – 109 (101)	7 (5)	
		0.01 *(348 → 175 m/z)*	0.01	78 – 112 (96)	16 (5)	0.02 – 2.0 ng/mL r = 0.9970 (n = 8) Y = 16945.8843x + 137.4208
			0.1	98 – 107 (103)	3.8 (5)	

Table B.5.1.2.5-44: Analytical validation data for the determination of DFPA-$CONH_2$ in beer

Analyte	Matrix	LOQ (mg/kg)	Recovery fortification level (mg/kg)	Recoveries % range (mean)	Repeatability % RSD (n)	Linearity
DFPA-$CONH_2$	Beer	0.01 *(176 → 136 m/z)*	0.01	73 – 109 (88)	16 (5)	0.02 – 2.0 ng/mL r = 0.9986 (n = 8) y = 91641.1601x + 301.1648
			0.1	98 – 103 (102)	2.0 (5)	
		0.01 *(176 → 156 m/z)*	0.01	72 – 91 (82)	9.2 (5)	0.02 – 2.0 ng/mL r = 0.9992 (n = 8) y = 75449.6555x + 550.0309
			0.1	96 – 105 (101)	3.3 (5)	

Table B.5.1.2.5-45: Analytical validation data for the determination of N-des-Me-DFPA in beer

Analyte	Matrix	LOQ (mg/kg)	Recovery fortification level (mg/kg)	Recoveries % range (mean)	Repeatability % RSD (n)	Linearity
N-des-Me-DFPA	Beer	0.01 *(161 → 141 m/z)*	0.01	88 – 100 (92)	9.2 (5)	0.04 – 4.0 ng/mL r = 0.9990 (n = 8) y = 57351.4347x -337.9435
			0.1	75 – 91 (83)	8.5 (5)	
		0.01 *(161 → 66 m/z)*	0.01	92 (97)	4.7 (5)	0.04 – 4.0 ng/mL r = 0.9994 (n = 8) y = 4531.6518x + 21.7564
			0.1	74 – 96 (85)	12 (5)	

Table B.5.1.2.5-46: Analytical validation data for the determination of DFPA in beer

Analyte	Matrix	LOQ (mg/kg)	Recovery fortification level (mg/kg)	Recoveries % range (mean)	Repeatability % RSD (n)	Linearity
DFPA	Beer	0.01 *(177 → 137 m/z)*	0.01	99 – 116 (107)	6.5 (5)	0.02 – 2.0 ng/mL r = 0.9998 (n = 8) y = 446510.9877x -605.0685
			0.1	90 – 115 (102)	11 (5)	
		0.01 *(177 → 137 m/z)*	0.01	96 – 120 (107)	11 (5)	0.02 – 2.0 ng/mL r = 0.9997 (n = 8) y = 425457.6777x -1852.5532
			0.1	94 – 113 (102)	8.0 (5)	

Table B.5.1.2.5-47: Analytical validation data for the determination of 1'-COOH-S-2840A in beer

Analyte	Matrix	LOQ (mg/kg)	Recovery fortification level (mg/kg)	Recoveries % range (mean)	Repeatability % RSD (n)	Linearity
1'-COOH-S-2840A	Beer	0.005 *(364 → 278 m/z)*	0.005	93 – 96 (95)	1.6 (5)	0.01 – 1.0 ng/mL. r = 0.9996 (n = 8) y = 575217.7997x -657.1403
			0.05	85 – 99 (92)	5.5 (5)	
		0.005 *(364 → 318 m/z)*	0.005	79 – 97 (93)	8.6 (5)	0.01 – 1.0 ng/mL r = 0.9992 (n = 8) y = 616398.2596x -290.2654
			0.05	84 – 94 (90)	4.7 (5)	

Table B.5.1.2.5-48: Analytical validation data for the determination of 1'-COOH-S-2840B in beer

Analyte	Matrix	LOQ (mg/kg)	Recovery fortification level (mg/kg)	Recoveries % range (mean)	Repeatability % RSD (n)	Linearity
1'-COOH-S-2840B	Beer	0.005 *(364 → 278 m/z)*	0.005	87 – 97 (91)	4.4 (5)	0.01 – 1.0 ng/mL r = 0.9996 (n = 8) y = 368211.9373x -800.9076
			0.05	78 – 85 (82)	3.3 (5)	
		0.005 *(364 → 318 m/z)*	0.005	78 – 96 (89)	7.6 (5)	0.01 – 1.0 ng/mL r = 0.9993 (n = 8) y = 1193443.0388x -2784.7629
			0.05	71 – 88 (83)	8.5 (5)	

Table B.5.1.2.5-49: Analytical validation data for the determination of 1'-CH_2OH-S-2840A in beer

Analyte	Matrix	LOQ (mg/kg)	Recovery fortification level (mg/kg)	Recoveries % range (mean)	Repeatability % RSD (n)	Linearity
1'-CH_2OH-S-2840A	Beer	0.005 *(350 → 292 m/z)*	0.005	79 – 100 (92)	9.1 (5)	0.01 – 1.0 ng/mL r = 0.9977 (n = 8) y = 1320344.4753x -2144.1558
			0.05	86 – 94 (90)	3.4 (5)	
		0.005 *(350 → 312 m/z)*	0.005	75 – 95 (88)	9.2 (5)	0.01 – 1.0 ng/mL r = 0.9995 (n = 8) y = 471564.3533x -225.7919
			0.05	82 – 92 (88)	4.3 (5)	

Table B.5.1.2.5-50: Analytical validation data for the determination of 1'-CH_2OH-S-2840B in beer

Analyte	Matrix	LOQ (mg/kg)	Recovery fortification level (mg/kg)	Recoveries % range (mean)	Repeatability % RSD (n)	Linearity
1'-CH_2OH-S-2840B	Beer	0.005 *(350 → 292 m/z)*	0.005	86 – 99 (91)	6.0 (5)	0.01 – 1.0 ng/mL r = 0.9994 (n = 8) y = 1277782.5774x -1957.3542
			0.05	82 – 92 (87)	4.8 (5)	
		0.005 *(350 → 312 m/z)*	0.005	84 – 98 (89)	6.2 (5)	0.01 – 1.0 ng/mL r = 0.9995 (n = 8) y = 513300.4902x -160.8428
			0.05	81 – 93 (88)	5.1 (5)	

Specificity

LC-MS/MS with primary and confirmatory ion transitions is considered a highly specific technique. Therefore, additional methods to confirm the identity of the analytes are not considered necessary.

Matrix peaks at the retention time of interest were observed for DFPA mass transition 175 → 91 *m/z* in wheat (flour and whole-grain bread). Therefore, two HPLC methods, applying C_{30} or Phenyl-Hexyl as the stationary phase, were used in combination with mass transition 177 → 137 *m/z* for quantitation and confirmation.

For the metabolites 1'-CH_2OH-S-2840A and B and 1'-COOH-S-2840A and B, isomeric peak identity was confirmed by measuring single standard solutions of 1'-CH_2OH-S-2840A and B and 1'-COOH-S-2840A and B. *Cis* and *trans* isomer peaks were separated chromatographically by their different retention times.

Chromatograms for standards, reagent blank, control samples and samples fortified at the LOQ have been provided for all matrices. No significant interference (>30% of the LOQ) between the analyte peaks and the matrix was observed. Analyte identity was confirmed by retention time match with reference standard.

Matrix effects

Matrix effects were investigated by comparing peak areas of matrix-matched standards (90 % matrix amount) with solvent standards at identical concentrations.

Significant matrix effects (>±20%) was observed in 3'-OH-S-2840, DFPA-$CONH_2$, N-des-Me-DFPA and 1'-CH_2OH-S-2840A in beer. Matrix effects for all other analytes were <±20%. Regardless of matrix effects, matrix matched standards were used for quantification throughout.

Linearity:

Linearity was demonstrated by the analysis of eight standards of increasing concentration. For inpyrfluxam, 3'-OH-S-2840, DFPA-$CONH_2$ and DFPA, the range of standard concentrations used was 0.02 – 2.0 ng/mL. For N-des-Me-DFPA a range of 0.04 – 4.0 ng/mL was used. For 1'-CH_2OH-S-2840A, 1'-CH_2OH-S-2840B, 1'-COOH-S-2840A and 1'-COOH-S-2840B the range of standard concentrations used was 0.01 – 1.0 ng/mL.

The responses were linear with correlation coefficients (r) of ≥ 0.99.

Accuracy:

Recovery samples were prepared by spiking beer with the target analyte. The analytes were fortified in two groups:

- Group A: Inpyrfluxam, 3'-OH-S-2840, DFPA-$CONH_2$ and N-des-Me-DFPA
- Group B: DFPA, 1'-CH_2OH-S-2840A and B, and 1'-COOH-S-2840A and B

Two different spiking groups were necessary as DFPA-$CONH_2$ is susceptible to DFPA transformation. Within each group the analytes were fortified jointly and but quantified separately by the method described. The spike concentrations for inpyrfluxam, 3'-OH-S-2840, DFPA-$CONH_2$, DFPA and N-des-Me-DFPA was 0.01 – 0.1 mg/kg, and the spike concentration for 1'-CH_2OH-S-2840A, 1'-CH_2OH-S-2840B, 1'-COOH-S-2840A and 1'-COOH-S-2840B was 0.005 – 0.05 mg/kg. Five samples were prepared at each fortification level. The mean recovery for all analytes in beer are within the acceptable ranges of 60 – 120% (≤0.01 mg/kg) and 70 – 120% (>0.01 - ≤0.1 mg/kg).

Precision:

Precision was determined from the accuracy recovery data. Five samples were prepared at each fortification level. The calculated %RSD is within the acceptable limits of ≤30% (≤0.01 mg/kg) and ≤20% (>0.01 - ≤0.1 mg/kg). Therefore, acceptable precision has been demonstrated.

Stock stability

The stability of stock solutions has not been addressed as part of the current study.

Stability of calibration solution

The stability of calibration solutions has not been addressed as part of the current study.

Stability of extract

Stability of analytes in extracts were not investigated as matrix matched standards used for quantification were prepared the same day as the work up residue samples. Acceptable procedural recoveries also support the conclusion that stability was sufficient for the purpose of the validation.

Extraction efficiency:

Consideration of extraction efficiency from beer is not required as beer is a liquid matrix.

Conclusion

Validation data was generated prior to the introduction of SANTE/2020/12830 Rev.1. The method can be considered sufficiently validated to the requirements as outlined in SANCO/3029/99 rev.4 for risk assessment. The method meets the requirements outlined under Section 4.2 of SANTE/2020/12830 Rev.1 "minimum validation requirements for the assessment of existing methods for risk assessment". Therefore, the method can be considered sufficiently validated in accordance with SANTE/2020/12830 for risk assessment purposes.

The method is acceptable in the areas of specificity, accuracy, precision and linearity. Extraction efficiency is considered sufficiently addressed. The LOQ of the method is 0.01

mg/kg (inpyrfluxam, 3'-OH-S-2840, DFPA-$CONH_2$, DFPA and N-des-Me-DFPA) and 0.005 mg/kg (1'-CH_2OH-S-2840A, 1'-CH_2OH-S-2840B, 1'-COOH-S-2840A and 1'-COOH-S-2840B).

Matrix effects were investigated in the study, with significant matrix effects observed for 3'-OH-S-2840, DFPA-$CONH_2$, N-des-Me-DFPA and 1'-CH_2OH-S-2840A. Matrix matched standards were used for quantification for all analytes. Stability of stock and calibration solutions has not been addressed as part of the current study.

B.5.1.2.6. Methods in soil, water, sediment, feed and any additional matrices used in support of ecotoxicology studies

Table B.5.1.2.6-1: Summary of risk assessment methods supporting ecotoxicology studies

Data point	Study	Conclusion	Studies relied on
KCA 4.1.2/33	Analytical Method Verification for the Determination of S-2399 TG in Avian Diet. [redacted] 2014 Report No.: TPA-0004	HPLC/UV Analyte: Inpyrfluxam LOQ: 50 mg/kg in avian diet Acceptable	KCA 8.1.1.3/01 Report no: TPW-0018 KCA 8.1.1.3/02 TPW-0019. KCA 8.1.1.2/03 TPW-0071
KCA 4.1.2/39	S-2399 – Fish Short-Term Reproduction Assay with Fathead Minnow (*Pimephales promelas*).	HPLC-MS/MS Analyte: Inpyrfluxam LOQ: 0.1 µg/L in aqueous solution.	KCA 8.2.3/01 Report no.: TPW-0137

	[redacted] 2021 Report no.: TPW-0137	Acceptable	KCA 8.1.4/01 Report no.: TPW-0138
KCA 4.1.2/34	S-2399 TG - Acute Toxicity Test with Rainbow Trout (Oncorhynchus mykiss) Under Static Conditions Following OECD Guideline #203 [redacted] 2014a Report no.: TPW-0006	HPLC-MS/MS Analyte: Inpyrfluxam LOQ: 0.6 µg/L in freshwater. Acceptable	KCA 8.2.1/01 Report no.: TPW-0006
			KCA 8.2.1/02 Report no.: TPW-0003
			KCA 8.2.1/03 Report no.: TPW-0004
			KCA 8.2.1/04 Report no.: TPW-0005
			KCA 8.2.1/05 Report no.:TPW-0014
			KCA 8.2.2.1/01 Report no.: TPW-0012
			KCA 8.2.2.1/02 Report no.: TPW-0085
			KCA 8.2.4.1/01 Report no.: TPW-0002
			KCA 8.2.4.2/01 Report no.: TPW-0013

			KCA 8.2.5.2/01 Report no.: TPW-0041
			KCA 8.2.5.1/01 Report no.: TPW-0007
			KCA 8.2.5.4/01 Report no.: TPW-0031
			KCA 8.2.5.4/02 Report no.: TPW-0034
			KCA 8.2.5.4/03 Report no.: TPW-0086
			KCA 8.2.6.1/01 Report no.: TPW-0017
			KCA 8.2.6.2/01 Report no.: TPW-0020
			KCA 8.2.6.2/03 Report no.: TPW-0030
			KCA 8.2.7/01 Report no.: TPW-0022
			KCA 8.2.8/01 Report no.: TPW-0021

			KCA 8.3.1.3/01 Report no.: TPW-0066
KCA 4.1.2/35	Acute Toxicity Study of S-2399 TG with Guppy (Poecilia reticulata). [redacted] 2016a Report No.: TPW-0053	HPLC-UV Analyte: Inpyrfluxam LOQ: 0.02 mg/L in water. Acceptable	KCA 8.2.1/06 Report no.: TPW-0053
			KCA 8.2.1/07 Report no.: TPW-0054
			KCA 8.2.1/08 Report no.: TPW-0055
KCA 4.1.2/36	Acute Toxicity Study of 3'-OH-S-2840 with Rainbow trout (*Oncorhynchus mykiss*). [redacted] 2016a Report No.: TPW-0035	HPLC-UV Analyte: 3'-OH-S-2840 LOQ: 0.1 mg/L in aquatic test solutions Acceptable	KCA 8.2.1/10 Report No.: TPW-0035
KCA 4.1.2/37	Acute Toxicity Study of 1'-COOH-S-2840 with Rainbow trout (*Oncorhynchus mykiss*) [redacted] 2016b Report No.: TPW-0036	HPLC-UV Analyte: 1'COOH-S-2840 LOQ: 1 mg/L in aquatic test solutions Acceptable	KCA 8.2.1/11 Report no: TPW-0036
KCA 4.1.2/38	Life-Cycle Toxicity Test Exposing Midges (Chironomus dilutus)	HPLC-UV Analyte: Inpyrfluxam	KCA 8.2.5.4/01 Report no: TPW-0031

	to S-2399 TG Applied to Sediment Under Static-Renewal Conditions Following EPA Test Methods. [redacted] 2015 Report No.: TPW-0031	LOQ: 200 µg/kg in sediment. Acceptable.	KCA 8.2.5.4/02 Report no.: TPW-0034
			KCA 8.2.5.4/03 Report no.: TPW-0086

Reference:	KCA 4.1.2/33
Report Title:	Analytical Method Verification for the Determination of S-2399 TG in Avian Diet
Author(s) & Year:	[redacted] [redacted] [redacted] 2014
Document No, Authority registration No	Study No.: 166C-119; Report No.: TPA-0004
Guideline(s):	SANCO/3029/99 rev.4 OPPTS 860.1340
Deviations:	No
GLP or GEP:	Yes
Acceptability:	Yes
Study relied upon:	Yes

The purpose of the study was to demonstrate the validity of a HPLC-UV method for the determination of inpyrfluxam in avian diet.

Principle of the method

Subsamples of avian diet (2.00 g) were weighed into 8 oz. French square bottles. Using a volumetric pipet (or equivalent), 25 mL of acetonitrile was added to each sample. The samples were placed on a Geno/Grinder for 5 minutes at 1500 rpm. An aliquot (~20 mL) was transferred to a scintillation vial and centrifuged at 1500 rpm for ~10 minutes. Samples were diluted with acetonitrile, as necessary, filtered and analysed by HPLC/UV.

HPLC conditions

HPLC system Waters Alliance 2690 high performance chromatograph equipped with a Waters Alliance 2489 variable wavelength detector

Column YMC-PACK ODS-AM (150 mm x 4.6 mm I.D. x 5 µm particle size)

Mobile phase A = 0.1% phosphoric acid
B = Acetonitrile

Time (minutes)	%A	%B
0.00	90.0	10.0
1.00	90.0	10.0
10.00	10.0	90.0
11.00	10.0	90.0
11.10	90.0	10.0
15.00	90.0	10.0

Column temperature 40 °C
Injection volume 25 µL
Flow rate 1.00 mL/min
Detector wavelength 254 nm
Retention time ~10.6 minutes

Table B.5.1.2.6-2: Summary of method validation for the determination of Inpyrfluxam in avian diet

Analyte	LOQ (mg/kg)	Recovery fortification level (mg/kg)	Recoveries % range (mean)	Repeatability % RSD (n)	Linearity	Specificity
Inpyrfluxam	50.0	50.0	91.8 – 121 (102) N = 5	12.7 @ ~50.0 mg/kg (n=5)	0.500 – 5.00 µg/mL (n = 5)	Retention time match to reference standard. No significant

		7000	95.2 – 101 (98) N = 5	2.67 @ ~7000 mg/kg (n=5)	y = 39152x – 62.263 R^2 = 0.9998	interfering peaks observed in the blank matrix.

Matrix effects

The matrix effects have not been addressed.

Specificity

Specificity was demonstrated by retention time match with a reference standard and the absence of significant (>30% LOQ) interfering peaks in the chromatogram of a matrix blank sample. Analyte identity was confirmed by comparison of the retention time of the analyte with that of a reference standard. In addition, the UV spectra of the fortified samples were consistent with that of a reference standard.

Linearity

Linearity was demonstrated by the analysis of five standards of increasing concentration. The range of standard concentrations used was 0.500 – 5.00 µg inpyrfluxam /mL. The concentrations extend over an appropriate range, and the response was linear with a coefficient of determination (R^2) of 0.9998. If necessary, samples were further diluted to within linear range.

Precision (repeatability)

The precision of the method was assessed via analysis of the accuracy samples. The reported %RSDs were <20%.

Accuracy (recovery)

The accuracy of the method was assessed by analysing five samples fortified with inpyrfluxam at concentrations of 50.0 and 7000 mg/kg The samples were diluted to fit within the linear range. The lower fortification level was diluted by a factor of 5 and the higher fortification level was diluted by a factor of 200. Recovery values were calculated as a percentage of measured concentration relative to fortified concentration. Acceptable mean recovery levels are within the range 70 to 120%.

Procedural recoveries

The recovery of the method was also determined during the analysis of the test item samples in all studies using this method. The fortification levels used are similar to the levels found

in the test samples. The procedural recoveries for each study using this method are given below.

Study	Fortification Levels (mg/kg)	Recoveries % (Mean)
KCA 8.1.1.3/01 TPW-0018 KCA 8.1.1.3/02 TPW-0019	50	79, 128, 86, 111, 99, 92, 103 (99.7)
	2000	101, 108, 109, 107, 103, 105, 98 (104.4)
KCA 8.1.1.2/03 TPW-0071	50	93.2, 93.8 (93.5)
	1000	92.3, 98.6 (95.5)

The recovery of the method was supported by the procedural recoveries provided in each study at all fortification levels (it is noted that TPW-0018 and TPW-0019 were performed together).

Stability of extracts and standards

The stability of standards and final extracts has not been addressed.

LOQ

It is noted that the applicant claims an LOQ of 31.3 mg/kg based on the lowest analytical standard. However, this is not acceptable way to derive the LOQ.
The limit of quantitation (LOQ), defined as the lowest fortification level where acceptable precision and accuracy data were obtained, has been demonstrated to be 50.0 mg/kg

Conclusion

The analytical method is not acceptably validated according to SANTE/2020/12830 rev. 1 for the determination of the active substance inpyrfluxam in avian feed from the Mallard and Northern Bobwhite reproduction studies as the matrix effects have not been determined and the stability of standards and extracts has not been addressed. However, the studies were generated prior to the implementation of SANTE 2020/12830 rev.1 and SANCO 3029/99 rev.4 did not require matrix effects and the stabilities of standards and extracts to be addressed. As all other validation requirements have been met (including the minimum validation requirements outlined in SANTE 2020/12830 rev.1), the method is fit for purpose.

Reference:	KCA 4.1.2/39
Report Title:	S-2399 – Fish Short-Term Reproduction Assay with Fathead Minnow (*Pimephales promelas*)
Author(s) & Year:	[redacted] 2021
Document No, Authority registration No	Study No.: 13048.7149 Report no.: TPW-0137
Guideline(s):	SANCO/3029/99 rev.4 OCSPP 860.1340
Deviations:	No
GLP or GEP:	Yes
Acceptability:	Yes
Study relied upon:	Yes

The purpose of the study was to demonstrate the validity of a HPLC-MS/MS method for the determination of inpyrfluxam in aqueous solutions.

Principle of the method

The samples are diluted 50:50 with methanol. Further dilutions can be performed as necessary with 50:50 methanol: laboratory well water (v/v) (LOQ samples were diluted by a factor of 2 and 'high' samples were diluted by a factor of 571. The samples were analysed by HPLC-MS/MS under the conditions shown below.

HPLC conditions

Column Waters XBridge BEH C18, 2.5 µm, 2.1 mm x 50 mm.

Mobile phase A = 0.1% acetic acid in purified reagent water
B = 100% methanol

Time (minutes)	Flow rate (mL/min)	%A	%B
0.10	0.400	50.0	50.0
0.50	0.400	50.0	50.0
5.00	0.400	10.0	90.0
6.00	0.400	10.0	90.0
6.10	0.400	50.0	50.0
7.10	0.400	50.0	50.0

Column temperature 40 °C
Injection volume 50.0 µL
Retention time ~3.8 minutes

MS conditions

Instrument MDX Sciex API 5000 mass spectrometer
Ionization Mode Positive ESI
Ion Spray Voltage 4500 V
Scan Type MRM
Q1/Q3 Masses 334.0/238.0 da
Dwell Time 500 milliseconds
Source temperature 400 °C

Table B.5.1.2.6-3: Summary of method validation for the determination of inpyrfluxam in aqueous solutions

Analyte	LOQ (µg/L)	Recovery fortification level (µg/L)	Recoveries % range (mean)	Repeatability % RSD (n)	Linearity	Specificity
Inpyrfluxam	0.100	0.100	108 – 111 (110) N = 5	1.39 @ ~0.1 µg/L (n=5)	0.0250 – 0.250 µg/L (n = 6*2†) y = 293198.7x - 1104.2 R^2 = 0.998	Retention time match of fortified sample to the reference standard. No significant interfering peaks observed in the blank matrix.
		100	108 – 110 (108)	0.882 @ ~100 µg/L (n=5)		

			N = 5			

†One outlier excluded by Q test.

Matrix effects

Matrix effects were determined by measuring the primary peak area with both matrix-matched standards and solvent standards. The matrix effects were not significant (<20%). Furthermore, the linearity was determined using matrix-matched calibration standards.

Specificity

Specificity was demonstrated by retention time match with a reference standard and the absence of significant (>30% LOQ) interfering peaks in the chromatogram of a blank sample. The reference standard was also compared to the fortified samples where a similar retention time match was observed.

Linearity

Linearity was demonstrated by the analysis of six standards of increasing concentration in duplicate. The range of standard concentrations used was 0.0250 – 0.250 µg/L. The concentrations extend over an appropriate range, and the response was linear with a coefficient of determination (R^2) of 0.998. Samples were further diluted to within linear range. It is noted that one of the determinations at 0.250 µg/L was excluded after it was identified as an outlier by a Dixon's Q test. No further consideration is required.

Precision (repeatability)

The precision of the method was assessed via analysis of the accuracy samples. The reported %RSDs were <20%.

Accuracy (recovery)

The accuracy of the method was assessed by analysing five samples fortified with inpyrfluxam at concentrations of 0.1 and 100 µg/L. The samples were diluted to fit within the linear range, the lower fortification level was diluted by 2 and the higher fortification level was diluted by 571. Recovery values were calculated as a percentage of measured concentration relative to fortified concentration. Acceptable mean recovery levels are within the range 70 to 120%.

Procedural recoveries

The recovery of the method was also determined during the analysis of the test item samples in both studies using this method. The fortification levels used are similar to the levels found in the test samples. The procedural recoveries for each study using this method are given below.

Study	Fortification Levels (µg/L)	Recoveries % (Mean)
KCA 8.1.4/01 Report no.: TPW-0138	2.00	96.5, 105, 110, 118 (107.4)
	14.0	97.4, 105, 109, 116 (106.9)
	45.0	95.0, 98.7, 109, 110 (103.2)
KCA 8.2.3/01 Report no.: TPW-0137	1.00	96.4, 103, 103, 111 (103.4)
	6.30	98.0, 98.9, 102, 106 (101.2)
	20.0	101, 102, 105, 913[1] (102.7)

[1]This result is likely due to a fortification error. The other samples at this fortification level are acceptable. This has been excluded from the mean.

The mean of the recoveries from both studies ranged from 101.2-107.4% and therefore considered acceptable.

Stability of extracts and standards

In study TPW-0137, the procedural recovery samples were stored under the same conditions with the final extracts and analysed at the same time, as the procedural recoveries are acceptable, the stability of final sample extracts has been demonstrated.

The stability of standards and stock solutions has not been addressed.

LOQ

The limit of quantitation (LOQ), defined as the lowest fortification level where acceptable precision and accuracy data were obtained, has been demonstrated to be 0.1 µg/L.

Conclusion

The analytical method is not acceptably validated according to SANTE/2020/12830 rev. 1 for the determination of inpyrfluxam in aqueous solutions from the fish short-term reproduction assay with Fathead Minnow (*Pimephales promelas*) and the amphibian

metamorphosis assay with African Clawed Frog (*Xenopus laevis*) studies due to the stability of standards and stock solutions has not been addressed. However, the studies were generated prior to the implementation of SANTE 2020/12830 rev.1 and SANCO 3029/99 rev.4 did not require stabilities of standards and extracts to be addressed. As all other validation requirements have been met (including the minimum validation requirements outlined in SANTE 2020/12830 rev.1), the method is fit for purpose.

Reference:	KCA 4.1.2/34
Report Title:	S-2399 TG - Acute Toxicity Test with Rainbow Trout (Oncorhynchus mykiss) Under Static Conditions Following OECD Guideline #203
Author(s) & Year:	[redacted] 2014a
Document No, Authority registration No	Study No.: 13048.6776 Report no.: TPW-0006
Guideline(s):	SANCO/3029/99 rev.4
Deviations:	No
GLP or GEP:	Yes
Acceptability:	Yes
Study relied upon:	Yes

The purpose of the study was to demonstrate the validity of a HPLC-UV method for the determination of inpyrfluxam in freshwater.

Principle of the method

Aqueous test samples are diluted with methanol then further diluted with methanol/water (reconstituted for hardness, 50/50, v/v), as necessary. The samples are analysed by HPLC-MS/MS under the conditions shown below.

HPLC conditions

Column XBridge C18, 2.5 µm, 2.1 mm x 50 mm.

Mobile phase A = 0.1% acetic acid in purified reagent water
B = 100% methanol

Time (minutes)	Flow rate (µL/min)	%A	%B
0.01	400	50.0	50.0
0.50	400	50.0	50.0
5.00	400	10.0	90.0
5.10	400	50.0	50.0
7.00	400	50.0	50.0

Column temperature 40.0 °C
Injection volume 15.0 µL
Retention time ~4.3 minutes

MS conditions

Instrument MDS Sciex API 3000 mass spectrometer
Ionization Mode Positive ESI
Ion Spray Voltage 4500 V
Scan Type MRM
Q1/Q3 Masses 334.00/238.00 amu
Dwell Time 500 milliseconds
Source temperature 400 °C

Table B.5.1.2.6-4: Summary of method validation for the determination of inpyrfluxam in freshwater

Analyte	LOQ (µg/L)	Recovery fortification level (µg/L)	Recoveries % range (mean)	Repeatability % RSD (n)	Linearity	Specificity
Inpyrfluxam	0.6	0.6	102 – 106 (104) N = 5	1.88 @ ~0.6 µg/L (n=5)	0.1 – 2.00 µg/L (n = 6*2) y = 42796x – 765.2 R^2 = 0.99299	Retention time match of fortified sample to the reference standard. No significant interfering peaks observed in the blank matrix.
		10000	106 – 113 (110) N = 5	2.62 @ ~10000 µg/L (n=5)		

Matrix Effects

The matrix effects have not been addressed.

Specificity

Specificity was demonstrated by retention time match with a reference standard and the absence of significant (>30% LOQ) interfering peaks in the chromatogram of a blank sample. The reference standard was also compared to the fortified samples where a similar retention time match was observed.

Linearity

Linearity was demonstrated by the analysis of six standards of increasing concentration in duplicate. The range of standard concentrations used was 0.1 – 2.00 µg/L. The concentrations extend over an appropriate range, and the response was linear with a coefficient of determination (R^2) of 0.99299. If necessary, samples were further diluted to within linear range.

Precision (repeatability)

The precision of the method was assessed via analysis of the accuracy samples. The reported %RSDs were <20%.

Accuracy (recovery)

The accuracy of the method was assessed by analysing five samples fortified with inpyrfluxam at concentrations of 0.6 and 10000 µg/L. The samples were diluted to fit within the linear range, the lower fortification level was diluted by two and the higher fortification level was diluted by 7500. Recovery values were calculated as a percentage of measured concentration relative to fortified concentration. Acceptable mean recovery levels are within the range 70 to 120 %.

Procedural Recoveries

The recovery of the method was also determined during the analysis of the test item samples in all studies using this method. The fortification levels used are similar to the levels found in the test samples. The procedural recoveries for each study using this method are given below.

Study	Fortification Levels	Recoveries % (Mean)
KCA 8.2.1/01 TPW-0006	0.0120 mg/L	105, 106 (105.5)
	0.0600 mg/L	109, 118 (113.5)

	0.150 mg/L	106, 113 (109.5)
KCA 8.2.1/02 TPW-0003	0.0120 mg/L	109, 114 (111.5)
	0.0400 mg/L	109, 114 (111.5)
	0.150 mg/L	109, 113 (111)
KCA 8.2.1/03 TPW-0004	0.0120 mg/L	111, 114 (112.5)
	0.0600 mg/L	111, 115 (113)
	0.150 mg/L	110, 119 (114.5)
KCA 8.2.1/04 TPW-0005	0.0120 mg/L	111, 115 (113)
	0.0400 mg/L	115, 115 (115)
	0.150 mg/L	109[1]
KCA 8.2.1/05 TPW-0014	0.0300 mg/L	101, 108 (104.5)
	0.250 mg/L	103, 108 (105.5)
	1.00 mg/L	104, 106 (105)
KCA 8.2.2.1/01 TPW-0012	0.600 µg/L	105, 111, 111, 112, 113, 116 (111.3)
	3.00 µg/L	104, 105, 107, 112, 113, 114 (109.2)
	12.0 µg/L	96.9, 101, 103, 105, 109, 111 (104.3)
KCA 8.2.2.1/02 TPW-0085	4.00 µg/L	104, 104, 106, 106, 106, 114, 120 (108.6)
	30.0 µg/L	97.0, 97.7, 98.0, 98.6, 99.1, 104, 108 (100.3)
	120 µg/L	92.8, 97.0, 97.8, 102, 102, 103, 107 (100.1)
KCA 8.2.4.1/01 TPW-0002	0.0470 mg/L	104, 114 (109)
	0.380 mg/L	105, 108 (106.5)
	3.00 mg/L	101, 111 (106)
KCA 8.2.4.2/01 TPW-0013	0.100 mg/L	103,112 (107.5)
	0.750 mg/L	105, 113 (109)
	3.00 mg/L	104,107

		(105.5)
KCA 8.2.5.1/01 TPW-0007	0.0150 mg/L	108, 109, 110, 112, 113, 119 (111.8)
	0.130 mg/L	107, 110, 113, 115, 116, 118 (113.2)
	0.500 mg/L	103, 109, 110, 113, 115, 117 (111.2)
KCA 8.2.5.2/01 TPW-0041	0.0100 mg/L	89.7, 105, 110, 111, 115 (106.1)
	0.0800 mg/L	89.7, 96.9, 100, 102, 102 (98.1)
	0.360 mg/L	99.0, 103, 106, 109, 117 (106.8)
KCA 8.2.5.4/01 TPW-0031	0.000600 mg/L	98.9, 113, 119 (110.3)
	1.00 mg./L	87, 116, 119 (107.3)
	10.0 mg/L	117, 118, 128 (121)
KCA 8.2.5.4/02 TPW-0034	0.000600 mg/L	105, 117, 119 (113.7)
	1.00 mg/L	115, 116, 118 (116.3)
	10.0 mg/L	106, 119, 120 (115)
KCA 8.2.5.4/03 TPW-0086	0.002 mg/L	91.8, 97.2, 101 (96.7)
	1.00 mg/L	101, 103, 106 (103.3)
	10.0 mg/L	102, 106, 124 (110.7)
KCA 8.2.6.1/01[2,3] TPW-0017	0.00100 mg/L	82.5
	0.00750 mg/L	80.8
	1.00 mg/L	98.2, 108 (103.1)
	7.50 mg/L	113
	7.52 mg/L	96.1
	30.0 mg/L	98.5, 99.2, 117 (104.9)
KCA 8.2.6.2/01[4] TPW-0020	0.0140 mg/L	102, 111, 125 (112.7)
	0.920 mg/L	80.1, 101, 114 (98.4)
	30.0 mg/L	106, 107, 125 (112.7)
KCA 8.2.6.2/03 TPW-0030	0.150 mg/L	105, 109, 117 (110.3)
	1.50 mg/L	102, 106, 116 (106.7)
	7.40 mg/L	97.7, 102, 118 (105.9)
KCA 8.2.7/01 TPW-0022	1.50 mg/L	101, 106, 116, 117 (110)
	13.0 mg/L	103, 103, 114, 116 (109)

	50.0 mg/L	105, 111, 115, 122 (113.3)
KCA 8.2.8/01 TPW-0021	0.0300 mg/L	101, 114 (107.5)
	0.250 mg/L	105, 116 (110.5)
	1.00 mg/L	104, 107 (105.5)
KCA 8.3.1.3/01 TPW-0066	4.67 mg/mL	119
	38.2 mg/mL	111
	148 mg/mL	113

[1]Peak area of second QC sample was outside the standard curve due to error in processing therefore it will not be reported.
[2]There are an extra three recovery levels for this study as the concentrations were adjusted for the 96-hour analysis to more closely match the exposure concentrations.
[3]Six of the samples were uncentrifuged while three of the samples were centrifuged. The recoveries of the centrifuged samples were within the range 80.8-117%. The recoveries of the uncentrifuged samples were within the range 96.1-99.2%.
[4]Six of the samples were uncentrifuged while three of the samples were centrifuged. The recoveries of the centrifuged samples were within the range 80.1-106%. The recoveries of the uncentrifuged samples were within the range 101-125%.

The recovery of the method was supported by the procedural recoveries provided in each study at all fortification levels.

Stability of extracts and standards

The stability of extracts and standards has not been addressed.

LOQ

The limit of quantitation (LOQ), defined as the lowest fortification level where acceptable precision and accuracy data were obtained, has been demonstrated to be 0.6 µg/L.

Conclusion

The analytical method is not acceptably validated according to SANTE/2020/12830 rev. 1 for the determination of the active substance inpyrfluxam in freshwater as the matrix effects have not been determined and the stability of standards and extracts has not been addressed. However, the studies were generated prior to the implementation of SANTE 2020/12830 rev.1 and SANCO 3029/99 rev.4 did not require matrix effects and the stabilities of standards and extracts to be addressed. As all other validation requirements have been met (including the minimum validation requirements outlined in SANTE 2020/12830 rev.1), the method is considered to be fit for purpose.

Reference:	KCA 4.1.2/35
Report Title:	Acute Toxicity Study of S-2399 TG with Guppy (Poecilia reticulata)
Author(s) & Year:	[redacted] 2016a
Document No, Authority registration No	Study No.: 1603EFAG Report No.: TPW-0053
Guideline(s):	SANCO/3029/99 rev.4 OPPTS 860.1340
Deviations:	No
GLP or GEP:	Yes
Acceptability:	Yes
Study relied upon:	Yes

The purpose of the study was to demonstrate the validity of a HPLC-UV method for the determination of inpyrfluxam in water.

Principle of the method

A 4.00 mL aliquot of test solution was taken into a 5 mL volumetric flask and diluted to volume with acetonitrile. The samples can be diluted with solvent mixture (1:4 acetonitrile: water (v:v)) if needed. The solution was filtered through a disk filter (0.45 µm). The samples are analysed by HPLC-UV under the conditions shown below.

HPLC conditions

Column Develosil ODS-UG-5, 4.6 mm x 150 mm

Mobile phase A = Distilled Water
B = Acetonitrile

Time (minutes)	%A	%B
0	50	50
15	50	50
15.01	10	90

25	10	90
25.01	50	50
35	50	50

Column temperature 40 °C
Injection volume 100 µL
Flow Rate 1 mL/min
Detector UV 264 nm
Run time 35 minutes
Retention time ~13.1 minutes

Table B.5.1.2.6-5: Summary of method validation for the determination of inpyrfluxam in water

Analyte	LOQ (mg/L)	Recovery fortification level (mg/L)	Recoveries % range (mean)	Repeatability % RSD (n)	Linearity	Specificity
Inpyrfluxam	0.0200	0.0200	90.5 – 97.5 (94.9) N = 5	3.24 @ ~0.0200 mg/L (n=5)	0.01 – 0.5 mg a.i./L (n = 5) y = 164929x – 83.6 R^2 = 1	Retention time match of fortified sample to the reference standard. No significant interfering peaks observed in the blank matrix.
		5.00	96.2 – 98.2 (97.2) N = 5	0.913 @ ~5.00 mg/L (n=5)		

Matrix Effects

The matrix effects have not been addressed.

Specificity

Specificity was demonstrated by retention time match with a reference standard and the absence of significant (>30% LOQ) interfering peaks in the chromatogram of a blank

sample. The reference standard was also compared to the fortified samples where a similar retention time match was observed.

Linearity

Linearity was demonstrated by the analysis of five standards of increasing concentration. The range of standard concentrations used was 0.0100 – 0.500 mg/L. The concentrations extend over an appropriate range, and the response was linear with a coefficient of determination (R^2) of 1. The calibration curve was replotted as no y-intercept was reported. If necessary, samples were further diluted to within linear range.

Precision (repeatability)

The precision of the method was assessed via analysis of the accuracy samples. The reported %RSDs were <20%. System precision was determined at 0.0500 mg/L by injecting the same solution five times into the HPLC, this has been excluded from the table above.

Accuracy (recovery)

The accuracy of the method was assessed by analysing five samples fortified with inpyrfluxam at concentrations of 0.0200 and 5.00 mg/L. The higher fortification level samples were diluted by a factor of 20 to fit within the linear range. Recovery values were calculated as a percentage of measured concentration relative to fortified concentration. Acceptable mean recovery levels are within the range 70 to 120 %.

Procedural recoveries

The recovery of the method was also determined during the analysis of the test item samples in all studies using this method. The fortification levels used are similar to the levels found in the test samples. The procedural recoveries for each study using this method are given below.

Study	Fortification Levels (mg/L)	Recoveries %
KCA 8.2.1/06 TPW-0053	0.0200	96.0
	5.00	102
KCA 8.2.1/07 TPW-0054	0.0200	89.5
	5.00	102
KCA 8.2.1/08 TPW-0055	0.0200	95.5
	5.00	102

The recovery of the method was supported by the procedural recoveries provided in each study at all fortification levels.

Stabilities of extracts and standards

In report TPW-0053, the procedural recovery samples were stored under the same conditions with the final extracts and analysed at the same time, as the procedural recoveries are acceptable, the stability of final sample extracts has been demonstrated.

The stability of standards and stock solutions has not been addressed.

LOQ

The limit of quantitation (LOQ), defined as the lowest fortification level where acceptable precision and accuracy data were obtained, has been demonstrated to be 0.02 mg/L.

Conclusion

The analytical method is not acceptably validated according to SANTE/2020/12830 rev. 1 for the determination of the active substance inpyrfluxam in water as the matrix effects have not been determined and the stability of standards has not been addressed. However, the studies were generated prior to the implementation of SANTE 2020/12830 rev.1 and SANCO 3029/99 rev.4 did not require matrix effects and the stabilities of standards to be addressed. As all other validation requirements have been met (including the minimum validation requirements outlined in SANTE 2020/12830 rev.1), the method is considered to be fit for purpose.

Reference:	KCA 4.1.2/36
Report Title:	Acute Toxicity Study of 3'-OH-S-2840 with Rainbow trout (*Oncorhynchus mykiss*)
Author(s) & Year:	[redacted] 2016a
Document No, Authority registration No	Study No.: 1512EFAR Report No.: TPW-0035
Guideline(s):	SANCO/3029/99 rev.4 OPPTS 860.1340
Deviations:	No
GLP or GEP:	Yes
Acceptability:	Yes
Study relied upon:	Yes

The purpose of the study was to demonstrate the validity of a HPLC-UV method for the determination of 3'-OH-S-2840 in aquatic test solutions.

Principle of the method

A 4.00 mL aliquot of test solution was taken into a 5 mL volumetric flask and diluted to volume with acetonitrile. The samples can be diluted with solvent mixture (1:4 acetonitrile: water (v:v)) if needed. The solution was filtered through a disk filter (0.45 µm). The samples are analysed by HPLC-UV under the conditions shown below.

HPLC conditions

Column Develosil ODS-UG-5, 4.6 mm x 150 mm

Mobile phase A = 0.1 % (v/v) trifluoroacetic acid aqueous solution
B = Acetonitrile

Time (minutes)	%A	%B
0	50	50
20.0	10	90
20.1	50	50
30	50	50

Column temperature 40 °C
Injection volume 25 µL
Flow Rate 1 mL/min
Detector UV 254 nm
Run time 30 minutes
Retention time ~7.8 minutes

Table B.5.1.2.6-6: Summary of method validation for the determination of 3'-OH-S-2840 in aquatic test solutions

Analyte	LOQ (mg/L)	Recovery fortification level (mg/L)	Recoveries % range (mean)	Repeatability % RSD (n)	Linearity	Specificity
3'-OH-S-2840	0.100	0.100	97.9 – 104 (99.6) N = 5	2.50 @ ~0.100 mg/L (n=5)	0.05 – 2.50 mg/L (n = 5) y = 53963x + 57.3 R^2 = 1	Retention time match of fortified sample to the reference standard. No significant interfering peaks observed in
		10.0	98.4 – 99.5 (99.1) N = 5	0.437 @ ~10.0 mg/L (n=5)		

						the blank matrix.

Matrix Effects

The matrix effects have not been addressed.

Specificity

Specificity was demonstrated by retention time match with a reference standard and the absence of significant (>30% LOQ) interfering peaks in the chromatogram of a blank sample. The reference standard was also compared to the fortified samples where a similar retention time match was observed.

Linearity

Linearity was demonstrated by the analysis of five standards of increasing concentration. The range of standard concentrations used was 0.05 – 2.50 mg/L. The concentrations extend over an appropriate range, and the response was linear with a coefficient of determination (R^2) of 1. The calibration curve was replotted as no y-intercept was reported. If necessary, samples were further diluted to within linear range.

Precision (repeatability)

The precision of the method was assessed via analysis of the accuracy samples. The reported %RSDs were <20%. System precision was determined at 0.250 mg/L by injecting the same solution five times into the HPLC, this has been excluded from the table above.

Accuracy (recovery)

The accuracy of the method was assessed by analysing five samples fortified with 3'-OH-S-2840 at concentrations of 0.100 and 10.0 mg/L. The higher fortification level samples were diluted by a factor of 10 to fit within the linear range. Recovery values were calculated as a percentage of measured concentration relative to fortified concentration. Acceptable mean recovery levels are within the range 70 to 120 %.

Procedural recoveries

Procedural recoveries were conducted using fortification levels of 0.100 and 10.0 mg/L. The recoveries were 99.5 and 99.9% respectively. The procedural recoveries are acceptable at both fortification levels.

Stabilities of extracts and standards

In report TPW-0035, the procedural recovery samples were stored under the same conditions with the final extracts and analysed at the same time, as the procedural recoveries are acceptable, the stability of final sample extracts has been demonstrated.

The stability of standards and stock solutions has not been addressed.

LOQ

The limit of quantitation (LOQ), defined as the lowest fortification level where acceptable precision and accuracy data were obtained, has been demonstrated to be 0.100 mg/L.

Conclusion

The analytical method is not acceptably validated according to SANTE/2020/12830 rev. 1 for the determination of 3'-OH-S-2840 in aquatic test solutions as the matrix effects have not been determined and the stability of standards has not been addressed. However, the studies were generated prior to the implementation of SANTE 2020/12830 rev.1 and SANCO 3029/99 rev.4 did not require matrix effects and the stabilities of standards to be addressed. As all other validation requirements have been met (including the minimum validation requirements outlined in SANTE 2020/12830 rev.1), the method is considered to be fit for purpose.

Reference:	KCA 4.1.2/37
Report Title:	Acute Toxicity Study of 1'-COOH-S-2840 with Rainbow trout (*Oncorhynchus mykiss*)
Author(s) & Year:	[redacted] 2016b
Document No, Authority registration No	Study No.: 1513EFAR Report No.: TPW-0036
Guideline(s):	SANCO/3029/99 rev.4 OPPTS 860.1340
Deviations:	No
GLP or GEP:	Yes
Acceptability:	Yes

Study relied upon:	Yes

The purpose of the study was to demonstrate the validity of a HPLC-UV method for the determination of 1'-COOH-S-2840 in aquatic test solutions.

Principle of the method

A 4.00 mL aliquot of test solution was taken into a 5 mL volumetric flask and diluted to volume with acetonitrile. The samples can be diluted with solvent mixture (1:4 acetonitrile: water (v:v)) if needed. The solution was filtered through a disk filter (0.45 µm). The samples are analysed by HPLC-UV under the conditions shown below.

HPLC conditions

Column Develosil ODS-UG-5, 4.6 mm x 150 mm

Mobile phase A = 0.1 % (v/v) trifluoroacetic acid aqueous solution
B = Acetonitrile

Time (minutes)	%A	%B
0	70	30
20.0	30	70
20.1	70	30
30	70	30

Column temperature 40 °C

Injection volume 50 µL

Flow Rate 1 mL/min

Detector UV 254 nm

Run time 30 minutes

Retention time ~10.5 and 11.7 minutes

Table B.5.1.2.6-7: Summary of method validation for the determination of 1'-COOH-S-2840 in aquatic test solutions

Analyte	LOQ (mg/L)	Recovery fortification level (mg/L)	Recoveries % range (mean)	Repeatability % RSD (n)	Linearity	Specificity
1'-COOH-S-2840	1.00	1.00	99.3 – 99.9 (99.7) N = 5	0.250 @ ~1.00 mg/L (n=5)	0.500 – 25.0 mg/L (n = 5) y = 82099x + 96.98 R^2 = 1	Retention time match of fortified sample to the reference standard. No significant interfering peaks observed in the blank matrix.
		100	98.5 – 102 (99.7) N = 5	1.36 @ ~100 mg/L (n=5)		

Matrix Effects

The matrix effects have not been addressed.

Specificity

Specificity was demonstrated by retention time match with a reference standard and the absence of significant (>30% LOQ) interfering peaks in the chromatogram of a blank sample. The reference standard was also compared to the fortified samples where a similar retention time match was observed.

Linearity

Linearity was demonstrated by the analysis of five standards of increasing concentration. The range of standard concentrations used was 0.500 – 25.0 mg/L. The concentrations extend over an appropriate range, and the response was linear with a coefficient of determination (R^2) of 1. The calibration curve was replotted as no y-intercept was reported. If necessary, samples were further diluted to within linear range.

Precision (repeatability)

The precision of the method was assessed via analysis of the accuracy samples. The reported %RSDs were <20%. System precision was determined at 2.50 mg/L by injecting the same solution five times into the HPLC, this has been excluded from the table above.

Accuracy (recovery)

The accuracy of the method was assessed by analysing five samples fortified with 1'-COOH-S-2840 at concentrations of 1.00 and 100 mg/L. The higher fortification level samples were diluted by a factor of 10 to fit within the linear range. Recovery values were calculated as a percentage of measured concentration relative to fortified concentration. Acceptable mean recovery levels are within the range 70 to 120 %.

Procedural recoveries

Procedural recoveries were conducted using fortification levels of 1.00 and 100 mg/L. The recoveries were 101 and 102% respectively. The procedural recoveries are acceptable at both fortification levels.

Stabilities of extracts and standards

In study TPW-0036, the procedural recovery samples were stored under the same conditions with the final extracts and analysed at the same time, as the procedural recoveries are acceptable, the stability of final sample extracts has been demonstrated.

The stability of standards and stock solutions has not been addressed.

LOQ

The limit of quantitation (LOQ), defined as the lowest fortification level where acceptable precision and accuracy data were obtained, has been demonstrated to be 1.00 mg/L.

Conclusion

The analytical method is not acceptably validated according to SANTE/2020/12830 rev. 1 for the determination of the 1'-COOH-S-2840 in in aquatic test solutions as the matrix effects have not been determined and the stability of standards has not been addressed. However, the studies were generated prior to the implementation of SANTE 2020/12830 rev.1 and SANCO 3029/99 rev.4 did not require matrix effects and the stabilities of standards to be addressed. As all other validation requirements have been met (including the minimum validation requirements outlined in SANTE 2020/12830 rev.1), the method is considered to be fit for purpose.

Reference:	KCA 4.1.2/38
Report Title:	Life-Cycle Toxicity Test Exposing Midges (Chironomus dilutus) to S-2399 TG Applied to Sediment Under Static-Renewal Conditions Following EPA Test Methods
Author(s) & Year:	[redacted] 2015
Document No, Authority registration No	Study No.: 12709.6364 Report No.: TPW-0031
Guideline(s):	SANCO/3029/99 rev.4
Deviations:	No
GLP or GEP:	Yes
Acceptability:	Yes
Study relied upon:	Yes

The purpose of the study was to demonstrate the validity of a HPLC-UV method for the determination of inpyrfluxam in sediment.

Principle of the method

Sediment samples (5.00 g dry weight) are extracted with 20 mL of solvent mixture (acetonitrile/purified reagent water/formic acid (90:10:0.1 v/v/v)) on a shaker table for 30 minutes at 150 rpm. Samples were then centrifuged at 3000 rpm for 10 minutes. The extracts were transferred to 50 mL volumetric flasks. The extraction and centrifugation procedures were repeated with an additional 20 mL aliquot of solvent mixture. The extracts were combined, filled to volume with solvent mixture and mixed well. The samples were analysed by HPLC-UV under the conditions given below. Samples were diluted into calibration standard range with 50:50 methanol:purified reagent water (v:v).

HPLC conditions

Column	XBridge C18, 2.5 µm, 2.1 mm x 50 mm.
Mobile phase	A = 0.1% acetic acid in purified reagent water

B = 100% methanol

Time (minutes)	Flow rate (mL/min)	%A	%B
0.01	0.400	50.0	50.0
0.50	0.400	50.0	50.0
5.00	0.400	10.0	90.0
5.10	0.400	50.0	50.0
7.00	0.400	50.0	50.0

Column temperature 40.0 °C
Injection volume 20.0 µL
Retention time ~4.3 minutes

MS conditions

Instrument MDS Sciex API 3000 mass spectrometer
Ionization Mode Positive ESI
Ion Spray Voltage 4500 V
Scan Type MRM
Q1/Q3 Masses 334.00/238.00 amu
Dwell Time 500 milliseconds
Source temperature 400 °C

Table B.5.1.2.6-8: Summary of method validation for the determination of inpyrfluxam in sediment

Analyte	LOQ (µg/kg)	Recovery fortification level (µg/kg)	Recoveries % range (mean)	Repeatability % RSD (n)	Linearity	Specificity
Inpyrfluxam	200	200	85.8 – 88.5 (87.0) N = 5	1.35 @ ~200 µg/kg (n=5)	0.1 – 2.00 µg/L (n = 6*2) y = 8213.5x – 84.7 R^2 = 0.99950	Retention time match of fortified sample to the reference standard. No significant interfering peaks observed in
		100,000	96.2 – 101 (98.6) N = 5	1.74 @ ~100,000 µg/kg (n=5)		

						the blank matrix.

Matrix Effects

The matrix effects have not been addressed.

Specificity

Specificity was demonstrated by retention time match with a reference standard and the absence of significant (>30% LOQ) interfering peaks in the chromatogram of a blank sample. The reference standard was also compared to the fortified samples where a similar retention time match was observed.

Linearity

Linearity was demonstrated by the analysis of six standards of increasing concentration in duplicate. The range of standard concentrations used was 0.1 – 2.00 µg/L. The concentrations extend over an appropriate range, and the response was linear with a coefficient of determination (R^2) of 0.99950. If necessary, samples were further diluted to within linear range.

Precision (repeatability)

The precision of the method was assessed via analysis of the accuracy samples. The reported %RSDs were <20%.

Accuracy (recovery)

The accuracy of the method was assessed by analysing five samples fortified with inpyrfluxam at concentrations of 200 and 100,000 µg/kg. The samples were diluted with methanol/purified water (50:50 v/v) to fit within the linear range. The lower fortification level was diluted by 160 and the higher fortification level was diluted by 80,000. Recovery values were calculated as a percentage of measured concentration relative to fortified concentration. Acceptable mean recovery levels are within the range 70 to 120 %.

Procedural Recoveries

The recovery of the method was also determined during the analysis of the test item samples in all studies using this method. The fortification levels used are similar to the levels found in the test samples. The procedural recoveries for each study using this method are given below.

Study	Fortification Levels	Recoveries % (Mean)
KCA 8.2.5.4/01 TPW-0031	0.5 mg/kg	97.8, 103, 118 (106.3)
	10.0 mg/kg	104, 108, 113 (108.3)
	100 mg/kg	101, 108, 111 (106.7)
KCA 8.2.5.4/02 TPW-0034	3.00 mg/kg	88, 112, 113 (104.3)
	25.0 mg/kg	98.9, 114, 116 (109.6)
	100 mg/kg	92.1, 116, 119 (109.0)
KCA 8.2.5.4/03 TPW-0086	0.340 mg/kg	81.1, 83.9, 90.2, 104 (89.8)
	8.00 mg/kg	69.4, 89.9, 90.4, 96.8 (86.6)
	80.0 mg/kg	65.2, 90.4, 95.5, 96.8 (87.0)

The recovery of the method was supported by the procedural recoveries provided in each study at all fortification levels.

Stability of extracts and standards

The stability of extracts and standards has not been addressed.

LOQ

The limit of quantitation (LOQ), defined as the lowest fortification level where acceptable precision and accuracy data were obtained, has been demonstrated to be 200 µg/kg.

Conclusion

The analytical method is not acceptably validated according to SANTE/2020/12830 rev. 1 for the determination of the active substance inpyrfluxam in sediment as the matrix effects have not been determined and the stability of extracts and standards has not been addressed. However, the studies were generated prior to the implementation of SANTE 2020/12830 rev.1 and SANCO 3029/99 rev.4 did not require matrix effects and the stabilities of extracts and standards to be addressed. As all other validation requirements have been met (including the minimum validation requirements outlined in SANTE 2020/12830 rev.1), the method is considered to be fit for purpose.

B.5.1.2.7. Methods in water, buffer solutions, organic solvents and any additional matrices resulting from the physical and chemical properties tests

Table B.5.1.2.7-1: Summary of risk assessment methods supporting physical and chemical properties tests

Data point	Study	Conclusion	Studies relied on
KCA 4.1.2/40	S-2399: Determination of Water Solubility. [redacted] 2013a Report No.: TPP-0001	HPLC-UV Analyte: Inpyrfluxam LOQ: 10 mg/L in water. Acceptable.	KCA 2.5 Report No.: TPP-0001
KCA 4.1.2/41	S-2399: Determination of Partition Coefficient (n-Octanol/Water) [redacted] 2013b	HPLC-UV Analyte: Inpyrfluxam LOQ: 0.84 mg/L in water (aqueous phase) 2000 mg/L in n-	KCA 2.7/01 Report No.: TPP-0002

	Report No.: TPP-0002	octanol (organic phase) Acceptable.	
KCA 4.1.2/43	S-2399 TGAI: Method Validation for Determination of S-2399 in Solvent Solubility Test. [redacted] 2021 Report No.: TPA-0085	HPLC-UV Analyte: Inpyrfluxam LOQ: Acetone: 37.5% w/w DCM: 22.5% w/w Ethyl acetate: 22.5% w/w n-octanol: 3.75% w/w Methanol: 22.5% w/w n-hexane: 0.075% w/w Toluene: 3.75% w/w Acceptable	KCA 2.6 Report No.: TPP-0012

Reference:	KCA 4.1.2/40
Report Title:	S-2399: Determination of Water Solubility
Author(s) & Year:	[redacted] 2013a
Document No, Authority registration No	Study No.: 41303367; Report No.: TPP-0001
Guideline(s):	Method EC A.6 (EC 440/2008), Method 105 (OECD), Method 830.7840 (OCSPP)
Deviations:	No
GLP or GEP:	Yes
Acceptability:	Yes
Study relied upon:	Yes

The purpose of the study was to demonstrate the validity of a HPLC-UV method for the determination of inpyrfluxam in water.

Principle of the method

Samples (130 mL) from the solubility test are centrifuged twice at 13500 rpm for 15 minutes. Duplicate aliquots of the supernatant are sampled, excluding excess, undissolved test item. The samples are diluted by a factor of 2 using acetonitrile before being assayed by HPLC-UV.

HPLC conditions

HPLC system	Agilent Technologies 1100
Column	Waters Sunfire C_{18} 5 µm, 250 x 4.6 mm
Mobile phase	Acetonitrile: water (60:40, v/v)
Column temperature	40 °C
Injection volume	25 µL
Flow rate	1 mL/min
Detector wavelength	220 nm
Retention time	~10 minutes

Table B.5.1.2.7-2: Summary of method validation for the determination of inpyrfluxam in water solubility test

Analyte	Recovery fortification level (mg/L)	Recoveries % range (mean)	Repeatability % RSD (n)	Linearity	Specificity
Inpyrfluxam	10	100.4 – 102.7 (101.4)	1.07 @ ~10 mg/L (n=5)	2.5 – 20 mg/L (n = 8) y = 1404000x + 102600 R^2 = 0.9999	Retention time match to reference standard. No significant interfering peaks observed in the blank formulation.
			0.975 @ 14.7 mg/L (n=5)		

Specificity

Specificity was demonstrated by retention time match with a reference standard and the absence of significant (>30% LOQ) interfering peaks in the chromatogram of a matrix

blank sample. Analyte identity was confirmed by comparison of the retention time of the analyte with that of a reference standard. In addition, the UV spectra of the samples were consistent with that of a reference standard.

Linearity

Linearity was demonstrated by the analysis of eight standards of increasing concentration. The range of standard concentrations used was 2.5 – 20 mg/L. The concentrations extend over an appropriate range, and the response was linear with a coefficient of determination (R^2) of 0.9999.

Precision (repeatability)

The precision of the method was assessed via analysis of the accuracy samples. The reported %RSDs were <20%. Five precision samples were also determined at 14.7 mg/L which were reported acceptable precision.

Accuracy (recovery)

The accuracy of the method was assessed by analysing five water samples fortified with pure inpyrfluxam at a concentration of 10 mg/L. Recovery values were calculated as a percentage of measured concentration relative to fortified concentration. Acceptable mean recovery levels are within the range 70 to 120 %.

LOQ

The limit of quantitation (LOQ), defined as the lowest fortification level where acceptable precision and accuracy data were obtained, has been demonstrated to be 10 mg/L.

Conclusion

Based off section 4.1.5 of SANTE/2020/12830 rev. 1, the analytical method is fit for purpose for the determination of the active substance inpyrfluxam in solution from the solubility in water study.

Reference:	KCA 4.1.2/41
Report Title:	S-2399: Determination of Partition Coefficient (n-Octanol/Water)
Author(s) & Year:	[redacted] 2013b
Document No, Authority registration No	Study No.: 41303719; Report No.: TPP-0002
Guideline(s):	Method EC A.8 (EC 440/2008), Method 107 (OECD), Method 830.7550 (OCSPP)
Deviations:	No
GLP or GEP:	Yes
Acceptability:	Yes
Study relied upon:	Yes

The purpose of the study was to demonstrate the validity of a HPLC-UV method for the determination of inpyrfluxam in water and n-octanol.

Principle of the method

Aliquots of aqueous and octanol (organic) phases are separated by centrifugation for 30 minutes at 13500 pm and 25 °C. The organic and aqueous phases are diluted with acetonitrile by a factor of 20 and 2 respectively prior to analysis by HPLC-UV.

HPLC conditions

HPLC system	Agilent Technologies 1200
Column	Waters Sunfire C_{18} 5 µm, 250 x 4.6 mm
Mobile phase	Acetonitrile: water (60:40, v/v)
Column temperature	40 °C
Injection volume	5 µL (stock solution and organic phase analysis) 50 µL (aqueous phase analysis)
Flow rate	1 mL/min
Detector wavelength	220 nm
Retention time	~10 minutes

Table B.5.1.2.7-3: Summary of method validation for the determination of inpyrfluxam in n-octanol/water partition coefficient test

Analyte	Matrix	Recovery fortification level (mg/L)	Recoveries % range (mean)	Repeatability % RSD (n)	Linearity	Specificity
Inpyrfluxam	Organic phase (n-octanol)	2000	97.8 – 100.9 (99.1)	1.27 @ ~2000 mg/L (n=5)	25 – 155 mg/L (n = 7) y = 184800x – 102800 R^2 = 0.9997	Retention time match to reference standard. No significant interfering peaks observed in the blank formulation.
				1.98 @ 1999 mg/L (n=5)		
	Aqueous phase (water)	0.84	100.0 – 101.2 (100.6)	0.45 @ ~0.84 mg/L (n=5)	0.05 – 1.0 mg/L (n = 8) y = 2735000x + 11770 R^2 = 0.9993	Retention time match to reference standard. No significant interfering peaks observed in the blank formulation.
				0.399 @ 0.444 mg/L (n=5)		

Specificity

No significant interferences (>30% of the LOQ) were observed at the retention time of interest in any control matrix samples, demonstrating specificity of the method. Analyte identity was confirmed by retention time match with an analytical standard. Additionally, UV spectra of the organic samples and mass ion detected (m/z 334) in aqueous samples were compared with analytical standards to confirm identity.

Linearity

Organic phase

Linearity was demonstrated by the analysis of seven standards of increasing concentration. The range of standard concentrations used was 25 – 155 mg/L. The

concentrations extend over an appropriate range, and the response was linear with a coefficient of determination (R^2) of 0.9997.

Aqueous phase

Linearity was demonstrated by the analysis of eight standards of increasing concentration. The range of standard concentrations used was 0.5 – 1.0 mg/L. The concentrations extend over an appropriate range, and the response was linear with a coefficient of determination (R^2) of 0.9993.

Precision (repeatability)

The precision of the method was assessed via analysis of the relevant accuracy samples. The reported %RSDs were <20% for both the organic and aqueous phases. Further acceptable precision data was also reported at 1999 mg/L and 0.444 mg/L for organic and aqueous phase respectively.

Accuracy (recovery)

Organic phase

The accuracy of the method was assessed by analysing five test samples fortified with pure inpyrfluxam at a concentration of 2000 mg/L. Recovery values were calculated as a percentage of measured concentration relative to fortified concentration. Acceptable mean recovery levels are within the range 70 to 120 %.

Aqueous phase

The accuracy of the method was assessed by analysing five test samples fortified with pure inpyrfluxam at a concentration of 0.84 mg/L. Recovery values were calculated as a percentage of measured concentration relative to fortified concentration. Acceptable mean recovery levels are within the range 70 to 120 %.

LOQ

The limit of quantitation (LOQ), defined as the lowest fortification level where acceptable precision and accuracy data were obtained, has been demonstrated to be 2000 mg/L and 0.84 mg/L in the organic and aqueous phases respectively.

Conclusion

Based off section 4.1.5 of SANTE/2020/12830 rev. 1, the analytical method is fit for purpose for the determination of the active substance inpyrfluxam in the aqueous and organic phases from the partition coefficient (n-octanol/water) study.

Reference:	KCA 4.1.2/43
Report Title:	S-2399 TGAI: Method Validation for Determination of S-2399 in Solvent Solubility Test
Author(s) & Year:	[redacted] 2021
Document No, Authority registration No	Study No.: 8456378; Report No.: TPA-0085
Guideline(s):	SANCO/3029/99 rev.4
Deviations:	No
GLP or GEP:	Yes
Acceptability:	Yes
Study relied upon:	Yes

The purpose of the study was to demonstrate the validity of a HPLC-UV method for the determination of inpyrfluxam in different solvents.

Principle of the method

Aliquots of inpyrfluxam were weighed into separate 10 mL flasks with prescribed amounts of acetone, dichloromethane (DCM), ethyl acetate or methanol. The flasks were then diluted to volume with acetonitrile. These solutions were then further diluted by a factor of 100 using acetonitrile.

Aliquots of inpyrfluxam were weighed into separate 100 mL flasks with prescribed amounts of *n*-Octanol or toluene. The flasks were then diluted to volume with acetonitrile.

n-Hexane samples were prepared by diluting an aliquot of inpyrfluxam with *n*-Hexane in a 50 mL flask and sonicating until the test item was fully dissolved. A 5 mL aliquot of each sample was pipetted into separate 50 mL round-bottomed flasks and evaporated to dryness using a rotary evaporator (30 °C). Finally, the residues were then dissolved by ultrasonication and swirling in 5 mL of acetonitrile.

For each solvent, 5 replicate samples were prepared at two known levels and analysed by HPLC-UV.

HPLC conditions

HPLC system	Agilent Technologies 1200
Column	Daicel Chiralpak ID 5 µm, 150 x 4.6 mm
Mobile phase	Acetonitrile: purified water (35:60, v/v)
Column temperature	40 °C
Injection volume	10 µL
Flow rate	1 mL/min
Detector wavelength	240 nm
Retention time	~16 minutes

Table B.5.1.2.7-4: Summary of method validation for the determination of inpyrfluxam in the solvent stability test

Analyte	Matrix	Recovery fortification level (%w/w)	Recoveries % range (mean)	Repeatability % RSD (n)	Linearity	Specificity
Inpyrfluxam	Acetone	50	96.8 – 99.7 (98.0)	1.16 @ ~50 %w/w (n=5)	1 – 1000 mg/L n = 7 y = 290890x + 473040 r = 1.000	Retention time match to reference standard. No significant interfering peaks observed in the blank formulation.
		37.5	91.1 – 97.4 (94.6)	2.49 @ ~37.5 %w/w (n=5)		

	DCM	30	94.4 – 97.05 (95.6)	1.07 @ ~30 %w/w (n=5)		Retention time match to reference standard. No significant interfering peaks observed in the blank formulation.
		22.5	92.6 – 94.5 (93.9)	0.8 @ ~22.5 %w/w (n=5)		
	Ethyl acetate	30	95.6 – 97.7 (96.7)	0.78 @ ~30 %w/w (n=5)	1 – 1000 mg/L n = 7 y = 291830x + 363560 r = 1.000	Retention time match to reference standard. No significant interfering peaks observed in the blank formulation.
		22.5	94.2 – 96.2 (95.4)	0.81 @ ~22.5 %w/w (n=5)		
	n-Octanol	5	99.6 – 102.2 (100.9)	1.04 @ ~5 %w/w (n=5)		Retention time match to reference standard. No significant interfering peaks observed in the

		3.75	98.9 – 102.6 (101.2)	1.38 @ ~3.75 %w/w (n=5)		blank formulation.
	Methanol	30	94.9 – 96.5 (95.9)	0.67 @ ~30 %w/w (n=5)		Retention time match to reference standard. No significant interfering peaks observed in the blank formulation.
		22.5	89.1 – 99.8 (94.9)	4.15 @ ~22.5 %w/w (n=5)		
	n-Hexane	0.1 %w/v	94.7 – 103.9 (99.2)	3.66 @ ~1 %w/v (n=5)	1 – 1000 mg/L n = 7 y = 293740x + 115480 r = 1.000	Retention time match to reference standard. No significant interfering peaks observed in the blank formulation.
		0.075 %w/v	96.0 – 100.3 (98.3)	1.66 @ ~0.75 %w/v (n=5)		

	Toluene	5	101.6 – 101.8 (101.7)	0.09 @ ~5 %w/w (n=5)	1 – 1000 mg/L n = 7 y = 293420x + 78721 r = 1.000	Retention time match to reference standard. No significant interfering peaks observed in the blank formulation.
		3.75	101.3 – 103.5 (102.3)	0.97 @ ~3.75 %w/w (n=5)		

Specificity

No significant interferences (>30% of the LOQ) were observed at the retention time of interest in any control matrix samples, demonstrating specificity of the method. Analyte identity was confirmed by retention time match with an analytical standard.

Linearity

Linearity was demonstrated by the analysis of seven standards of increasing concentration. The range of standard concentrations used was 1 – 1000 mg/L. The concentrations extend over an appropriate range, and the response was linear with a correlation coefficient (r) of 1.00 for all matrices.

Precision (repeatability)

The precision of the method was assessed via analysis of the relevant accuracy samples. The reported %RSDs were <20% for all matrices.

Accuracy (recovery)

Acetone

The accuracy of the method was assessed by analysing five test samples fortified with acetone at a concentration of 37.5 %w/w and 50 %w/w. Recovery values were calculated as a percentage of measured concentration relative to fortified concentration. Acceptable mean recovery levels are within the range 70 to 110 %.

DCM, ethyl acetate and methanol

The accuracy of the method was assessed by analysing five test samples fortified with either DCM, ethyl acetate or methanol at a concentration of 22.5 %w/w and 30 %w/w. Recovery values were calculated as a percentage of measured concentration relative to fortified concentration. Acceptable mean recovery levels are within the range 70 to 110 %.

n-octanol and toluene

The accuracy of the method was assessed by analysing five test samples fortified with either *n*-octanol or toluene at a concentration of 3.75 %w/w and 5 %w/w. Recovery values were calculated as a percentage of measured concentration relative to fortified concentration. Acceptable mean recovery levels are within the range 70 to 110 %.

n-hexane

The accuracy of the method was assessed by analysing five test samples fortified with *n*-hexane at a concentration of 0.075 %w/v and 0.1 %w/v. Recovery values were calculated as a percentage of measured concentration relative to fortified concentration. Acceptable mean recovery levels are within the range 70 to 110 %.

Recovery data in study TPP-0012

Fortified solutions were prepared at a nominal concentration of 100 mg/L test item in acetonitrile spiked with each solvent (1.0 %). The mean percentage recovery obtained was found to be acceptable (91.5-106%).

LOQ

The limit of quantitation (LOQ), defined as the lowest fortification level where acceptable precision and accuracy data were obtained, has been demonstrated to be:

- 37.5 %w/w for acetone
- 22.5 %w/w for DCM, ethyl acetate and methanol
- 3.75 %w/w for *n*-octanol and toluene
- 0.075 %w/v for *n*-hexane

Conclusion

Based off section 4.1.5 of SANTE/2020/12830 rev. 1, the analytical method is fit for purpose for the determination of the active substance inpyrfluxam in acetone, DCM, ethyl acetate, methanol, *n*-octanol, toluene and *n*-hexane from the solvent solubility test.

B.5.2. Methods for post-approval control and monitoring purposes

B.5.2.1. Methods for residues in or on food and feed of plant origin

Reference:	KCA 4.2/01
Report Title:	Validation of the Multi-Residue Method QuEChERS for the determination of S-2399 in Matrices of Plant Origin.
Author(s) & Year:	[redacted]
Document No, Authority registration No	Study No.: S16-03372; Report No.: TPA-0027
Guideline(s):	SANCO/825/00 rev. 8.1
Deviations:	No
GLP or GEP:	Yes
Acceptability:	Yes
Study relied upon:	Yes

Report KCA 4.2/01 has been submitted to support the monitoring of residues of inpyrfluxam in/on wheat (grain) (dry/high starch commodity), soybean (seed) (high oil commodity), cucumber (high water commodity) and grapes (high acid commodity).

The standard used for the validation procedure is noted to be 99.9 % R-isomer.

Primary validation was performed by Eurofins Agroscience Services, Hamburg, Germany. The independent laboratory validation reported under KCA 4.2/02 was performed at Eurofins Agroscience Services, Niefern-Öschelbronn, Germany.

Principle of the method

Homogenized samples (5 g wheat grain or soybean seeds, 10 g cucumber or grapes) are weighed into centrifuge tubes (50 mL). Water (10 mL) is added to wheat grain and soybean seeds only and the samples are allowed to soak for 20 minutes at room

temperature. All the samples are extracted with acetonitrile (10 mL) on a platform shaker for 15 minutes before the addition of magnesium sulphate (4.0 g), sodium chloride (1.0 g), trisodium citrate dihydrate (1.0 g) and disodium hydrogen citrate sesquihydrate (0.5 g). The tubes are shaken by hand for approximately 1 minute and centrifuged for 5 minutes at approximately 3200 x g.

For wheat grain and soybean seed samples, after complete phase separation, aliquots (8 mL) of the upper acetonitrile phase are transferred to test tubes (10 mL) and frozen overnight at ≤ -18°C. The samples are then centrifuged for 3 minutes at approximately 3200 x g.

For all matrices, aliquots of acetonitrile phase (1.5 mL) are transferred to safe-lock tubes (2 mL) containing 40 mg of primary secondary amine (PSA) and 225 mg of magnesium sulphate. The samples are vortex mixed, shaken by hand for 30 seconds and centrifuged for 5 minutes at approximately 3500 x g. Aliquots of the extracts (200 µL for wheat grain and soybean seeds and 100 µL for cucumber and grapes) are diluted to 10 mL with acetonitrile/0.1 % formic acid (7:3, v/v). The samples are decanted into an HPLC-vial and stored at 1 – 10°C in the dark prior to analysis. The samples are analysed by high performance liquid chromatography with tandem mass selective detection (HPLC-MS/MS) in positive ion mode using an Accucore Phenyl-Hexyl column (50 mm x 4.6 mm, 2.6 µm particle size) and gradient elution with mobile phases of 0.1% formic acid in acetonitrile and 0.1% formic acid in water.

Nominal concentration: 0.01 g/mL

Quantification is performed using external standards. The ion transition m/z 334 > 258 is used for quantification and the ion transition m/z 334>238 is used for confirmation.

Analytical Parameters

HPLC System:	1200 Binary Rapid Resolution LC System, Agilent technologies (HPLC, ≤ 600 bar)			
Pre-column:	Not used			
Column:	Accucore Phenyl-Hexyl (500 mm x 4.6 mm, 2.6 µL, Thermo Scientific, Art. No. 17926-054630)			
Column oven temperature:	40 °C			
Injection Volume:	7 µL			
Mobile phase:	Eluent A: Acetonitrile containing 0.1 % (v/v) formic acid Eluent B: Water containing 0.1 % (v/v) formic acid			
Gradient:	Time [Min]	% Eluent A	% Eluent B	Flow [µL/min]
	0.0	30	70	1000
	4.5	90	10	1000
	5.0	90	10	1000
	5.1	30	70	1000

	6.5	30	70	1000		
Divert value:	0.0 min to 1.7 to waste to 4.3 to MS; 4.3 min to 6.5 to waste					
Retention time:	Approx. 3.2 min					
MS system:	API 500 System, SCIEX (Triple quadrupole mass spectrometer)					
Ionisation type:	Electrospray ionization (ESI, Turbolon Spray)					
Polarity:	Positive ion mode					
Scan tyee:	MS/MS, Multiple Reaction Monitoring (MRM)					
Capillary voltage:	5000 V		Ionspray turbo heater (TEM)	500 °C		
Curtain gas (CUR):	25 (arbitrary units)		Gas flow 1 (GS1)	40 (arbitrary units)		
Collision (CAD):	7 (arbitrary units)		Gas flow 2 (GS)	60 (arbitrary units)		
Analyte Monitored:	Mass Transition (m/z)	Declustering potential (DP) [V]	Entrance potential (EP) [V]	Collision energy (CE) [eV]	Cell exit potential (CXP) [V]	Dwell time [ms]
inpyrfluxam	334 - 258	60	10	25	15	75
	334 - 238	60	10	40	15	75

Table 5.2.1-1. Analytical validation data for the determination of inpyrfluxam in wheat (grain), cucumber, soybean (seed) and grapes.

Matrix	m/z	LOQ (mg/kg)	Recovery fortification level (mg/kg)	Recoveries % range (mean)	Repeatability % RSD (n)	Linearity
Wheat (grain)	334 - 258	0.01	0.01	106 – 111 (109)	1.9 (5)	0.02 – 2.0 ng/ml (0.002 – 0.2 mg/kg). r = 0.9989 (n = 8) y = 191097.3636x + 263.2958
			0.1	95 – 109 (102)	5.1 (5)	
	334 - 238	0.01	0.01	106 – 113 (108)	3.1 (5)	

			0.1	98 – 106 (101)	3.2 (5)	0.02 – 2.0 ng/ml (0.002 – 0.2 mg/kg). r = 0.9999 (n = 8) y = 314191.7447x - 147.7682
Cucumber	334 - 258	0.01	0.01	99 – 105 (103)	2.1 (5)	0.02 – 2.0 ng/ml (0.002 – 0.2 mg/kg). r = 0.9999 (n = 8) y = 215883.5407x - 39.9439
			0.1	93 – 104 (99)	4.1 (5)	
	334 - 238	0.01	0.01	99 – 106 (103)	2.8 (5)	0.02 – 2.0 ng/ml (0.002 – 0.2 mg/kg). r = 0.9995 (n = 8) y = 346401.8993x + 581.5453
			0.1	96 – 103 (100)	3.0 (5)	
Soybean (seeds)	334 - 258	0.01	0.01	103 – 114 (110)	4.1 (5)	

			0.1	101 – 108 (104)	3.1 (5)	0.02 – 2.0 ng/ml (0.002 – 0.2 mg/kg). r = 0.9990 (n = 8) y = 195164.1126x - 277.7564
	334 - 238	0.01	0.01	107 – 114 (110)	3.0 (5)	0.02 – 2.0 ng/ml (0.002 – 0.2 mg/kg). r = 0.9996 (n = 8) y = 302553.0529x + 1019.5027
			0.1	105 – 108 (107)	1.4 (5)	
Grapes	334 - 258	0.01	0.01	89 – 107 (99)	6.5 (5)	0.02 – 2.0 ng/ml (0.002 – 0.2 mg/kg). r = 0.9995 (n = 8) y = 219508.3479x - 92.1655
			0.1	90 – 105 (101)	6.2 (5)	
	334 - 238	0.01	0.01	88 – 109 (102)	8.1 (5)	

			0.1	99 – 106 (103)	2.8 (5)	0.02 – 2.0 ng/ml (0.002 – 0.2 mg/kg). r = 0.9997 (n = 8) y = 338183.0368x + 405.2504

Specificity:

Specificity was demonstrated by retention time match with a reference standard. Analysis of unfortified control samples and reagent blanks demonstrated no significant interference (> 30% of the LOQ) at the retention time of interest.

Two mass transitions were validated for the method. Product ion spectrum was provided demonstrating the acceptability of the mass transitions used.

Matrix Effects:

Matrix effect was investigated and is reported below.

Matrix / Commodity	Standard Concentration (ng/mL)	Matrix effect for inpyrfluxam (%)	
		Quantification (334-258 m/z)	Confirmation (334-238)
Wheat (grain)	0.1	(-) 9.4	(-) 0.6
	1.0	(-) 2.1	(-) 1.9
Cucumber	0.1	(-) 1.4	(-) 4.9
	1.0	(-) 9.5	(+) 0.3
Soybean (seed)	0.1	(+) 6.6	(-) 2.3
	1.0	(-) 4.3	(-) 1.6
Grapes	0.1	(-) 10	(-) 7.0
	1.0	(-) 3.3	(-) 3.3

Matrix effect was demonstrated to be <20% therefore solvent based standards were used for quantification.

Linearity:

Linearity was demonstrated by the analysis of eight standards of increasing concentration. The range of standard concentrations used was 0.02 – 2 ng/mL, equivalent to 0.002 – 0.2 mg/kg active substance in the samples. The response was linear with a correlation coefficient (r) of ≥0.9989. Residual plots were not submitted.

Accuracy:

Recovery samples were prepared by spiking blank wheat (grain), cucumber, soybean (seed) and grapes with inpyrfluxam standard and analysing them by the method described. The spike concentrations were in the range 0.01 to 0.1 mg/kg. Five samples were prepared at each fortification level. Mean recovery levels were within the range 99 - 110 % and are acceptable.

Precision:

Precision was determined from the accuracy recovery data. Five samples were prepared at each fortification level, and the % RSD at each fortification level was < 20%.

Stability of stock and fortification solution

Stock solutions were prepared in methanol were stored at 1 °C to 10 °C for 104 days in the dark. After storage the stored stock solutions were compared to freshly prepared solutions, The results are reported below

Analyte	Solvent of stock solution	Standard conc. Of diluted stock solution (ng/mL)	Storage period (Days)	Recovery of stored stock solution (Freshly prepared stock solution = 100 %)
Inpyrfluxam	Methanol	20	104	98

The quantified values of inpyrfluxam in methanol compared to freshly prepared solutions was ≤20 % therefore it can be concluded acceptable stability is demonstrated for up to 104 days at 1 – 10 °C in the dark.

Stability in calibration solutions

Calibration solutions were prepared in acetonitrile/0.1 % formic acid in water (7:3, v/v), and stored at 1 – 10 °C for up to 20 days in the dark. After storage the stored samples were compared to freshly prepared standards to determine stability. The results are reported below.

Analyte	Solvent for calibration solutions	Standard conc. (ng/mL)	Storage period (days)	Difference (%) of stored solute compared to a freshly prepared solution
Inpyrfluxam	Acetonitrile/0.1 % formic acid in water (7/3, v/v)	0.1	20	-5.3
		1.0	20	-9.6

The quantified values of inpyrfluxam in acetonitrile/0.1 % formic acid in water (7/3, v/v) compared to freshly prepared solutions was ≤ 20 % therefore it can be concluded acceptable stability is demonstrated for up to 20 days at 1 – 10 °C in the dark.

Extract Stability

Extract stability was investigated as part of the method validation. After first analysis extracts were stored at 1 – 10 °C for at least 14 days in the dark along with one control. After this period, the extracts were re-analysed against freshly prepared calibration standards at the m/z of 334 – 258 m/z. Results are reported below

Matrix	Fortification level (mg/kg)	Mean Recovery 1st injection (n)	Mean recovery 2nd injection (n)	Days of storage (1st to 2nd injection)	Percent of the initial recovery calculated from unrounded mean recovery values
Wheat (grain)	0.01	109	120	14	110
Cucumber	0.01	103	107	15	104
Soybean (seeds)	0.01	110	120	14	109
Grapes	0.01	99	112	15	113

The mean recoveries of the stored extracts were within 70 – 120 %, and within ±20 % of the original result. Therefore, it can be concluded inpyrfluxam is stable in wheat (grain), cucumber, soybean (seeds) and grape extracts for 14, 15, 14, and 15 days, respectively.

LOQ

The limit of quantitation (LOQ), defined as the lowest fortification level where acceptable precision and accuracy data were obtained, has been demonstrated to be 0.01 mg/kg for all matrices.

Extraction efficiency:

A study has been provided to compare the QuEChERS extraction procedure with the metabolism study extraction procedure. Extraction efficiency has been demonstrated for the QuEChERS method for the determination of inpyrfluxam in the dry/high starch, high oil and high-water commodity groups. See KCA 4.2/05 for full details.

There is no extraction efficiency data for high acid commodities. Nevertheless, bridging between high water commodities and high acid commodities is acceptable for slightly acidic matrices, e.g. apple, tomato, grapes. As extraction efficiency is validated in apples, it can be bridged to high acid commodities.

Conclusion

The method is acceptably validated in accordance with SANTE/2020/12830 rev. 1
2 for the determination of inpyrfluxam in wheat (grain) (dry commodity - high protein/high starch), cucumber (high water), soybean (seed) (high oil) and grapes (high acid).

Reference:	KCA 4.2/02
Report Title:	Independent Laboratory Validation of the Multi-Residue Method QuEChERS for the Determination of S-2399 in Matrices of Plant Origin.
Author(s) & Year:	[redacted] 2017a
Document No, Authority registration No	Study No.: S17-02966; Report No.: TPA-0048
Guideline(s):	SANCO/825/00 rev. 8.1
Deviations:	No
GLP or GEP:	Yes
Acceptability:	Yes
Study relied upon:	Yes

Report KCA 4.2/02 has been submitted to support the monitoring of residues of inpyrfluxam in/on wheat (grain) (dry/high starch commodity), soybean (seed) (high oil commodity), cucumber (high water commodity) and grapes (high acid commodity). KCA 4.2/01 contains validation from an independent laboratory, supporting the acceptability of the method for monitoring in combination with the method report under KCA 4.2/02.

The standard used for the validation procedure is noted to be 99.9% R-isomer.

The independent laboratory validation was performed at Eurofins Agroscience Services, Niefern-Öschelbronn, Germany. Primary validation was performed by Eurofins Agroscience Services, Hamburg, Germany.

Principle of the method

Homogenized samples (5 g wheat grain or soybean seeds, 10 g cucumber or grapes) are weighed into centrifuge tubes (50 mL). Water (10 mL) is added to wheat grain and soybean seeds only and the samples are allowed to soak for 20 minutes at room temperature. All the samples are extracted with acetonitrile (10 mL) on a platform shaker for 15 minutes before the addition of magnesium sulphate (4.0 g), sodium chloride (1.0 g), trisodium citrate dihydrate (1.0 g) and disodium hydrogen citrate sesquihydrate (0.5 g). The tubes are shaken by hand for approximately 1 minute and centrifuged for 5 minutes at approximately 3200 x g.

For wheat grain and soybean seed samples, after complete phase separation, aliquots (8 mL) of the upper acetonitrile phase are transferred to test tubes (10 mL) and frozen overnight at ≤ -18°C. The samples are then centrifuged for 3 minutes at approximately 3200 x g.

For all matrices, aliquots of acetonitrile phase (1.5 mL) are transferred to safe-lock tubes (2 mL) containing 40 mg of primary secondary amine (PSA) and 225 mg of magnesium sulphate. The samples are vortex mixed, shaken by hand for 30 seconds and centrifuged for 5 minutes at approximately 3500 x g. Aliquots of the extracts (200 µL for wheat grain and soybean seeds and 100 µL for cucumber and grapes) are diluted to 10 mL with acetonitrile/0.1 % formic acid (7:3, v/v). The samples are decanted into an HPLC vial and stored at 1 – 10°C in the dark prior to analysis. The samples are analysed by high performance liquid chromatography with tandem mass selective detection (HPLC-MS/MS) in positive ion mode using an Accucore Phenyl-Hexyl column (50 mm x 4.6 mm, 2.6 µm particle size) and gradient elution with mobile phases of 0.1% formic acid in acetonitrile and 0.1% formic acid in water.

Nominal concentration: 0.01 g/mL

Quantification is performed using external standards. The ion transition m/z 334 > 258 is used for quantification and the ion transition m/z 334>238 is used for confirmation.

Analytical Parameters

HPLC System:	1290 Infinity HPLC, Agilent Technologies			
Pre-column:	Not used			
Column:	Accucore Phenyl-Hexyl (500 mm x 4.6 mm, 2.6 µL, Thermo Scientific, Art. No. 17926-054630)			
Column oven temperature:	40 °C			
Injection Volume:	5 µL			
Mobile phase:	Eluent A: Water containing 0.1 % (v/v) formic acid Eluent B: Acetonitrile containing 0.1 % (v/v) formic acid			
Gradient:	Time [Min]	% Eluent A	% Eluent B	Flow [µL/min]
	0.0	70	30	1000
	4.5	10	90	1000
	5.0	10	90	1000
	5.1	70	30	1000
	6.5	70	30	1000
Divert value:	0.0 min to 1.7 to waste to 4.3 to MS; 4.3 min to 6.5 to waste			
Retention time:	Approx. 2.8 min			
MS system:	API 6500 System, SCIEX			
Ionisation type:	Electrospray ionization (ESI, Turbolon Spray)			
Polarity:	Positive ion mode			
Scan tyee:	MS/MS, Multiple Reaction Monitoring (MRM)			
Capillary voltage:	5000 V	Ionspray turbo heater (TEM)	500 °C	
Curtain gas (CUR):	40 (arbitrary units)	Gas flow 1 (GS1)	50 (arbitrary units)	
Collision (CAD):	10 (arbitrary units)	Gas flow 2 (GS)	60 (arbitrary units)	

Analyte Monitored:	Mass Transition (m/z)	Declustering potential (DP) [V]	Entrance potential (EP) [V]	Collision energy (CE) [eV]	Cell exit potential (CXP) [V]	Dwell time [ms]
Inpyrfluxam	334 - 258	90	10	29	28	75
	334 - 238	90	10	41	22	75

Table 5.2.1-2 Analytical validation data for the determination of inpyrfluxam in wheat (grain), cucumber, soybean (seed) and grapes.

Matrix	m/z	LOQ (mg/kg)	Recovery fortification level (mg/kg)	Recoveries % range (mean)	Repeatability % RSD (n)	Linearity
Wheat (grain)	334 - 258	0.01	0.01	104 – 110 (107)	2 (5)	Solvent based calibration. *m/z = 334 – 258* 0.02 – 2 ng/mL (equivalent to 0.002-0.2 mg/kg) r = 0.9998 (n = 8) Y = 3.01 x 10^5x +719 *m/z = 334 – 238* 0.02 – 2 ng/mL (equivalent to 0.002-0.2 mg/kg) r = 0.9998
			0.1	105 – 114 (109)	4 (5)	
	334 - 238	0.01	0.01	101 – 111 (107)	4 (5)	
			0.1	107 – 113 (110)	2 (5)	
Soybean (seed)	334 - 258	0.01	0.01	99 – 113 (106)	5 (5)	
			0.1	101 – 107 (104)	2 (5)	
	334 - 238	0.01	0.01	101 – 109 (105)	3 (5)	
			0.1	101 – 105 (103)	2 (5)	
Grape	334 - 258	0.01	0.01	97 – 101 (99)	2 (5)	
			0.1	98 – 105 (101)	3 (5)	

	334 - 238	0.01	0.01	99 – 102 (101)	1 (5)	Y = 4.01 x 10^5x + 769
			0.1	97 – 103 (100)	3 (5)	
Cucumber	334 - 258	0.01	0.01	98 – 106 (101)	3 (5)	
			0.1	97 – 104 (100)	3 (5)	
	334 - 238	0.01	0.01	98 – 104 (101)	2 (5)	
			0.1	97 – 104 (100)	3 (5)	

Specificity and Selectivity:

Specificity was demonstrated by retention time match with a reference standard. Analysis of unfortified control samples and reagent blanks demonstrated no significant interference (> 30% of the LOQ) at the retention time of interest.

Two mass transitions were investigated (> 100 m/z) and are considered acceptable. HPLC-MS/MS is considered a highly specific technique. The method demonstrated acceptable specificity and selectivity.

Matrix Effects:

Matrix effect was investigated by comparing the peak area of matrix matched standards with solvent standards at identical concentrations. Results are reported below.

Matrix / Commodity	Standard Concentration (ng/mL)	Matrix effect for inpyrfluxam (%)	
		Quantification (334-258 m/z)	Confirmation (334-238)
Wheat (grain)	2.0	(+) 6.6	(+) 6.3
	1.2	(-) 0.8	(+) 4.8
	1.0	(+) 5.5	(+) 4.6
	0.5	(+) 1.1	(+) 0.9

	0.2	(+) 4.2	(+) 3.7
	0.1	(+) 2.6	(+) 3.2
	0.05	(+) 0.2	(+) 9.5
	0.02	(+) 2.9	(+) 1.6
Soybean (seed)	2.0	(+) 0.2	(+) 1.7
	1.2	(+) 2.5	(+) 3.9
	1.0	(+) 1.9	(+) 2.0
	0.5	(+) 1.0	(+) 4.0
	0.2	(-) 7.2	(-) 4.8
	0.1	(+) 2.8	(+) 1.0
	0.05	(-) 0.8	(-) 2.9
	0.02	(+) 7.1	(+) 5.0
Grapes	2.0	(+) 5.9	(+) 5.8
	1.2	(-) 0.1	(+) 2.6
	1.0	(-) 4.6	(-) 4.2
	0.5	(+) 3.7	(+) 0.3
	0.2	(-) 3.8	(+) 0.6
	0.1	(+) 3.4	(+) 0.3
	0.05	(+) 0.1	(+) 1.5
	0.02	(-) 4.0	(+) 0.7
Cucumber	2.0	(-) 0.6	(+) 0.5
	1.2	(-) 1.6	(-) 1.3
	1.0	(-) 4.5	(-) 5.3
	0.5	(-) 0.4	(-) 2.2
	0.2	(-) 1.6	(+) 2.0
	0.1	(-) 2.0	(-) 2.4
	0.05	(-) 0.9	(-) 0.7
	0.02	(+) 8.3	(+) 3.4

Matrix effect was demonstrated to be <20% therefore solvent based standards were used for quantification.

Linearity:

Linearity was demonstrated by the analysis of eight standards of increasing concentration. The range of standard concentrations used was 0.02 – 2 ng/mL, equivalent to 0.002 – 0.2

mg/kg active substance in the samples. The response was linear with a correlation coefficient (r) of ≥0.9998. Residual plots were not provided.

Accuracy:

Recovery samples were prepared by spiking blank wheat (grain), cucumber, soybean (seed) and grapes with inpyrfluxam standard and analysing them by the method described. The spike concentrations were in the range 0.01 to 0.1 mg/kg. Five samples were prepared at each fortification level. Mean recovery levels were within the range 99 - 110 % and are acceptable.

Precision:

Precision was determined from the accuracy recovery data. Five samples were prepared at each fortification level, and the % RSD at each fortification level was < 20%.

Stability of stock and fortification solution

Stability in calibration solutions

Extract Stability

Stability of stock, fortification, calibration solutions and the stability in extracts was investigated in Study S16-03372 (KCA 4.2/01).

Extraction efficiency:

A study has been provided to compare the QuEChERS extraction procedure with the metabolism study extraction procedure. Extraction efficiency has been demonstrated for the QuEChERS method for the determination of inpyrfluxam in the dry (high protein/high starch), high oil and high-water commodity groups. See KCA 4.2/05 for full details.

There is no extraction efficiency data for high acid commodities. Nevertheless, bridging between high water commodities and high acid commodities is acceptable for slightly acidic matrices, e.g. apple, tomato, grapes. As extraction efficiency is validated in apples, it can be bridged to high acid commodities.

Conclusion

The method is acceptably validated in accordance with SANTE/2020/12840 Rev.1 for the determination of inpyrfluxam in wheat (grain) (dry – high protein/high starch), cucumber (high water), soybean (seed) (high oil) and grapes (high acid).

Both the primary method valdation (Study S16-03372) and the independent laboratory validation for the QuEChERS method for the quantification of analyte inpyrfluxam in wheat

wheat (grain) (dry -high protein/high starch), cucumber (high water), soybean (seed) (high oil) and grapes (high acid) are considered acceptable, therefore the reported QuEChERS method is sufficient for the monitoring of residues of inpyrfluxam.

B.5.2.2. Methods for residues in or on food and feed of animal origin

Reference:	KCA 4.2/03
Report Title:	Validation of the Multi-Residue Method QuEChERS for the Determination of S-2399 in Different Matrices of Animal Origin and Body Fluids
Author(s) & Year:	[redacted] 2017b
Document No, Authority registration No	Study No.: S17-03967; Report No.: TPA-0049
Guideline(s):	SANCO/825/00 rev. 8.1
Deviations:	No
GLP or GEP:	Yes
Acceptability:	Yes
Study relied upon:	Yes

KCA 4.2/03 has been submitted to support the monitoring of residues of inpyrfluxam in milk, eggs, fat, meat, liver, blood and urine. Primary validation reported in the current study report was conducted at Eurofins Agroscience Services, Niefern-Öschelbronn, Germany. ILVs reported under KCA 4.2/04 were validated by EAG Laboratories GmbH, Ulm, Germany.

The standard used for the validation procedure is noted to be 99.9% R-isomer.

Validation has been performed on validation on milk, eggs, fat, muscle, liver and kidney. ILVs reported under 4.2/04 has been performed on fat and muscle.

Principle of the method

Homogenized samples (5.0 g bovine whole milk, poultry eggs, bovine muscle meat, 5.0 mL bovine blood, 10.0 mL urine or 2.0 g bovine fat) are weighed into centrifuge tubes (50 mL). For bovine whole milk, poultry eggs, bovine muscle meat, bovine liver and bovine blood, water (6.0 mL) and acetonitrile (10 mL) are added. For urine, acetonitrile (10 mL) is added. For bovine fat, water (10 mL) and acetonitrile (10 mL) are added. The samples are shaken for 15 minutes on a platform shaker (with heating in a water bath at 60°C for bovine fat).

The contents of QuEChERS Bekolut Citrate Kit 01 (magnesium sulphate (4.0 g), sodium chloride (1.0 g), trisodium citrate dehydrate (1.0 g) and disodium hydrogen citrate sesquihydrate (0.5 g)) is added and the samples shaken by hand vigorously for 1 minute. The upper acetonitrile phase is transferred to a new centrifuge tube. Bovine whole milk, poultry's eggs, bovine muscle meat, bovine liver and bovine fat samples are frozen overnight at -18°C. All samples are then centrifuged for 5 minutes at 4000 rpm.

Aliquots of the acetonitrile extracts (1 mL) are cleaned up using PSA-KIT-03 (Bekolut) containing PSA (25 mg), C_{18} (25 mg) and magnesium sulphate (150 mg). The tubes are vortex mixed, shaken by hand for 30 seconds and centrifuged for 5 minutes at 4000 rpm. Aliquots of the final extract (0.10 mL for bovine whole milk, poultry's eggs, bovine muscle meat, bovine liver and bovine blood, 0.05 mL urine and 0.25 mL bovine fat) are diluted to 10 mL with acetonitrile/0.1 % formic acid (1:1, v/v).

The samples are analysed by high performance liquid chromatography with tandem mass selective detection (HPLC-MS/MS) in positive ion mode using a Thermo Betasil C18 column (100 mm x 2.1 mm, 5 µm particle size) fitted with a Phenomenex C18 cartridge guard column and gradient elution with mobile phases of 0.1% formic acid in water and 0.1% formic acid in acetonitrile. Quantification is performed using external standards. The ion transition m/z 334 > 258 is used for quantification and the ion transition m/z 334 > 238 is used for confirmation.

Nominal Concentration: 0.005 g/mL

Analytical parameters

HPLC System:	Shimadzu HPLC System (LC-30 AD pumps, SIL 30 ACMP autosampler, column oven and vacuum solvent degasser)
Pre-column:	HPLC guard column (KJ0-4282, Phenomenex) with C_{18} cartridge (AJ0-4287, Phenomenex)
Column:	Thermo Betasil $C_{18,}$ No. 70105-102130, 100 mm x 2.1 mm, 5 µm
Column oven temperature:	40 °C
Injection Volume:	5 µL
Mobile phase:	Eluent A: Water containing 0.1% formic acid

	Eluent B: Acetonitrile containing 0.1% formic acid			
Gradient:	Time [min]	% Eluent A	% Eluent B	Flow [µL/min]
	0.0	70	30	600
	1.0	5	95	600
	2.5	5	95	600
	2.6	70	30	600
	4.0	70	30	600
Divert value:	0.0 min to 1.3 min to waste; 1.3 min to 2.0 min to MS; 2.0 min to 4.0 min to waste			
Retention time:	Inpyrfluxam: Approx 1.5 min			
MS system:	API 5500™ LC-MS/MS System (Sciex)			
Ionisation type:	Electrospray ionisation (ESI, Turbolon Spray)			
Polarity:	Positive ion mode			
Scan tyee:	MS/MS, Multiple Reaction Monitoring (MRM)			
Capillary voltage:	5500 V	Isonspray turbo heater (TEM)	500 °C	
Curtain gas (CUR):	50 (arbitrary units)	Gas flow 1 (GS1)	50 (arbitrary units)	
Collision (CAD):	10 (arbitrary units)	Gas flow 2 (GS2)	60 (arbitrary units)	

Analyte Monitored:	Mass transition monitored (m/z)	Declustering potential (DP) [V]	Extrance potential (EP) [V}	Collision energy (CE) [V]	Cell exit potential (CXP) [V]	Dwell Time [ms]
Inpyrfluxam	334 - 258	80	10	29	28	75
	334 - 238	80	10	41	22	75

Table 5.2.1-3: Analytical validation data for the determination of inpyrfluxam in animal matrices (bovine whole milk, poultry eggs, bovine fat, bovine muscle meat and bovine liver):

Matrix	m/z	LOQ (mg/kg)	Recovery fortification level (mg/kg)	Recoveries % range (mean)	Repeatability % RSD (n)	Linearity
Bovine whole milk	334 - 258	0.01	0.01	84 – 96 (90)	6 (5)	Solvent based linearity *m/z 334 – 258* 0.01 – 1.0 ng/mL (0.002
			0.1	88 – 100 (93)	5 (5)	
	334 - 238	0.01	0.01	85 – 94 (90)	4 (5)	
			0.1	89 – 99 (93)	5 (5)	

Poultry eggs	334 - 258	0.01	0.01	91 – 97 (94)	2 (5)	– 0.2 mg/kg / mg/L) r = 0.9997 (N = 7) Y = 8.04 x 10^5x + 1.01 x 10^3
			0.1	90 – 94 (93)	2 (5)	
	334 - 238	0.01	0.01	90 – 92 (91)	1 (5)	
			0.1	91 – 94 (92)	1 (5)	
Bovine Fat	334 - 258	0.01	0.01	72 – 81 (77)	5 (5)	*m/z = 334 – 238* 0.01 – 1.0 ng/mL (0.002 – 0.2 mg/kg / mg/L) r = 0.9997 (N = 7) Y = 9.58 x 10^5x +1.41 x 10^3
			0.1	83 – 94 (88)	5 (5)	
	334 - 238	0.01	0.01	72 – 79 (75)	4 (5)	
			0.1	82 – 95 (88)	5 (5)	
Bovine Muscle meat	334 - 258	0.01	0.01	89 – 98 (93)	4 (5)	
			0.1	88 – 96 (94)	3 (5)	
	334 - 238	0.01	0.01	90 – 97 (94)	3 (5)	
			0.1	89 – 97 (95)	3 (5)	
Bovine Liver	334 - 258	0.01	0.01	92 – 98 (95)	2 (5)	
			0.1	100 – 106 (103)	2 (5)	
	334 - 238	0.01	0.01	92 – 98 (95)	2 (5)	
			0.1	102 – 107 (104)	2 (5)	
Bovine blood	334 - 258	0.01	0.01	83 – 96 (91)	5 (5)	
			0.1	98 – 105 (102)	2 (5)	

	334 - 238	0.01	0.01	84 – 96 (91)	5 (5)	
			0.1	98 – 106 (102)	3 (5)	
Urine	334 - 258	0.01	0.01	99 – 103 (100)	2 (5)	
			0.1	99 – 105 (101)	3 (5)	
	334 - 238	0.01	0.01	98 – 103 (101)	2 (5)	
			0.1	98 – 105 (101)	3 (5)	

Specificity:

Specificity was demonstrated by retention time match with a reference standard. Analysis of unfortified control samples and reagent blanks demonstrated no significant interference (> 30% of the LOQ) at the retention time of interest.

Two mass transitions were validated for the method. A product ion spectrum was provided demonstrating the acceptability of the mass transitions used.

Matrix Effects:

Matrix effect was investigated by comparing the peak area of matrix matched standards with solvent standards at identical concentrations. Results are reported below.

Matrix / Commodity	Standard Concentration (ng/mL)	Matrix effect for inpyrfluxam (%)	
		Quantification (334-258 m/z)	Confirmation (334-238)
Bovine whole milk	1.0	(+) 0.0	(-) 0.5
	0.5	(+) 3.8	(+) 5.0
	0.2	(+) 0.4	(+) 2.3
	0.1	(+) 0.3	(+) 3.0
	0.05	(+) 1.8	(-) 3.0
	0.02	(+) 0.7	(-) 1.9
	0.01	(-) 5.1	(-) 3.3
Poultry eggs	1.0	(-) 1.8	(-) 2.1
	0.5	(-) 0.3	(+) 0.0
	0.2	(-) 3.4	(-) 2.5

	0.1	(+) 2.4	(+) 0.6
	0.05	(-) 2.8	(+) 0.5
	0.02	(-) 2.5	(+) 1.2
	0.01	(+) 0.8	(+) 7.3
Bovine fat	1.0	(-) 1.3	(-) 2.4
	0.5	(+) 0.4	(+) 2.1
	0.2	(-) 3.4	(-) 2.2
	0.1	(-) 5.1	(-) 4.7
	0.05	(-) 5.3	(-) 3.2
	0.02	(-) 6.3	(-) 5.4
	0.01	(-) 13.4	(-) 8.6
Bovine muscle meat	1.0	(-) 8.3	(-) 10.5
	0.5	(-) 9.4	(-) 10.1
	0.2	(-) 11.4	(-) 12.8
	0.1	(-) 9.2	(-) 9.5
	0.05	(-) 10.0	(-) 8.0
	0.02	(-) 13.7	(-) 11.4
	0.01	(-) 2.5	(-) 2.1
Bovine liver	1.0	(+) 11.6	(+) 10.9
	0.5	(+) 10.9	(+) 11.8
	0.2	(+) 6.9	(+) 8.1
	0.1	(+) 8.5	(+) 8.8
	0.05	(+) 11.6	(+) 11.3
	0.02	(+) 2.7	(+) 4.4
	0.01	(+) 12.2	(+) 10.6
Bovine blood	1.0	(-) 1.5	(-) 1.8
	0.5	(-) 3.8	(-) 4.3
	0.2	(-) 10.3	(-) 12.0
	0.1	(-) 7.7	(-) 6.6
	0.05	(-) 9.5	(-) 6.2
	0.02	(-) 7.2	(-) 8.0
	0.01	(-) 11.1	(-) 2.9
Urine	1.0	(-) 1.0	(+) 0.4
	0.5	(+) 4.6	(+) 4.3
	0.2	(-) 3.7	(-) 3.7

	0.1	(-) 4.0	(-) 2.2
	0.05	(-) 3.5	(-) 3.5
	0.02	(-) 5.8	(-) 8.3
	0.01	(-) 2.5	(-) 2.3

Matrix effect was demonstrated to be <20% therefore solvent based standards were used for quantification.

Linearity:

Linearity was demonstrated by the analysis of seven standards of increasing concentration. The range of standard concentrations used was 0.01 – 1 ng/mL, equivalent to 0.002 – 0.2 mg/kg active substance in milk, eggs, fat, muscle and liver and 0.002 – 0.2 mg/L in blood and urine. The response was linear with a correlation coefficient (r) of ≥0.9997. No residual plots were submitted.

Accuracy:

Recovery samples were prepared by spiking blank products of animal origin with inpyrfluxam standard and analysing them by the method described. The spike concentrations were in the range 0.01 to 0.1 mg/kg. Five samples were prepared at each fortification level. Mean recovery levels were within the range 75 - 104% and are acceptable.

Precision:

Precision was determined from the accuracy recovery data. Five samples were prepared at each fortification level, and the % RSD at each fortification level was < 20%.

Stability of stock and fortification solution

Stock solutions were prepared in methanol were stored at 1 °C to 10 °C for 31 days in the dark. After storage the stored stock solutions were compared to freshly prepared solutions, The results are reported below

Analyte	Solvent of stock solution	Standard conc. Of diluted stock solution (ng/mL)	Storage period (Days)	Recovery of stored stock solution (Freshly prepared stock solution = 100 %)
Inpyrfluxam	Methanol	10	31	104

The quantified values of inpyrfluxam in methanol compared to freshly prepared solutions was ≤20 % therefore it can be concluded acceptable stability is demonstrated for up to 31 days at 1 – 10 °C in the dark.

Stability in calibration solutions

Calibration solutions were prepared in acetonitrile/0.1 % formic acid in water (7:3, v/v), and stored at 1 – 10 °C for 23 days in the dark. After storage the stored samples were compared to freshly prepared standards to determine stability. The results are reported below. Analysis was performed in triplicate for each fortification level at a single mass transition.

Analyte	Solvent for calibration solutions	Standard conc. (ng/mL)	Storage period (days)	Difference (%) of stored solute compared to a freshly prepared solution
Inpyrfluxam	Acetonitrile/0.1 % formic acid in water (7/3, v/v)	0.05	23	108
		0.1	23	110
		0.5	23	105
		1.0	23	106

The quantified values of inpyrfluxam in acetonitrile/0.1 % formic acid in water (7/3, v/v) compared to freshly prepared solutions was ≤20 % therefore it can be concluded acceptable stability is demonstrated for up to 23 days at 1 – 10 °C in the dark.

Extract Stability

Extract stability was investigated as part of the method validation. After first analysis extracts were stored at 1 – 10 °C for at least 8 days in the dark along with one control. After this period, the extracts were re-analysed against freshly prepared calibration standards at the m/z of 334 – 258 m/z. Results are reported below

Matrix	Fortification level (mg/kg)	Mean Recovery 1st injection (n)	Mean recovery 2nd injection (n)	Days of storage (1st to 2nd injection)	Percent of the initial recovery calculated from unrounded mean recovery values
Bovine whole milk	0.1	93	95	9	102
Poultry eggs	0.1	93	91	8	98
Bovine fat	0.1	88	84	8	95
Bovine meat	0.1	94	93	8	99
Bovine liver	0.1	103	99	8	96

Bovine blood	0.1	102	95	8	93
Urine	0.1	101	97	8	96

The mean recoveries of the stored extracts were within 70 – 120 %, and within ±20 % of the original result. Therefore, it can be concluded inpyrfluxam is stable in bovine (milk), poultry eggs, bovine fat, bovine meat, bovine liver, bovine blood and urine for up to 8 days.

LOQ

The limit of quantitation (LOQ), defined as the lowest fortification level where acceptable precision and accuracy data were obtained, has been demonstrated to be 0.01 mg/kg for all matrices.

Extraction efficiency:

A study has been provided to compare the QuEChERS extraction procedure with the metabolism study extraction procedure. Extraction efficiency has been demonstrated for the QuEChERS method for the determination of inpyrfluxam in goat liver, goat fat, goat milk, hen composite muscle and hen composite egg. See KCA 4.2/06 for full details.

This is sufficient to address the extraction efficiency in poultry eggs and bovine whole milk, fat, meat and liver. Extraction efficiency is not required for blood and urine.

Conclusion

The method is acceptably validated in accordance with SANTE/2020/12830 rev. 1 for the determination of inpyrfluxam in bovine fat, meat, liver, milk, blood and urine and poultry eggs.

Reference:	KCA 4.2/04
Report Title:	Independent Laboratory Validation of an Analytical Method for the Determination of S-2399 in Matrices of Animal Origin, using LC/MS/MS
Author(s) & Year:	[redacted] 2018a
Document No, Authority registration No	Study No.: P 4665 G; Report No.: TPA-0061

Guideline(s):	SANCO/825/00 rev. 8.1
Deviations:	No
GLP or GEP:	Yes
Acceptability:	Yes
Study relied upon:	Yes

KCA 4.2/04 has been submitted to support the monitoring of residus of inpyfluxam in milk, eggs, fat, meat, liver, blood and urine. ILVs were validated by EAG Laboratories GmbH, Ulm, Germany. Primary validation reported under KCA 4.2/03 was conducted at Eurofins Agroscience Services, Niefern-Öschelbronn, Germany.

Validation has been performed on fat and muscle, with primary validation on milk, eggs, fat, muscle, liver and kidney.

The standard used for the validation procedure is noted to be 99.9% R-isomer.

Principle of the method

The sample preparation used for bovine liver and fat as part of the independent laboratory validation is reported under KCA 4.2/03 above.

Analytical parameters

HPLC System:	Agilent Infinity 1290 HPLC System (vacuum solvent degasser, binary HPLC pump, column oven) and HTC PAL Autosampler			
Pre-column:	Phenomenex C18, 4 mm x 3 mm			
Column:	Thermo Betasil C18, 100 mm length x 2.1 mm i.d., 5.0 µm particle size			
Column oven temperature:	40 °C			
Injection Volume:	10 µL			
Mobile phase:	Solvent A: 0.1 % formic acid in water Solvent B: 0.1 % formic acid in acetonitrile			
Gradient:	Time (min)	Flow rate (mL/min)	% A	% B
	0.0	0.6	70	30
	1.0	0.6	5	95
	2.5	0.6	5	95
	2.6	0.6	70	30

	4.0	0.6	70	30

Retention time: Approx 1.6 min
MS system: Applied Biosystems MDS Sciex APO 5500 Q-Trap system with TurboIonspray (ESI) source
Source Temperature: 550 °C
Gas supply GS1: 50 (arbitrary units)
Gas supply GS2: 60 (arbitray units)
Curtain Gas: 20 (arbitrary units)
Entrance potential: 10 V
IonSpray voltage: 5500 V
CAD: Medium
Declustering Potential 116 V
Resolution Q1/Q3 Unit/low
Inpyrfluxam: Mass transition 344 m/z – 258 (quantitation)
CE: 27 V
CXP: 22 V
Dwell time: 110 ms
DFB: Transition 334 m/z – 238 m/z (Confirmation)
CE: 35 V
CXP: 12 V
Dwell time: 100 ms

Table 5.2.1-4: Analytical validation data for the determination of inpyrfluxam in animal matrices (bovine liver and bovine fat):

Matrix	m/z	LOQ (mg/kg)	Recovery fortification level (mg/kg)	Recoveries % range (mean)	Repeatability % RSD (n)	Linearity
Bovine Fat	334 - 258	0.01	0.01	102 – 144* 102 – 107 (104)	2 (4)	Solvent based linearity *m/z = 334 – 258* 0.01 – 1.0 ng/mL (0.002 – 0.2 mg/kg) r = 0.9996
			0.1	97 – 102 (100)	2 (5)	
	334 - 238	0.01	0.01	103 – 155* 103 – 110 (106)	3 (4)	
			0.1	97 – 102 (100)	2 (5)	

Bovine Liver	334 - 258	0.01	0.01	94 – 98 (96)	2 (5)	(n = 7) Y = 1.08 x 10^5x - 2.64 x 10^3 *m/z = 334 – 238* 0.01 – 1.0 ng/mL (0.002 – 0.2 mg/kg) r = 0.9994 (n = 7) Y = 1.2 x 10^6x -1.1 x 10^3
			0.1	88 – 96 (92)	4 (5)	
	334 - 238	0.01	0.01	95 – 99 (97)	2 (5)	
			0.1	90 – 98 (95)	4 (5)	

* Dixon Outlier, excluded from calculation

Specificity and Selectivity:

Specificity was demonstrated by retention time match with a reference standard. Analysis of unfortified control samples and reagent blanks demonstrated no significant interference (> 30% of the LOQ) at the retention time of interest.

Two mass transitions were validated for the method.

Matrix Effects:

Matrix effect was investigated by comparing the peak area of matrix matched standards with solvent standards at identical concentrations. Results are reported below.

Matrix / Commodity	Standard Concentration (ng/mL)	Matrix effect for inpyrfluxam (%)	
		Quantification (334-258 m/z)	Confirmation (334-238)
Bovine Liver	0.05	(+) 9	(+) 6
		(-) 3	(-) 2
Bovine Fat	0.05	(+) 2	(+) 1
		(-) 2	(-) 5

Matrix effect was demonstrated to be <20% therefore solvent based standards were used for quantification.

Linearity:

Linearity was demonstrated by the analysis of eight standards of increasing concentration. The range of standard concentrations used was 0.01 – 1 ng/mL, equivalent to 0.002 – 0.2 mg/kg active substance in the samples. The response was linear with a correlation coefficient (r) of ≥0.9994. No residual plots were submitted.

Accuracy:

Recovery samples were prepared by spiking blank bovine liver and fat with inpyrfluxam standard and analysing them by the method described. The spike concentrations were in the range 0.01 to 0.1 mg/kg. Five samples were prepared at each fortification level.

Outliers were identified in bovine fat at the fortification level of 0.01 at both mass transitions. Identified outliers were removed from mean and %RSD calculations. As only a single value was identified as a mean at each fortification this is considered acceptable.

Mean recovery levels were within the range 92-106% and are acceptable.

Precision:

Precision was determined from the accuracy recovery data. Five samples prepared at each fortification level, and the % RSD at each fortification level was < 20%. For bovine fat due to identified outliers four values were used to calculate the %RSD at the fortification of 0.01 mg/kg at both mass transitions.

Stability of stock and fortification solution

Stability in calibration solutions

Extract Stability

Stability of standard solutions and extracts was demonstrated in the original method validation and considered acceptable to support the current validation. See KCA 4.2/03.

Extraction efficiency:

A study has been provided to compare the QuEChERS extraction procedure with the metabolism study extraction procedure. Extraction efficiency has been demonstrated for the QuEChERS method for the determination of inpyrfluxam in goat liver, goat fat, goat milk, hen composite muscle and hen composite egg. See KCA 4.2/06 for full details.

This is sufficient to address the extraction efficiency in bovine fat and liver.

Conclusion

The method is acceptably validated in accordance with SANTE 2020/12830 rev.1 for the determination of inpyrfluxam in bovine liver and fat.

Acceptable primary method validation is available in bovine fat, meat, liver, milk, blood and urine and poultry eggs. The method has been independently validated as part of the current study in bovine liver and fat. The QuEChERS method has be sufficiently validated to the requirements outlined in SANTE/2020/12830 rev. 1 and can be considered an acceptable monitoring method for inpyrfluxam in products of animal origin.

Reference:	KCA 4.2/05
Report Title:	Extraction Efficiency of the Analytical Method of [C^{14}]S-2399 in Various Crops
Author(s) & Year:	[redacted] 2018a
Document No, Authority registration No	Study No: 3031W Report No: TPA-0062
Guideline(s):	SANTE/2017/10632 rev. 3
Deviations:	No
GLP or GEP:	Yes
Acceptability:	Yes
Study relied upon:	Yes

Samples from the metabolism studies conducted with rice (EAG study 2508W), soybean (EAG study 2506W) and apples (EAG study 2508W) were used to investigate the extraction efficiency of the multi-residue QuEChERS analytical method, validated under KCA 4.2/01 and KCA 4.2/02, for the determination of residues of inpyrfluxam. The extraction procedure used in the QuEChERS method were compared to the extraction procedures used in the metabolism studies.

Only samples that contained quantifiable concentrations of [C^{14}]inpyrfluxam were selected for the investigation.

Extraction: Original Metabolism Method.

In the original metabolism studies, aliquots of processed rice grain, rice straw, apple peel, apple fruit pulp (20-50 g) and mature soybean pods (20-30 g) were weighed into centrifuge bottles. Initial extractions used neutral solvent mixtures of acetonitrile and water. Apple peel and pulp were extracted once with acetonitrile/water (1:1 v/v) and once with acetonitrile. Rice grain, rice straw, and mature soybean pod matrices were extracted twice with acetonitrile/water (1:1 v/v) and once with acetonitrile. For each extraction the solvent was added and then mechanically shaken for 30 minutes. The mixture was centrifuged at 10,000 rpm for 10 minutes. The supernatant was measured, and aliquots were analysed by liquid scintillation counting (LSC). No further extractions were performed on apple pulp or peel. Soybean and rice matrices were further extracted with other solvents.

Extraction: QuEChERS Method.

Aliquots of processed rice grain (~5 g), rice straw (~2 g), mature soybean pod (~2 g) or whole apple (~10 g) were weighed into centrifuge tubes. The amount of moisture in each aliquot was calculated using a Mettler Toledo Moisture Analyzer. 9.4 mL of water was added to rice grain resulting in a total moisture content of 10 mL. Acetonitrile (10 mL) was added to give a 1:1 (v/v) solution.

To rice straw (~2 g), 14.4 mL of water was added resulting in a total moisture content of 15 mL. 15 mL of acetonitrile was added to give a 1:1 (v/v) solution. An initial attempt to extract rice straw used ~5 g of rice straw with 8.5 mL water added to result in a total moisture content of 10 mL. 10 mL of acetonitrile was subsequently added. However, the initial attempt resulted in low extraction efficiency and was replaced by the method described above. In the initial method, much of the solvent was soaked up by the straw resulting in less extract recovered and a lower extraction efficiency.

To mature soybean pods (~2 g) 14.7 mL of water was added resulting in a total moisture content of 15 mL.15 mL of acetonitrile was added to give a 1:1 (v/v) solution. An initial attempt to extract added 14.4 mL water to also result in a total moisture content of 15 mL but used ~5 g of soybean pods. Similarly to rice straw, the greater mass of sample resulted in more liquid being soaked up by the matrix resulting in less extract being recovered and lower extraction efficiency.

To whole processed apple, 1.8 mL water was added to result in a total moisture content of 10 mL. Acetonitrile (10 mL) was added to give a 1:1 (v/v) solution.

The rice grain, rice straw, and soybean samples were left to soak for 20 minutes at room temperature between the addition of water and acetonitrile. Acetonitrile was added to the apple samples directly after the addition of water.

Samples were shaken vigorously for 15 minutes then centrifuged at 3200 rpm for 5 minutes. The supernatant volume was measured, and aliquots were analysed by LSC.

Quantification

Radioactivity in sample extracts samples was determined by LSC. Post extraction solid (PES) residues were determined by combustion. Total radioactive residues (TRR) were determined as the sum of QuEChERS extraction and PES.

Whole apple fruit was extracted in the QuEChERS method however, in the original apple metabolism study, peel and pulp were extracted separately. To compare the two extraction methods, a whole fruit TRR has been calculated by adding together the original peel and pulp metabolism data.

HPLC-UV-Vis was used to determine the level of inpyrfluxam in extracts.

Table 5.2.1-5: TRR obtained from the metabolism study

Crop/matrix	Metabolism Study				
	TRR			Identified as inpyrfluxam	
	TRR – Total (mg/kg)	TRR – extracted (mg/kg)	%TRR extracted	TRR (mg/kg)	%TRR
Rice grain	0.049	0.047	95.9	0.038	78.6
Rice straw	0.927	0.755	81.4	0.534	57.6
Soybean mature pod (rinsed pod)[1]	0.687	0.436	58.8	0.170	23.0
Apple pulp + peel (Whole apple)[2]	0.104	0.094	90.4	0.060	57.7

[1] TRR in mature pods (including rinses) was 0.742 mg/kg. Rinses accounted for 0.055 mg/kg (7.4% TRR), of which 0.046 mg/kg (6.2% TRR) was inpyrfluxam.
[2] A whole fruit TRR has been calculated by adding together the original peel and pulp metabolism data so the extraction methods can be compared.

Table 5.2.1-6: TRR obtained from the QuEChERS extraction method

Crop/matrix	QuEChERS				
	TRR			Identified as inpyrfluxam	
	TRR – Total (mg/kg)	TRR – extracted (mg/kg)	%TRR extracted	TRR (mg/kg)	%TRR
Rice grain	0.051	0.038	74.5	0.037	72.5
Rice straw	0.909	0.510	56.1	0.447	49.2
Soybean mature pod (rinsed pod)	0.918	0.404	44.0	0.210	22.9
Whole apple	0.154	0.082	53.2	0.067	43.5

Conclusion

The HPLC profiles of each matrix is sufficiently similar to the HPLC analysis conducted in the original metabolism studies, indicating sufficient stability over the course of the study; stability is considered sufficient only for the purposes of this study.

Both methods extracted >70% TRR from rice grain, with the majority of extracted residues accounting for inpyrfluxam. Therefore, extraction efficiency is sufficiently addressed in rice grain for both methods.

For rice straw, >70% TRR was extracted from the metabolism study, the majority of extracted residue attributed to inpyrfluxam. However, for the QuEChERS extraction method only 56.1% TRR was extracted, 49.2% TRR for inpyrfluxam. It is noted that the levels of inpyrfluxam extracted by the QuEChERS method are similar to those extracted by the metabolism extract procedure (0.534 mg/kg; 57.6 %TRR vs. 0.447 mg/kg; 49.2 %TRR). In the available metabolism study minimal levels of inpyrfluxam were found in other extract fractions, with the majority extracted via solvent extraction. As the %TRR extracted in the metabolism study was shown to be the majority of inpyrfluxam residues, and similar extraction was seen by the available QuEChERS method, extraction efficiency can be considered acceptable for the QuEChERS extraction procedure for rice straw.

For soybean mature pods, both the metabolism and QuEChERS extraction procedure showed <70 %TRR (0.436 mg/kg; 58.8 %TRR, 0.404 mg/kg; 44.0 %TRR, respectively). As similar levels were shown between the metabolism study and the QUEChERS extraction method, and the available metabolism study shows that the majority of inpyrfluxam residues were extracted via solvent extraction, it can be concluded that the available data demonstrates acceptable extraction efficiency for inpyrfluxam from mature soybean pods.

For whole apple, similar levels of inpyrfluxam were extracted by both the metabolism study and the QuEChERS method. As analysis of the PES and other extract fractions in the metabolism study demonstrated the majority of inpyrfluxam were extracted via solvent extraction and both the metabolism and QuEChERS method demonstrated similar extractability levels of inpyrfluxam it can be concluded that the QuEChERS extraction procedure demonstrated acceptable extraction efficiency.

Acceptable extraction efficiency has been demonstrated for the QuEChERS method for the determination of inpyrfluxam in rice grain and straw (dry commodity group), soyabean mature pods (high oil commodity) and whole apple (high water commodity group).

Reference:	KCA 4.2/06
Report Title:	Extraction Efficiency of the Analytical Method of [^{14}C]S-2399 in Animal Matrices
Author(s) & Year:	[redacted] 2018b
Document No, Authority registration No	Report No: TPA-0063 Study No: 3032W

Guideline(s):	SANTE/2017/10632 rev. 3
Deviations:	No
GLP or GEP:	Yes
Acceptability:	Yes
Study relied upon:	Yes

The extraction efficiency of the residue analytical method based on QuEChERS (as validated in Study S17-03967 and Study 3032W) for measurement of extractable radioactivity and determination of inpyrfluxam in animal matrices was tested using aged samples obtained from the metabolism studies for goat and hen, which were performed with [Phenyl-U-^{14}C]inpyrfluxam or [Pyrazolyl-4-^{14}C]inpyrfluxam (purity > 99%).

Only samples with quantifiable concentrates of [^{14}C]inpyrfluxam were selected for experiment, as such extraction efficiency was investigated in goat fat (omental, subcutaneous and renal), goat liver, goat milk, hen eggs and hen muscle (breast and thigh)

Extraction: Original Metabolism Method.

In the original metabolism studies, aliquots of processed animal matrices were weighed into centrifuge bottles for extraction. For each extraction, samples were shaken with extraction solvent for 45 minutes then centrifuged for 10 minutes at 10,000 rpm. Goat liver was extracted twice with acetonitrile/water (1:1, v/v) and once with acetonitrile. All three extracts were combined for concentration and analysis. Goat fat (subcutaneous, renal, and omental) was extracted with hexane/acetone (4:1, v/v) and then twice with acetone. All three extracts were combined for concentration and analysis. Whole goat milk was centrifuged prior to extraction to separate the skim milk from the milk fat. The skim milk was extracted once with acetone and then once with acetone/water (1:1, v/v). Only the first extract was used for analysis. Milk fat was extracted twice with hexane/acetone (4:1, v/v) and once with acetone. The three extracts were combined prior to concentration and analysis. Hen muscle (breast and thigh) and hen eggs were extracted twice with acetonitrile/water (1:1, v/v) and once with acetonitrile. Extracts were combined prior to concentration and analysis.

Extraction: QuEChERS Method

Composite samples were made of the goat fat (omental, subcutaneous and renal), goat milk, hen muscle and egg matrices. The animal samples were allowed to thaw and homogenize before composites were made. Aliquots of the animal matrices (5 g of goat liver, whole goat milk, and hen eggs, 0.5 g of goat fat) were weighed into 50 mL centrifuge tubes. 20 g of hen muscle was used for extraction therefore the sample was weighed into a 250 mL centrifuge bottle. Water was added to the samples (6 mL to goat liver, whole goat milk and hen eggs, 2.5 mL to goat fat, and 24 ml to hen muscle). Acetonitrile was added immediately after the addition of water (10 mL to goat liver, whole goat milk, and eggs, 2.5 mL to goat fat, and 40 mL to hen muscle). Samples were shaken for fifteen minutes and centrifuged for 5 minutes at 3200 rpm. The supernatant volume was measured, and aliquots were analysed by liquid scintillation counting (LSC).

Quantification

Radioactivity in sample extracts samples was determined by LSC. Post extraction solid (PES) residues were determined by combustion. Total radioactive residues (TRR) were determined as the sum of QuEChERS extraction and PES.

HPLC-UV/VIS was used to determine levels of inpyrfluxam in extracts.

Table 5.2.1-7: TRR obtained from the metabolism study

Crop/matrix	Metabolism Study				
	TRR			Identified inpyrfluxam	
	TRR – Total (mg/kg)	TRR – extracted (mg/kg)	%TRR extracted	TRR (mg/kg)	%TRR
Goat liver	0.313	0.285	91.1	0.019	5.9
Goat Fat:					
Omental	0.024	0.021	87.5	0.004	15.8
Subcutaneous	0.029	0.028	96.6	0.002	6.4
Rental	0.041	0.037	90.2	0.004	8.2
Goat milk	0.018	0.017	94.4	0.002	9.1
Hen Muscle:					
Breast	0.012	0.011	91.7	≤0.001	2.9
Thigh	0.013	0.012	92.3	0.001	4.9
Hen eggs	0.02	0.018	90.0	0.002	10.9

Table 5.2.1-8: TRR obtained from the QuEChERS extraction method

Crop/matrix	QuEChERS				
	TRR			Identified inpyrfluxam	
	TRR – Total (mg/kg)	TRR – extracted (mg/kg)	%TRR extracted	TRR (mg/kg)	%TRR
Goat liver	0.310	0.221	71.3	0.006	1.9
Goat fat	0.035	0.027	77.1	ND	ND
Goat milk	0.023	0.022	95.7	ND	ND
Hen composite muscle	0.013	0.009	69.2	0.001	5.3
Hen composite egg	0.022	0.016	72.7	0.006	25.7

ND = not detected

Conclusion

The HPLC profiles of goat liver, hen muscle and eggs are sufficiently similar to the HPLC analysis conducted in the original metabolism studies, indicating sufficient stability over the

course of the study; stability is considered sufficient only for the purposes of this study. Storage stability in goat fat and goat milk has not been demonstrated as inpyrfluxam was not detected using the QuEChERS method. Therefore, comparison of the HPLC profiles could not be conducted.

In all matrices (with the exception of hen composite muscle for the QuECHERS method) >70% TRR was extracted. For hen composite muscle the reported recovery was <70% TRR (69.2%) however, the overall TRR is low (<0.01 mg/kg) and levels of inpyrfluxam from the metabolism study extraction and the QuEChERS extraction are similar (≤0.002 mg/kg; 7.8 %TRR, 0.001 mg/kg; 5.3 %TRR). Therefore, it can be concluded that residues of inpyrfluxam have been sufficiently extracted despite the slightly lower overall %TRR extracted.

Due to the overall low residual concentrations of inpyrfluxam in goat fat and milk (≤0.01 mg/kg) in the metabolism study, inpyrfluxam was not detected using the QuEChERS extraction method.

While the levels of inpyrfluxam extracted from animal matrices is low for the QuEChERS method, it is similar to the levels extracted from the procedure used in the metabolism study. The available metabolism study on animal matrices shows that inpyrfluxam accounts for very little of the overall residues, therefore high levels are not expected. The overall %TRR extracted by both methods is acceptable.

It can be concluded that the extractability of inpyrfluxam is sufficiently addressed for the QuEChERS monitoring method in goat liver, goat fat, goat milk, hen composite muscle and hen composite egg.

B.5.2.3. Methods for residues in soil sediment

Reference:	KCA 4.1.2/03
Report Title:	Validation Study for the Determination of S-2399 and its Metabolites 3'-OH-S-2840, 1'-COOH-S-2840A and 1'-COOH-S-2840B in Soil
Author(s) & Year:	[redacted]
Document No, Authority registration No	Report No.: TPA-0043
Guideline(s):	SANCO/825/00 rev. 8.1
Deviations:	No
GLP or GEP:	Yes
Acceptability:	Yes
Study relied upon:	Yes

Report KCA 4.1.2/03 has been submitted to support the monitoring of residues of inpyrfluxam in soil. This method was validated in section B.5.1.2.1.

The inpyrfluxam standard used for the validation procedure is noted to be 99.9% Inpyrfluxam (R-isomer).

Conclusion

The analytical method is validated according to SANTE/2020/12830 rev. 1 for the determination of inpyrfluxam, 3'-OH-S-2840, 1'-COOH-S-2840A and 1'-COOH-S-2840B in soil and is suitable for the monitoring of inpyrfluxam and its metabolites in soil.

B.5.2.4. Methods for residues in water

Reference:	KCA 4.2/07
Report Title:	Validation of an Analytical Method for the Determination of S-2399 in Ground and Surface Water
Author(s) & Year:	[redacted] 2017c
Document No, Authority registration No	Study No.: S17-03968 Report No.: TPA-0050
Guideline(s):	SANCO/825/00, rev. 8.1
Deviations:	No
GLP or GEP:	Yes
Acceptability:	Yes
Study relied upon:	Yes

KCA 4.2/07 has been submitted to support the monitoring of residues of inpyrfluxam in ground and surface water. Primary validation reported in the current study report was conducted at Eurofins Agroscience Services, Niefern-Öschelbronn, Germany. ILVs reported under KCA 4.2/08 were validated by EAG Laboratories GmbH, Ulm, Germany.

The standard used for the validation procedure is noted to be 99.9% R-isomer.

Ground water was taken from Rotenbach, Neuenbürg on 19 February 2017 and Surface water was taken from Hanhofen, Pfalz on 10 May 2017. The water samples were stored at 1-10°C in the dark. The results of the water characterization (pH, TOC (total organic carbon), DOC (dissolved organic carbon), total hardness and electrical conductivity) are given in below:

Parameter	Ground Water (Rotenbach)	Surface Water (Hanhofen)
pH value	7.05 (at 16.7 °C)	8.13 (at 25.2 °C)
Electrical conductivity (µS/cm)	69.4 (at 25 °C)	475 (at 25 °C)
Total organic carbon (TOC) (mg/L)	0.78	13.5
Dissolved organic carbon (DOC) (mg/L)	0.70	10.4
Biochemical oxygen demand (mg/L)	<1	1.92
Total nitrogen (mg/L)	<1.5	<1.5
Total phosphorus (mg/L)	<0.02	0.08
Dissolved orthophosphate (mg/L)	0.03	0.19
Nitrite (mg/L)	<0.01	0.02
Nitrate (mg/L)	5.3	0.82
Ammonium (mg/L)	<0.02	0.19
Total hardness	1.1 °dH	10.7 °dH
	0.2 mmol/L	1.92 mmol/L

Principle of the method

Each 10 mL specimen of ground and surface water is added to a 20 mL screw neck vial. Acetonitrile (10 mL) is added for stabilization. The vial is capped and mixed with a vortex

mixer. An aliquot is added to a HPLC vial and stored at 1 -10 °C in the dark. The sample is then analysed by HPLC-MS/MS using the conditions outlined below.

HPLC-MS/MS conditions

HPLC system Shimadzu HPLC System (LC-30 AD pumps, SIL 30 ACMP Autosampler, column oven and vacuum solvent degasser)

Pre-column HPLC guard column (KJ0-4282, Phenomenex) with C18 cartridge (AJ0-4287, Phenomenex)

Column Thermo C18, No. 70105-102130, 100 mm x 2.1 mm, 5 µm

Mobile phase Eluent A = 0.1% formic acid in water
Eluent B = 0.1% formic acid in acetonitrile

Time (minutes)	%A	%B
0.0	70	30
1.0	5	95
2.5	5	95
2.6	70	30
4.0	70	30

Column oven temperature 40 °C

Injection volume 7 µL

Flow rate 0.600 mL/min

MS system API 5500™ LC-MS/MS System (Sciex)

Ionisation Electrospray ionisation (ESI)

Polarity Positive

Mass transition monitored 334 → 258 (*m/z*) (used for quantification)
334 → 238 (*m/z*)

Scan Type MS/MS, MRM

Retention time ~1.5 minutes

Table 5.2.1-9: Summary of method validation for the monitoring of inpyrfluxam in ground water and surface water

Analyte	Matrix	LOQ (µg/L)	Recovery fortification level (µg/L)	Recoveries % range (mean)	Repeatability % RSD (n)	Linearity	Specificity
Inpyrfluxam (334 → 258 *m/z*) Quantification	Ground Water	0.03	0.03	92 – 96 (94) N = 5	2 @ ~0.03 µg/L (N=5)	0.0030 – 1.0 ng/mL, corresponding to 0.0060 – 2.0 µg/L	Retention time match to reference standard. No
			0.3	84 – 88 (86) N = 5	2 @ ~0.3 µg/L (N=5)		

	Surface Water		0.03	95 – 101 (97) N = 5	3 @ ~0.03 µg/L (N=5)	inpyrfluxam in water (n = 8) y = 1400000x – 16.8 r = 0.9989	significant interfering peaks observed in the blank matrix.
			0.3	89 – 91 (90) N = 5	1 @ ~0.3 µg/L (N=5)		
Inpyrfluxam (334 → 238 *m/z*) Confirmation	Ground Water	0.03	0.03	91 – 96 (93) N = 5	2 @ ~0.03 µg/L (N=5)	0.0030 – 1.0 ng/mL, corresponding to 0.0060 – 2.0 µg/L inpyrfluxam in water (n = 8) y = 1700000x – 93 r = 0.9991	
			0.3	88 – 94 (91) N = 5	3 @ ~0.3 µg/L (N=5)		
	Surface Water		0.03	95 – 100 (98) N = 5	2 @ ~0.03 µg/L (N=5)		
			0.3	89 – 91 (90) N = 5	1 @ ~0.3 µg/L (N=5)		

Matrix effects

The effect of the matrix on the LC-MS/MS response was determined by comparing the peak areas of matrix-matched standards with solvent standards at identical concentrations. The effect of the matrix was not significant for ground water or surface water (<20%).

Specificity

Specificity was demonstrated by retention time match with a reference standard and the absence of significant (>30% LOQ) interfering peaks in the chromatogram of a matrix blank sample.

Two mass transitions were validated for the method. Product ion spectra were provided to justify selection of ions.

Linearity

Linearity of both mass transitions was demonstrated by the analysis of eight matrix-matched standards of increasing concentration. The range of standard concentrations used was 0.0030 – 1.0 ng/mL. The concentrations extend over an appropriate range, and the response was linear with a correlation coefficient (r) greater than 0.99 for both transitions. No residual plots were submitted.

Precision (repeatability)

The precision of the method was assessed via analysis of the accuracy samples for both transitions in both ground and surface water. The reported %RSDs were <20% for both transitions and matrices.

Accuracy (recovery)

The accuracy of the method was assessed by analysing five samples fortified with inpyrfluxam for both transitions in both ground and surface water at concentrations of 0.03 and 0.3 µg/L. Recovery values were calculated as a percentage of measured concentration relative to fortified concentration. The mean recovery levels are within the range 70 to 120%.

LOQ

The limit of quantitation (LOQ), defined as the lowest fortification level where acceptable precision and accuracy data were obtained, has been demonstrated to be 0.03 µg/L for both surface and ground water.

Stability of standards and extracts

The stability of extracts was demonstrated by injecting the same sample (fortified with 0.3 µg/L of inpyrfluxam) before and after storage at 1 – 10 °C for 7 days in the dark. After this period, the final extracts were re-analysed against freshly prepared calibration standards. The mean percentage of initial recoveries were calculated. All mean recoveries were within the range 70-120%.

The stability of stock and fortification solutions prepared in methanol and calibration solutions prepared in acetonitrile/0.1 % formic acid (1/1, v/v), was demonstrated in report TPA-0049. Here, it was reported that the stability of stock solutions diluted in methanol was 31 days and stability of solvent calibration solutions in acetonitrile/ 0.1 % formic acid (1/1, v/v) was 23 days when stored at 1 °C to 10 °C in the dark.

Conclusion

The analytical method is acceptably validated according to SANTE/2020/12830 rev. 1 for the monitoring of the active substance inpyrfluxam in ground water and surface water.

Reference:	KCA 4.2/08
Report Title:	Independent Laboratory Validation of an Analytical Method for the Determination of S-2399 in Water
Author(s) & Year:	[redacted] 2018b

Document No, Authority registration No	Study No.: P 4664 G Report No.: TPA-0060
Guideline(s):	SANCO/825/00, rev. 8.1
Deviations:	No
GLP or GEP:	Yes
Acceptability:	Yes
Study relied upon:	Yes

Study KCA 4.2/08 has been submitted to support the ILV for the monitoring of residues of inpyrfluxam in ground and surface water. The ILV reported under KCA 4.2/08 was validated by EAG Laboratories GmbH, Ulm, Germany.

Principle of the method

Each 10 mL specimen of ground and surface water is added to a 20 mL screw neck vial. Acetonitrile (10 mL) is added for stabilization. The vial is capped and mixed with a vortex mixer. An aliquot is added to a HPLC vial and stored at 1 -10 °C in the dark. The sample is then analysed by HPLC-MS/MS using the conditions outlined below.

HPLC-MS/MS conditions

HPLC system Agilent 1290 Series HPLC system (vacuum solvent degasser, binary HPLC pump, column oven) and CTC Analytics HTC PAL System Autosampler, Analyst 1.6.3 Instrument control and data acquisition software

Pre-column Phenomenex C18, 4 mm x 3 mm

Column Thermo Betasil C18, 100 mm length x 2.1 mm i.d., 5.0 µm particle

Mobile phase Eluent A = 0.1% formic acid in water
Eluent B = 0.1% formic acid in acetonitrile

Time (minutes)	%A	%B
0.0	70	30
1.0	5	95
2.5	5	95
2.6	70	30
4.0	70	30

Column oven temperature 40 °C

Injection volume 10 µL

Flow rate 0.600 mL/min

MS system Applied Biosystems MDS Sciex API 5500 Q-Trap system with TurboIonspray (ESI) source

Ionisation Electrospray ionisation (ESI)

Polarity Positive

Mass transition monitored 334 → 258 (*m/z*) (used for quantification)
334 → 238 (*m/z*)
Retention time ~1.6 minutes

Table 5.2.1-10: Summary of method validation for the ILV of inpyrfluxam in ground water and surface water

Analyte	Matrix	LOQ (µg/L)	Recovery fortification level (µg/L)	Recoveries % range (mean)	Repeatability % RSD (n)	Linearity	Specificity
Inpyrfluxam (334 → 258 *m/z*) Quantification	Ground Water	0.03	0.03	98 – 105 (100) N = 5	3 @ ~0.03 µg/L (N=5)	0.0030 – 1.0 ng/mL, corresponding to 0.0060 – 2.0 µg/L inpyrfluxam in water (n = 8) y = 536000x – 563 r = 0.9988	Retention time match to reference standard. No significant interfering peaks observed in the blank matrix.
			0.3	95 – 105 (100) N = 5	4 @ ~0.3 µg/L (N=5)		
	Surface Water		0.03	87 – 98 (91) N = 5	5 @ ~0.03 µg/L (N=5)		
			0.3	94 – 99 (96) N = 5	2 @ ~0.3 µg/L (N=5)		
Inpyrfluxam (334 → 238 *m/z*) Confirmation	Ground Water	0.03	0.03	97 – 110 (104) N = 5	4 @ ~0.03 µg/L (N=5)	0.0030 – 1.0 ng/mL, corresponding to 0.0060 – 2.0 µg/L inpyrfluxam in water (n = 8) y = 593000x – 398 r = 0.9989	
			0.3	98 – 108 (103) N = 5	4 @ ~0.3 µg/L (N=5)		
	Surface Water		0.03	87 – 97 (92) N = 5	4 @ ~0.03 µg/L (N=5)		
			0.3	97 – 102 (99) N = 5	2 @ ~0.3 µg/L (N=5)		

Matrix effects

The effect of the matrix on the LC-MS/MS response was determined by comparing the peak areas of matrix-matched standards with solvent standards at 0.030 and 0.50 ng/mL. The effect of the matrix was not significant for ground water or surface water (<20%).

Specificity

Specificity was demonstrated by retention time match with a reference standard and the absence of significant (>30% LOQ) interfering peaks in the chromatogram of a matrix blank sample.

Two mass transitions were validated for the method. Product ion spectra were provided to justify selection of ions.

Linearity

Linearity of both mass transitions was demonstrated by the analysis of eight standards of increasing concentration. The range of standard concentrations used was 0.0030 – 1.0 ng/mL. The concentrations extend over an appropriate range, and the response was linear with a correlation coefficient (r) greater than 0.99 for both transitions. No residual plots were submitted.

Precision (repeatability)

The precision of the method was assessed via analysis of the accuracy samples for both transitions in both ground and surface water. The reported %RSDs were <20% for both transitions and matrices.

Accuracy (recovery)

The accuracy of the method was assessed by analysing five samples fortified with inpyrfluxam in duplicate for both transitions in both ground and surface water at concentrations of 0.03 and 0.3 µg/L. Recovery values were calculated as a percentage of measured concentration relative to fortified concentration. The mean recovery levels are within the range 70 to 120%.

LOQ

The limit of quantitation (LOQ), defined as the lowest fortification level where acceptable precision and accuracy data were obtained, has been demonstrated to be 0.03 µg/L. The LOQ for ILV is the same as the LOQ for the monitoring method.

Stability of standards and extracts

The stability was demonstrated in the primary monitoring method for water (KCA 4.2/07).

Conclusion

The ILV method is validated according to SANTE/2020/12830 rev. 1 for the monitoring of the active substance inpyrfluxam in ground water and surface water.

Acceptable primary method validation is available in ground and surface water. The method has been independently validated as part of the current study also in ground and surface water. The method has been sufficiently validated to the requirements outlined in SANTE/2020/12830 rev. 1 and can be considered an acceptable monitoring method for determining inpyrfluxam in ground and surface water.

B.5.2.5. Methods for residues in air

Reference:	KCA 4.2/09
Report Title:	Validation of an Analytical Method for the Determination of S-2399 in Air
Author(s) & Year:	[redacted] [redacted] 2017
Document No, Authority registration No	Study No.: P 4391 G Report No.: TPA-0052
Guideline(s):	SANCO/825/00 rev. 8.1
Deviations:	No
GLP or GEP:	Yes
Acceptability:	Yes
Study relied upon:	Yes

Study KCA 4.2/09 has been submitted to support the monitoring of residues of inpyrfluxam in air.

The inpyrfluxam standard used for the validation procedure is noted to be 99.9% Inpyrfluxam (R-isomer).

Principle of the method

Air is sucked through XAD adsorption tubes at about 1.0 L/min for 6 hours (total air sampling ≈ 0.36 m^3). Adsorption portion A (sampling portion) with the first and second (middle) glass plug is added to a centrifuge vial. The empty glass tube was rinsed with 3 mL of acetonitrile. The rinse was collected in the centrifuge vial. The sample was sonicated for 3 minutes. The extract was then transferred into a graduated centrifuge vial. The extraction procedure was repeated twice more, each time with 3 mL of acetonitrile. The extracts were combined, and the volume is brought up to 10 mL with acetonitrile. The sample was then mixed. The sample was then diluted by a factor of 100: 10 µL of extract was added to 990 µL of water/acetonitrile (70/30, v/v) + 0.1% formic acid in an autosampler vial. The sample was then analysed by HPLC-MS/MS under the following conditions.

HPLC-MS/MS conditions

HPLC system Agilent 1290 HPLC system (vacuum solvent degasser, binary HPLC pump, column oven), and CTC Analytics HTC-Pal Autosampler

Column Thermo Accucore Phenyl-Hexyl column: Length: 50 mm, i.d.: 4.6 mm, particle size: 2.6 µm

Mobile phase Eluent A = 0.1% formic acid in water
Eluent B = 0.1% formic acid in acetonitrile

Time (minutes)	%A	%B
0.0	70	30
4.5	10	90
5.0	10	90
5.1	70	30
6.5	70	30

Column oven temperature 40 °C
Injection volume 10 µL
Flow rate 1.00 mL/min
MS system AB Sciex API 5500 Q-Trap LC-MS/MS system
Ionisation Electrospray ionisation (ESI)
Polarity Positive
Mass transition monitored 334 → 258 (*m/z*)
334 → 238 (*m/z*)
Retention time ~3.0 minutes

Table 5.2.1-11: Summary of method validation for the monitoring of inpyrfluxam in air

Analyte	Matrix	LOQ (µg/m³)	Recovery fortification level (µg/m³)	Recoveries % range (mean)	Repeatability % RSD (n)	Linearity	Specificity
Inpyrfluxam (334 → 258 *m/z*) Quantification	Air	0.83	0.83	81 – 102 (95) N = 5	8.3 @ ~0.83 µg/m³ (N=5)	0.10 – 30 ng/mL, equivalent to 0.3 to 83.5 µg/m³ (n = 6) y = 307000x + 16700 r = 0.9981	Retention time match to reference standard. No significant interfering peaks observed in the blank matrix.
			8.35	101 – 110 (108) N = 5	3.3 @ ~8.35 µg/m³ (N=5)		
Inpyrfluxam (334 → 238 *m/z*)	Air	0.83	0.83	82 – 103 (96) N = 5	8.4 @ ~0.83 µg/m³ (N=5)	0.10 – 30 ng/mL, equivalent to	

Confirmation			8.35	104 – 110 (109) N = 5	2.3 @ ~8.35 µg/m^3 (N=5)	0.3 to 83.5 µg/m^3 (n = 6) y = 393000x + 24400 r = 0.9959	

Matrix effects

The effect of the matrix on the LC-MS/MS response was determined by comparing the peak areas of matrix-matched standards with solvent standards at identical concentrations (0.3 and 3.0 ng/mL). The effect of the matrix was not significant (<20%).

Specificity

Specificity was demonstrated by retention time match with a reference standard and the absence of significant (>30% LOQ) interfering peaks in the chromatogram of a matrix blank sample. A retention time match of the analyte was observed between the test sample and the calibration standard.

Two mass transitions were validated for the method. Product ion spectra were provided to justify selection of ions.

Linearity

Linearity of both mass transitions was demonstrated by the analysis of six standards of increasing concentration. The range of standard concentrations used was 0.1 – 30 ng/mL, equivalent to 0.3-83.5 µg/m^3. The concentrations extend over an appropriate range, and the response was linear with a correlation coefficient (r) greater than 0.99 for both transitions. No residual plots were submitted.

Precision (repeatability)

The precision of the method was assessed via analysis of the accuracy samples for both transitions. The reported %RSDs were <20% for both transitions.

Accuracy (recovery)

The accuracy of the method was assessed by analysing five samples fortified with inpyrfluxam for both transitions at concentrations of 0.83 and 8.35 µg/m^3. Recovery values were calculated as a percentage of measured concentration relative to fortified concentration. The mean recovery levels are within the range 70 to 120%.

LOQ

The limit of quantitation (LOQ), defined as the lowest fortification level where acceptable precision and accuracy data were obtained, has been demonstrated to be 0.83 µg/m^3.

Breakthrough test.

Breakthrough was evaluated by fortifying sampling cartridges with inpyrfluxam at 3 µg (8.4 µg/m^3). No breakthrough (< 10 %) into the back layer of the adsorption tubes was observed. Minor breakthrough was observed in one sample, which is most likely because of contamination during the fortification procedure.

Sorbent characteristics.

Air sampling uses adsorption tubes filled with two layers of XAD porous polymer. Particles and aerosols are trapped by filtration or impact on the adsorbent material. After air sampling (approximately 6 hours) the XAD layer A was analysed for recovery. For all cartridges fortified at the higher level the rear layer B was analysed for breakthrough determination.

Extractability

Extraction efficiency was determined by fortifying the analyte (triplicates at 0.30 µg (LOQ)) onto adsorbent portions of sampling cartridges. Extraction efficiency was demonstrated with average recoveries of 93 % for both transitions.

Stability of analyte on sorbent material

Storage stability of adsorbed inpyrfluxam was demonstrated by fortifying the analyte onto adsorbent layers of sampling cartridges (duplicates fortified at 3.0 µg (10xLOQ)). Storage stability of the analyte on XAD adsorbent material under refrigerated condition was demonstrated for 7 days with average recoveries of 110 % and 108 on the respective mass transitions.

Stability of standards and extracts

The stability of diluted final extracts was demonstrated by injecting the same sample (fortified with 0.3 µg of Inpyrfluxam) before and after storage under refrigerated conditions for 10 days in the dark. After this period, the final extracts were re-analysed against freshly prepared calibration standards. The mean percentage of initial recoveries were calculated. All mean recoveries were within the range 70-120%.

The stability of stock solutions was demonstrated by comparing newly prepared stock solution diluted in acetone and a stock solution that had been stored in a refrigerator for 10 days (freshly diluted into the same solvent. The difference before and after storage was less than 2% and therefore not considered significant.

The stability of calibration standards diluted in water/acetonitrile (7/3, v/v) + 0.1 % formic acid was demonstrated by reinjecting standards after storing for 10 days under refrigerated conditions and comparing to freshly prepared solutions. The difference before and after

storage was not significant (10%) in the quantification transition (334 → 258 *m/z*). It is noted that the difference was +13% in the confirmation transition but this is acceptable.

Conclusion

The analytical method is validated according to SANTE/2020/12830 rev. 1 for the monitoring of the active substance inpyrfluxam in air.

B.5.2.6. Methods for residues in body fluids and tissues

1'-COOH-S-2840 contains two diastereomers (1'-COOH-S-2840 A and 1'-COOH-S-2840 B) which, in turn, both have two enantiomers. Two studies have been submitted for the analytical method for monitoring in body tissue (These are summarised in study KCA 4.1.2/28 (report no.: TPR-0015) and KCA 4.1.2/29 (report no.: TPR-0013). These methods determine the sum of 1'-COOH-S-2840 A and 1'-COOH-S-2840 B. ~~These methods are not acceptably validated in accordance with SANTE/2020/12830 rev. 1 for monitoring of 1'-COOH-S-2840 in body tissue and therefore further information has been requested.~~

The methods are acceptably validated for monitoring as data was provided to address the extract and standard stability for 1'-COOH-S-2840.

Reference:	KCA 4.2/11
Report Title:	Validation of an Analytical Method for the Determination of Residues of 1'-COOH-S-2840 in Urine
Author(s) & Year:	[redacted] [redacted] 2017
Document No, Authority registration No	Study No.: S25-101865 Report No.: TPA-0094
Guideline(s):	SANTE/2020/12830 rev. 1
Deviations:	No
GLP or GEP:	Yes
Acceptability:	Yes
Study relied upon:	Yes

Study TPA-0094 has been submitted to support the for the monitoring of residues of 1'-COOH-S-2840 in body fluid (urine). In this method 1'-COOH-S-2840A and B were mixed at 1:1 in weight basis and applied for validation. 1'-COOH-S-2840A and 1'-COOH-S-2840B were fortified and quantified jointly.

Principle of the method

5.0 mL of porcine urine was filled into a 50 mL centrifuge tube. 5 mL of water was added, and the tube was shaken shortly. For extraction, 10 mL of acetonitrile was added. The centrifuge tube was capped and shaken vigorously by hand for one minute followed by shaking on a platform shaker for 15 minutes and 250 rpm. 4.0 g of magnesium sulphate, 1.0 g of sodium chloride, 1.0 g of trisodium citrate dihydrate and 0.5 g of disodium hydrogen citrate sesquihydrate were added. The centrifuge tube was capped again and immediately shaken by hand for one minute. The sample tube was centrifuged for about 5 minutes at about 3200 xg. Before analysis, 100 µL of the extract was transferred to a calibrated test tube and filled up exactly to 5000 µL with water/methanol (3/1, v/v). The tube was mixed by vortex, and an aliquot was transferred into a HPLC vial. The extract was stored at 1 – 10 °C in the dark until analysis by HPLC-MS/MS using the conditions outlined below.

The chromatographic peaks were integrated separately, and the sum of both peak areas was calculated.

HPLC-MS/MS conditions

HPLC system 1290 Infinity II High Speed Pump LC System, Agilent Technologies (UHPLC, ≤ 1300 bar)

Pre-column None

Column ACQUITY UPLC HSS T3 (150 mm x 2.1 mm, 1.8 µm, Waters, Art. No. 186003540)

Mobile phase Eluent A = 0.1% formic acid in acetonitrile
Eluent B = 0.1% formic acid in water

Time (minutes)	%A	%B
0.0	5	95
3.5	30	70
7.2	50	50
7.3	95	5
9.0	95	5
9.1	5	95
11.0	5	95

Column oven temperature 60 °C

Injection volume 3 µL

Flow rate 400 µL/min

MS system TripleQuad 6500 System, SCIEX (Triple quadrupole mass spectrometer)

Ionisation Electrospray ionisation (ESI)

Polarity	Positive
Mass transition monitored	364 → 318 (*m/z*) (used for quantification) 364 → 278 (*m/z*)
Scan Type	MS/MS, MRM
Retention time	1'-COOH-S-2840A ~6.8 minutes 1'-COOH-S-2840B ~7.1 minutes

Table 5.2.1-12: Summary of method validation for the monitoring of 1'-COOH-S-2840 in urine

Analyte	Matrix	LOQ (mg/L)	Recovery fortification level (mg/L)	Recoveries % range (mean)	Repeatability % RSD (n)	Linearity	Specificity
1'-COOH-S-2840 (364 → 318 *m/z*)	Urine	0.01	0.01	97 – 104 (101) N = 5	3.0 @ ~0.01 mg/L (N=5)	0.030 – 3.0 ng/mL, corresponding to 0.0030 – 0.30 mg/L 1'-COOH-S-2840 in urine (n = 8) y = 263930x – 952 r = 0.9999	Retention time match to reference standard. No significant interfering peaks observed in the blank matrix.
1'-COOH-S-2840 (364 → 278 *m/z*)	Urine	0.01	0.01	97 – 103 (100) N = 5	2.4 @ ~0.01 mg/L (N=5)	0.030 – 3.0 ng/mL, corresponding to 0.0030 – 0.30 mg/L 1'-COOH-S-2840 in urine (n = 8) y = 242476x – 575	

						r = 0.9999	

Matrix effects

The effect of the matrix on the LC-MS/MS response was determined by comparing the peak areas of matrix-matched standards with solvent standards at identical concentrations. The effect of the matrix was not significant (<20%). Furthermore, matrix-matched standards were used for the calibration.

Specificity

Specificity was demonstrated by retention time match with a reference standard and the absence of significant (>30% LOQ) interfering peaks in the chromatogram of a matrix blank sample.

Two mass transitions were validated for the method. Product ion spectra were provided to justify selection of ions.

Linearity

Linearity of both mass transitions was demonstrated by the analysis of eight matrix-matched standards of increasing concentration. The range of standard concentrations used was 0.030 – 3.0 ng/mL. The concentrations extend over an appropriate range, and the responses were linear with a correlation coefficient (r) of 0.9999 for both transitions.

Precision (repeatability)

The precision of the method was assessed via analysis of the accuracy samples for both transitions. The reported %RSDs were <20% for both transitions.

Accuracy (recovery)

The accuracy of the method was assessed by analysing five samples fortified with 1'-COOH-S-2840 for both transitions at 0.01 mg/L. Recovery values were calculated as a percentage of measured concentration relative to fortified concentration. The mean recovery levels are within the range 70 to 120%. Two control samples were also analysed alongside the 0.01 mg/L fortification which addresses the requirement in SANTE 2020/12830 rev.1.

LOQ

The limit of quantitation (LOQ), defined as the lowest fortification level where acceptable precision and accuracy data were obtained, has been demonstrated to be 0.01 mg/L which is sufficiently low enough.

Stability of standards and extracts

The stability of extracts was demonstrated by analysing five samples fortified with 0.01 mg/L of 1'-COOH-S-2840 after storage for 8 days at 1 to 10 °C in the dark. The final extracts were analysed against freshly prepared standards and the recoveries were determined. The mean recovery was within the range 70-120%. The final extracts have been demonstrated to be stable for 8 days when stored at 1 to 10 °C in the dark.

The stability of methanol stock solutions of 1'-COOH-S-2840 was determined by comparing the response of a freshly prepared dilution of a stock solution stored for 8 days at 1 to 10 °C in the dark, against a freshly prepared stock solution by five-fold injection. The difference between the response of the two samples was negligible (-0.8%). The methanol stock solutions of 1'-COOH-S-2840 have been demonstrated to be stable for 8 days when stored at 1 to 10 °C in the dark.

The stability of water/methanol (3/1, v/v) standard solutions of 1'-COOH-S-2840 was determined by comparing the response standard solution stored for 8 days at 1 to 10 °C in the dark, against a freshly prepared standard solution by five-fold injection. The difference between the response of the two samples was negligible (1.0%). The water/methanol (3/1, v/v) standard solutions of 1'-COOH-S-2840 have been demonstrated to be stable for 8 days when stored at 1 to 10 °C in the dark. It is noted that matrix-matched calibration standards were used. The final extract data above shows the analyte is stable for 8 days at 1 to 10 °C in the dark and therefore this is suitable to support the stability of analyte in calibration solutions.

Conclusion

The analytical method is validated according to SANTE/2020/12830 rev. 1 for the monitoring of 1'-COOH-S-2840 in urine.

B.5.3. References Relied On

Literature search

A literature review has been carried out for the active substance inpyrfluxam. One literature search was submitted to address all areas of the risk assessment. HSE has assessed the suitability of the mechanics of the literature search in line with EFSA guidance on conducting literature searches (EFSA Journal 2011). The literature review was conducted in accordance with Article 8(5) of Regulation No. 1107/2009 at the time of completion, and was conducted to comply with the EFSA guidance document as published in EFSA Journal 2011; 9(2):2092.

The process of selection of relevant scientific peer-reviewed open literature was based on a single-concept search in the CAS and Dialog platform databases. The time period was limited to studies published July 2013 up to July 2023 using the search criteria of inpyrfluxam, metabolites and mixtures and related CAS numbers, common names, trade names and lab codes.

A stepwise process for selection of relevant scientific peer-reviewed open literature was undertaken:

- A rapid assessment of the summary records references (e.g., titles, abstracts, index terms, keywords) was conducted.
- Summary records which appeared to be relevant went to the next level of evaluation.
- These were further evaluated and categorized into “reliable without restriction”, “reliable with restriction”, “not reliable” and “not assignable”.

The results of the literature review are as follows:

Summary of the review	n	Justification
Total number of summary records retrieved from search	352	Appendix 1
Number of summary records excluded after rapid assessment for relevance (by title/abstract)	349	Appendix 6

Number of summary records of potential/unclear relevance assessed in further detail (by abstract/full-text)	3	Appendices 4 and 5
Number of studies excluded from the risk assessment after detailed assessment of full-text documents (i.e. not relevant)	2	
Number of studies not excluded for relevance after detailed assessment (i.e. relevant studies and studies of unclear relevance)	1	
Number of studies included in the dossier as supporting information (reliability criteria 1-2)	1	

In the area of analytical method data no records were considered relevant for the assessment of analytical methods of the active substance inpyrfluxam.

Conclusion

Regarding the literature search undertaken by the applicant, it is considered that the search is acceptable in terms of databases searched and the search criteria applied. No references of relevance to this assessment were identified.

Data Point	Author(s)	Year	Title Company Report No. Source (where different from company) GLP or GEP status Published or not	Vertebrate Study Y/N	Data Protection Claimed Y/N	Justification if Data Protection is claimed	Owner	Previous evaluation
KCA 4.1.1/01	[redacted]	2016	Enforcement Analytical Methods of S-2399 Technical Grade Sumika Chemical Analysis Service Ltd., Japan, Study No. GP16057, Sumitomo Chemical Co., Ltd. Report No: TPA-0019. GLP, unpublished	N	Y	The study is necessary for this regulatory decision and is eligible for data protection	SUM	N
KCA 4.1.2/01	[redacted]	2017a	S-2399: Validation of Valent's Method RM-50S, "Determination of Residues of S-	N	Y	The study is necessary for this regulatory decision and is eligible for	SUM	N

			2399, 3'-OH-S-2840, 1'-COOH-S-2840-A, and 1'-COOH-S-2840-B in Soil", Valent USA Corporation, USA, Study ID: VP-38934, Sumitomo Chemical Co., Ltd, Report No.: TPA-0028 GLP, unpublished			data protection		
KCA 4.1.2/02	[redacted]	2017b	S-2399: Validation of Valent's Method RM-50V, "Determination of Residues of S-2399 on Application Verification Pads", Valent USA Corporation,	N	Y	The study is necessary for this regulatory decision and is eligible for data protection	SUM	N

			USA, Study No.: VP-38872, Sumitomo Chemical Co., Ltd. Report No. TPA-0029 GLP, unpublished					
KCA 4.1.2/03	[redacted]	2017	Validation Study for the Determination of S-2399 and its Metabolites 3'-OH-S-2840, 1'-COOH-S-2840A and 1'-COOH-S-2840B in Soil, Eurofins Agroscience Services EcoChem GmbH/Eurofins Agroscience Services Ecotox GmbH, Germany, Study No.: S16-05522, Sumitomo Chemical Co., Ltd.,	N	Y	The study is necessary for this regulatory decision and is eligible for data protection	SUM	N

			Report No.: TPA-0043 GLP, unpublished					
KCA 4.1.2/05	[redacted]	2015	Validation of Analytical Method for S-2399 Technical Grade (Polyethylene Glycol 400 Solution) on Glass Filters, Amendment of final report Sumika Technoservice Corporation, Japan, Study No.: 14002VAL, Sumitomo Chemical Co., Ltd. Report No.: TPA-0044 GLP, unpublished	N	Y	The study is necessary for this regulatory decision and is eligible for data protection	SUM	N

KCA 4.1.2/07	[redacted]	2013	Validation of Analytical Method for S-2399 TG in the Diet for Rodents, The Institute of Environmental Toxicology, Japan, Study No.: IET 13-5030, Sumitomo Chemical Co., Ltd., Report No.: TPA-0002 GLP, unpublished	N	Y	The study is necessary for this regulatory decision and is eligible for data protection	SUM	N
KCA 4.1.2/08	[redacted]	2014b/2020	Validation of Analytical Method for S-2399 in Dog Plasma, Final Report Amendment The Institute of Environmental Toxicology,	N	Y	The study is necessary for this regulatory decision and is eligible for data protection	SUM	N

			Japan, Study No.: IET 14-5074, Sumitomo Chemical Co., Ltd. Report No. TPA-0008 GLP, unpublished					
KCA 4.1.2/11	[redacted]	2015/2020	Validation of Analytical Method for S-2399 in Mouse Plasma, Final Report Amendment, The Institute of Environmental Toxicology, Japan, Study No.: IET 14-5052, Sumitomo Chemical Co., Ltd. Report No.: TPA-0009	N	Y	The study is necessary for this regulatory decision and is eligible for data protection	SUM	N

			GLP, unpublished					
KCA 4.1.2/12	[redacted]	2014a/2020	Validation of Analytical Method for S-2399 in Rat Plasma, Final Report Amendment, The Institute of Environmental Toxicology, Japan, Study No.: IET 14-5051, Sumitomo Chemical Co., Ltd. Report No.: TPA-0005 GLP, unpublished	N	Y	The study is necessary for this regulatory decision and is eligible for data protection	SUM	N
KCA 4.1.2/15	[redacted]	2017	3'-OH-S-2840: Validation of an Analytical Method in the Diet for Rodents, The Institute of	N	Y	The study is necessary for this regulatory decision and is eligible for	SUM	N

			Environmental Toxicology, Japan, Study No.: IET 17-5003, Sumitomo Chemical Co., Ltd. Report No.: TPA-0032 GLP, unpublished			data protection		
KCA 4.1.2/44	[redacted]	2009	Reg. No. 5069089 (metabolite of BAS 700 F) Repeated dose 90-day oral toxicity study in Wistar rats; Administration in the diet. Report ID: 2009/1072503	Y	N	The study is necessary for this regulatory decision	BASF (Letter of access provided to SUM)	Y
KCA 4.1.2/45	[redacted]	2009	Reg. No. 5069089 (metabolite of BAS 700 F) Prenatal developmental	Y	N	The study is necessary for this regulatory decision	BASF (Letter of access	Y

			toxicity study in New Zealand white rabbits Oral administration (gavage). Report ID: 2009/1072507				provided to SUM)	
KCA 4.1.2/46	[redacted]	2009	Reg. No. 5435595 (metabolite of BAS 700 F) Repeated dose 90-day oral toxicity study in Wistar rats; Administration in the diet Report ID: 2009/1012026	Y	N	The study is necessary for this regulatory decision	BASF (Letter of access provided to SUM)	Y
KCA 4.1.2/47	[redacted]	2009	Reg. No. 5435595 (metabolite of BAS 700 F) Prenatal developmental toxicity study in New Zealand white rabbits Oral administration (gavage).	Y	N	The study is necessary for this regulatory decision	BASF (Letter of access provided to SUM)	Y

			Report ID: 2009/1072509					
KCA 4.1.2/19	[redacted]	2017a	Validation of an Analytical Method for the Determination of three Metabolites of S-2399 (N-des-Me-S-2840, N-des-Me-1´-CH_2OH-S-2840A and N-des-Me-1´-CH_2OH-S-2840B) in Cereal (Whole Plant and Grain), Potato (Tubers), Grapes and Soybean (Seeds) Eurofins Agroscience Services Chem	N	Y	The study is necessary for this regulatory decision and is eligible for data protection	SUM	N

			GmbH, Germany Study No. S17-00277 (SUM-1701V) Sumitomo Chemical Co., Ltd. Report No. TPA-0053 GLP, unpublished					
KCA 4.1.2/20	[redacted]	2017	Validation of an Analytical Method for the Determination of S-2399 and its Metabolites in Cereal (Whole Plant and Grain), Potato (Tubers), Grapes and Soybean (Seeds) – Amendment No. 1 to Final Report.	N	Y	The study is necessary for this regulatory decision and is eligible for data protection	SUM	N

			Eurofins Agroscience Services Chem GmbH, Germany Study No. S16-03371 (SUM-1601V) Sumitomo Chemical Co., Ltd. Report No. TPA-0057 GLP, unpublished					
KCA 4.1.2/21	[redacted]	2017	S-2399: Validation of Valent's Method RM-50C-1, Determination of Residues of S-2399, 3-OHS-2840, 1-CH2OH-S-2840-A,1-CH2OH-S-2840-B, DFPA-CONH2, 1-COOH-S-2840-	N	Y	The study is necessary for this regulatory decision and is eligible for data protection	SUM	N

			A, and 1-COOH-S-2840-B in Crops (including Conjugated Forms of the Metabolites Converted to their Aglycones)", Valent U.S.A. LLC, USA, Study No.:VP-39140, Sumitomo Chemical Co., Ltd., Report No.: 201700135 GLP, unpublished					
KCA 4.1.2/28	[redacted]	2017	Magnitude of S-2399 and Metabolites 1'-CH_2OH-S-2840 A&B (including conjugate) and 1'-COOH-S-2840 A&B Residues in Laying Hen	Y	Y	The study is necessary for this regulatory decision and is eligible for data protection	SUM	N

			Tissues and Eggs from a 28-Day Feeding Study [redacted] Study No. 2815W. Sumitomo Chemical Co., Ltd. Report No: TPR-0015 GLP, unpublished					
KCA 4.1.2/29	[redacted]	2016	Magnitude of S-2399 and Metabolites 1'-CH2OH-S-2840 A&B (including conjugate) and 1'-COOH-S-2840 A&B Residues in Bovine Tissues and Milk from a 28-Day Feeding Study [redacted] Study No. 2814W. Sumitomo Chemical Co., Ltd.	Y	Y	The study is necessary for this regulatory decision and is eligible for data protection	SUM	N

			Report No: TPR-0013 GLP, unpublished					
KCA 4.1.2/30	[redacted]	2018d	Magnitude of the Residue of S-2399 and its Metabolites in Wheat Processed Fractions in Northern and Southern Europe - 2016, TESTAPI, France, Study No.: 261-2016 (S16-05040-L1), Sumitomo Chemical Co., Ltd., Report No.: TPR-0081 GLP, unpublished	N	Y	The study is necessary for this regulatory decision and is eligible for data protection	SUM	N

KCA 4.1.2/31	[redacted]	2018b	Magnitude of the Residue of S-2399 and its Metabolites in Barley Processed Fractions in Northern and Southern Europe - 2016, TESTAPI, France, Study No.: 262-2016 (S16-05042-L1), Sumitomo Chemical Co., Ltd., Report No.: TPR-0082 GLP, unpublished	N	Y	The study is necessary for this regulatory decision and is eligible for data protection	SUM	N
KCA 4.1.2/33	[redacted]	2014	Analytical Method Verification for the Determination of S-2399 TG in Avian Diet Wildlife International, USA, Study No.166C-119	N	Y	The study is necessary for this regulatory decision and is eligible for data protection	SUM	N

			Sumitomo Chemical Co., Ltd. Report No. TPA-0004 GLP, unpublished					
KCA 4.1.2/34	[redacted]	2014a	S-2399 TG - Acute Toxicity Test with Rainbow Trout (*Oncorhynchus mykiss*) Under Static Conditions Following OECD Guideline #203, OPPTS Draft Guideline 850.1075, JMAFF 12 NohSan, No. 8147 Fish, Acute Toxicity Test (2-7-1-1) and The Official Journal of the European Communities, L383A, Method C.1, Acute Toxicity for Fish, [redacted]	Y	Y	The study is necessary for this regulatory decision and is eligible for data protection	SUM	N

			Study No.: 13048.6776, Sumitomo Chemical Co., Ltd., Report No. TPW-0006 GLP, unpublished					
CA 4.1.2/35	[redacted]	2016a	Acute Toxicity Study of S-2399 TG with Guppy (*Poecilia reticulata*), [redacted] Study No.: 1603EFAG, Sumitomo Chemical Co., Ltd., Report No. TPW-0053 GLP, unpublished	Y	Y	The study is necessary for this regulatory decision and is eligible for data protection	SUM	N

CA 4.1.2/36	[redacted]	2016a	Acute Toxicity Study of 3'-OH-S-2840 with Rainbow trout (*Oncorhynchus mykiss*), [redacted] Study No.: 1512EFAR, Sumitomo Chemical Co., Ltd., Report No. TPW-0035 GLP, unpublished	Y	Y	The study is necessary for this regulatory decision and is eligible for data protection	SUM	N
CA 4.1.2/37	[redacted]	2016b	Acute Toxicity Study of 1'-COOH-S-2840 with Rainbow trout (*Oncorhynchus mykiss*), [redacted], Study No.: 1513EFAR,	Y	Y	The study is necessary for this regulatory decision and is eligible for data protection	SUM	N

			Sumitomo Chemical Co., Ltd., Report No. TPW-0036 GLP, unpublished					
CA 4.1.2/38	[redacted]	2015	Amended Report: Life-Cycle Toxicity Test Exposing Midges (*Chironomus dilutus*) to S-2399 TG Applied to Sediment Under Static-Renewal Conditions Following EPA Test Methods, Smithers Viscient, USA, Study No.: 12709.6364, Sumitomo Chemical Co., Ltd., Report No. TPW-0031 GLP, unpublished	N	Y	The study is necessary for this regulatory decision and is eligible for data protection	SUM	N

CA 4.1.2/39	[redacted]	2021	S-2399 - Fish Short-Term Reproduction Assay with Fathead Minnow (*Pimephales promelas*) [redacted], Study No. 13048.7149 Sumitomo Chemical Co., Ltd. Report No: TPW-0137, GLP, unpublished	Y	Y	The study is necessary for this regulatory decision and is eligible for data protection	SUM	N
KCA 4.1.2/40	[redacted]	2013a	S-2399: Determination of Water Solubility, Harlan Laboratories Ltd., UK, Study No.: 41303367, Sumitomo Chemical Co., Ltd., Report No.: TPP-0001 GLP, unpublished	N	Y	The study is necessary for this regulatory decision and is eligible for data protection	SUM	N

KCA 4.1.2/41	[redacted]	2013b	S-2399: Determination of Partition Coefficient (n-Octanol/Water), Harlan Laboratories Ltd., UK, Study No.: 41303719, Sumitomo Chemical Co., Ltd., Report No.: TPP-0002 GLP, unpublished	N	Y	The study is necessary for this regulatory decision and is eligible for data protection	SUM	N
KCA 4.1.2/43	[redacted]	2021	Report Amendment 1, S-2399 TGAI: Method Validation for Determination of S-2399 in Solvent Solubility Test. Covance Laboratories Ltd., UK, Study No.: 8456378.	N	Y	The study is necessary for this regulatory decision and is eligible for data protection	SUM	N

			Sumitomo Chemical Co., Ltd. Report No.: TPA-0085 GLP, unpublished					
KCA 4.2/01	[redacted]	2016	Validation of the Multi-Residue Method QuEChERS for the Determination of S-2399 in Matrices of Plant Origin, Eurofins Agroscience Services Chem GmbH, Germany, Study No.: S16-03372 (SUM-1602V), Sumitomo Chemical Co., Ltd., Report No.: TPA-0027 GLP, unpublished	N	Y	The study is necessary for this regulatory decision and is eligible for data protection	SUM	N

KCA 4.2/02	[redacted]	2017a	Independent Laboratory Validation of the Multi-Residue Method QuEChERS for the Determination of S-2399 in Matrices of Plant Origin, Eurofins Agroscience Services EcoChem GmbH/ Eurofins Agroscience Services Ecotox GmbH, Germany, Study No.: S17-03966, Sumitomo Chemical Co., Ltd., Report No.: TPA-0048 GLP, unpublished	N	Y	The study is necessary for this regulatory decision and is eligible for data protection	SUM	N
KCA 4.2/03	[redacted]	2017b	Validation of the Multi-Residue Method QuEChERS for the	N	Y	The study is necessary for this regulatory decision and is	SUM	N

			Determination of S-2399 in Different Matrices of Animal Origin and Body Fluids – Report Amendment1, Eurofins Agroscience Services EcoChem GmbH/ Eurofins Agroscience Services Ecotox GmbH, Germany, Study No.: S17-03967, Sumitomo Chemical Co., Ltd., Report No.: TPA-0049 GLP, unpublished			eligible for data protection		
KCA 4.2/04	[redacted]	2018a	Independent Laboratory Validation of an Analytical Method for the Determination of S-2399 in Matrices of	N	Y	The study is necessary for this regulatory decision and is eligible for data protection	SUM	N

			Animal Origin, Using LC/MS/MS, EAG Laboratories GmbH, Germany, Study No.: P 4665 G, Sumitomo Chemical Co., Ltd., Report No.: TPA-0061 GLP, unpublished					
KCA 4.2/05	[redacted]	2018a	Extraction Efficiency of the Analytical Method of [^{14}C]S-2399 in Various Crops, EAG Laboratories, USA, Study No.: 3031W, Sumitomo Chemical Co., Ltd., Report No.: TPA-0062 GLP, unpublished	N	Y	The study is necessary for this regulatory decision and is eligible for data protection	SUM	N

KCA 4.2/06	[redacted]	2018b	Extraction Efficiency of the Analytical Method of [^{14}C]S-2399 in Animal Matrices, EAG Laboratories, USA, Study No.: 3032W, Sumitomo Chemical Co., Ltd., Report No.: TPA-0063 GLP, unpublished	N	Y	The study is necessary for this regulatory decision and is eligible for data protection	SUM	N
KCA 4.2/07	[redacted]	2017c	Validation of an Analytical Method for the Determination of S-2399 in Ground and Surface Water, Eurofins Agroscience Services EcoChem GmbH / Eurofins Agroscience Services Ecotox GmbH, Germany,	N	Y	The study is necessary for this regulatory decision and is eligible for data protection	SUM	N

			Study No.: S17-03968, Sumitomo Chemical Co., Ltd., Report No.: TPA-0050 GLP, Unpublished					
KCA 4.2/08	[redacted]	2018b	Independent Laboratory Validation of an Analytical Method for the Determination of S-2399 in Water, Using LC/MS/MS, EAG Laboratories GmbH, Germany, Study No.: P 4664 G, Sumitomo Chemical Co., Ltd., Report No.: TPA-0060 GLP, Unpublished	N	Y	The study is necessary for this regulatory decision and is eligible for data protection	SUM	N

KCA 4.2/09	[redacted]	2017	Validation of an Analytical Method for the Determination of S-2399 in Air, EAG Laboratories GmbH, Germany, Study No.: P 4391 G, Sumitomo Chemical Co., Ltd., Report No.: TPA-0052 GLP, unpublished	N	Y	The study is necessary for this regulatory decision and is eligible for data protection	SUM	N
KCA 4.2/11	[redacted]	2017	Validation of an Analytical Method for the Determination of Residues of 1'-COOH-S-2840 in Urine Sumitomo Chemical Co., Ltd., Report No.: TPA-0094 GLP, unpublished	N	Y	The study is necessary for this regulatory decision and is eligible for data protection	SUM	N

Further information

For information about health and safety, or to report inconsistencies or inaccuracies in this guidance, visit www.hse.gov.uk.

You can order HSE priced publications at https://books.hse.gov.uk HSE priced publications are also available from bookshops.

This publication is available on the HSE website at www.hse.gov.uk/xxx

This guidance is issued by the Health and Safety Executive. Following the guidance is not compulsory, unless specifically stated, and you are free to take other action. But if you do follow the guidance you will normally be doing enough to comply with the law. Health and safety inspectors seek to secure compliance with the law and may refer to this guidance.

First published 0X/26. Published by the Health and Safety Executive 0X/26.