



# **Draft Assessment Report**

## **Evaluation of Active Substances**

Plant Protection Products

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

**Aqueous extract from the germinated seeds  
of sweet *Lupinus albus***

**Volume 3 – B.7 (AS)**

**Residue Data**

Great Britain

February 2025

## Version History

<b>When</b>	<b>What</b>
<b>June 2024</b>	Initial DAR
<b>February 2025</b>	Updates made after ECP
<b>February 2025</b>	Updates made after additional information submitted post ECP
	Updates made after public consultation
	Updates made after additional information submitted post public consultation
	[Updates made after any additional steps not covered by the above]

# Contents

<b>B.7. RESIDUE DATA .....</b>	<b>4</b>
<b>B.7.1. STORAGE STABILITY OF RESIDUES .....</b>	<b>4</b>
<b>B.7.2. METABOLISM, DISTRIBUTION AND EXPRESSION OF RESIDUES.....</b>	<b>4</b>
B.7.2.1. Plants .....	5
B.7.2.2. Poultry .....	17
B.7.2.3. Lactating ruminants.....	17
B.7.2.4. Pigs .....	17
B.7.2.5. Fish .....	17
<b>B.7.3. MAGNITUDE OF RESIDUE TRIALS IN PLANTS.....</b>	<b>19</b>
B.7.3.1. Strawberries and Tomatoes .....	22
<b>B.7.4. FEEDING STUDIES .....</b>	<b>33</b>
<b>B.7.5. EFFECTS OF PROCESSING .....</b>	<b>34</b>
B.7.5.1. Nature of the residue.....	34
B.7.5.2. Distribution of the residue in inedible peel and pulp .....	34
B.7.5.3. Magnitude of residues in processed commodities.....	34
<b>B.7.6. RESIDUES IN SUCCEEDING OR ROTATIONAL CROPS.....</b>	<b>35</b>
<b>B.7.7. OTHER STUDIES.....</b>	<b>35</b>
B.7.7.1. Effect on the residue level in pollen and bee products .....	35
<b>B.7.8. LITERATURE REVIEW.....</b>	<b>36</b>
<b>B.7.9. REFERENCES RELIED ON .....</b>	<b>43</b>

## B.7. Residue data

Aqueous extract from the germinated seeds of sweet *Lupinus albus* is a plant extract with fungicidal properties that can be used on strawberries and tomatoes. The extract is obtained from the germinated seeds of sweet lupin. Aqueous extract from the germinated seeds of sweet *Lupinus albus* is an unknown or variable composition, complex reaction product or biological origin (UVCB) substance. The complete aqueous extract is recognized as the active substance, which contains 'BLAD' as a marker compound. The representative product PROBLAD PLUS has the same composition as the active substance (aqueous extract from the germinated seeds of sweet *Lupinus albus*). The lead component 'BLAD' comprises 20% w/w of the PROBLAD PLUS formulation.

The majority of residues data generated and evaluated here have tested PROBLAD PLUS. For further information on the differences in identity of the active substance, lead component and plant protection product, please refer to Volume 4.

The applicant has proposed uses in GB which are summarised in Table 7.3-1.

There may be references to PROBLAD PLUS within the DAR, however the applicant has confirmed that the tradename for the product will be PROBLAD in GB.

### B.7.1. Storage stability of residues

No data submitted.

Supervised residue trials were submitted in section Magnitude of Residue Trials in Plants (study number: S13-04129), and samples of strawberries, tomatoes and grapes were stored deep frozen at approximately -18°C for up to 117 days prior to analysis. Therefore storage stability data to support the stability of residues upon frozen storage should be provided. However, in this case, a scientific judgement has been made to take into account the uncertainty resulting from the lack absence of storage stability data (see Volume 1), therefore further data are not required at this time. Further data may be required in a future consideration where results from samples stored for extended time periods are relied upon.

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

### B.7.2. Metabolism, distribution and expression of residues

The applicant did not submit radiolabelled studies according to OECD guidelines. Since it is often not possible to radiolabel complex botanical active substances, it is reasonable that it is not feasible to perform studies based on radioactive detection.

However, three supplementary studies were submitted to address the nature of residues expected. It should be noted that these studies partly address the magnitude of residues expected also.

### B.7.2.1. Plants

<b>Reference:</b>	CA 6.2.1/01
<b>Report Title:</b>	BLAD residues test
<b>Author(s) &amp; Year:</b>	<del>unknown</del> Sara Monteiro, 2011
<b>Document No, Authority registration No</b>	<del>Not provided</del> CEV110310
<b>Guideline(s):</b>	None
<b>Deviations:</b>	No, no guidelines available
<b>GLP or GEP:</b>	No
<b>Acceptability:</b>	Supplementary
<b>Study relied upon:</b>	Yes

### Study design

The main aim is to investigate the fate of BLAD after one application of 0.4 mg/mL BLAD to grapevine leaves. Note: 0.4 mg BLAD/mL application solution equates to 0.4 g/L, which equates to 0.1 N considering the critical GAP for tomato (4 g BLAD/L water) and 0.22 N considering the critical GAP for strawberry (1.78 g BLAD/L water), but is within  $\pm 25\%$  of the lowest spray concentration (0.5 g BLAD/L water for both representative GAPs), but only one application appears to have been made whereas the GAPs state up to 6 applications are possible.

### Plant material

*Lupinus albus* seeds were obtained locally. They were allowed to germinate for eight days and the cotyledons collected and stored at  $-70^{\circ}\text{C}$ .

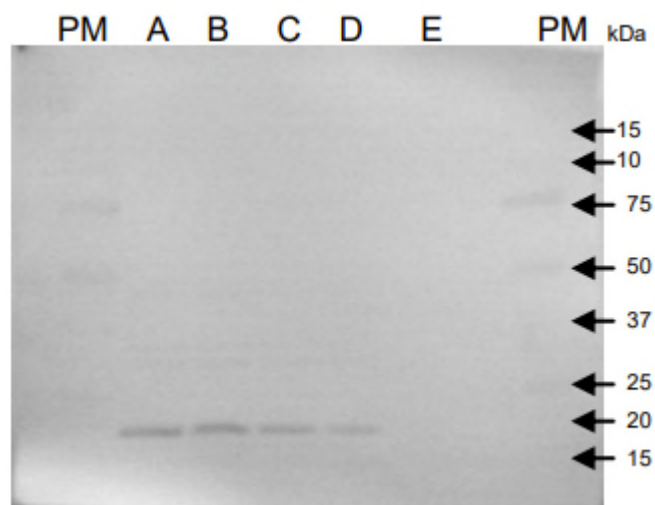
Grapevine (*Vitis vinifera*) plants were also used. The plants were sprayed with a solution of 0.4 mg/mL BLAD. Leaf samples were collected after 18 h incubation. It is not clear if the treatments were made indoors or outdoors. It is also unclear if the substance applied to grapevine leaves was purified BLAD or PROBLAD PLUS.

### Methods

Total protein from grapevine leaves was extracted according to Jacobs (Jacobs, A.K., et al., 1999) which involved extraction with 0.35 M Tris-acetate buffer, pH 8.0, containing 20 mM EDTA, 11 mM Na diethyldithiocarbamate, 15 mM cysteine-HCl and 6% polyethylene glycol. Protein from lupin eight days germinated cotyledons (BLAD), was extracted with water (5 mL/g fresh weight).

All protein extracts were desalted in PD-10 (GE Healthcare) columns into water at pH 7.5. Protein content was determined according to a modification of the Lowry method (Bensadoun, A.; Weinstein, D., 1976). To divide the protein extracts into polypeptides, a reducing and denaturing electrophoresis system in polyacrylamide gels, R-SDS-PAGE, was used in a discontinuous buffer system (Laemli, U.K., 1970). Proteins separated by R-SDS-PAGE were blotted onto a nitrocellulose (NC) membrane (previously soaked for 15 min in transfer buffer: 39 mM Tris, 48mM glycine, 0.1% (w/v) SDS, 20% (v/v) methanol, pH 8.3) at 15 V for 1 h 15 min using a semidry transfer unit (BIO-RAD). After protein transfer, the polypeptides in the membrane were fixed for 5 min in a solution containing 10% (v/v) acetic acid and 25% (v/v) 2-propanol. Total polypeptides in the membrane were visualized with Ponceau S (stain). The membrane was washed for 1 min with water, incubated for 15 min with 0.026 M Ponceau S (stain), 1.8 M trichloroacetic acid, and 1.2 M sulfosalicylic acid, and washed for 5 min with water. The proteins were separated by R-SDS-PAGE, blotted onto an NC membrane and subjected to immunoblotting. The blots were probed with anti-BLAD antibodies and processed as described by Ramos et al. (Ramos, P.C.R., et al., 1997); this identifies the proteins as being specific to BLAD. (Note: This literature provides useful background information regarding the isolation and identification of BLAD as an intermediate product of  $\beta$ -conglutin catabolism that abruptly accumulates in *L. albus* cotyledons between 4 and 14 d after the onset of germination. The source of anti-BLAD antibodies is from rats injected with purified BLAD and their blood serum sampled).

### **Results**



**Figure 7.1** Immunological detection results from treated grapevine and lupin cotyledons. Lanes: PM- molecular mass markers (BIO-RAD), kDa; A- BLAD (40 µg); B- BLAD (30 µg); C- BLAD (20 µg); D- BLAD (10 µg); E- grapevine leaf extract sprayed with BLAD (18 h incubation, 30 µg).

Note: it appears that the right hand labels in the figure should state 150 and 100 for the top two labels.

It appears that a range of sample weights of extracts from the eight day germinated cotyledons of lupin seeds were analysed at the same time as extracts from the treated grapevine leaves. The intensity of the response at 20 kDa associated with the sample size gives an indication of quantification using this method. There is a complete lack of response in column E, the test sample of treated grapevine leaves.

## Conclusion

There are no significant residues of BLAD or any proteins with molecular masses ~15-75 kDa in the samples of treated grapevine leaves. It should be noted that this test was underdosed considering the proposed GAPs (application of 0.4 g/L with critical GAP of 6 applications at 4 g BLAD/L water). It is unclear from the study whether PROBLAD PLUS or purified BLAD was applied during the test; either of these would be underdosed. It is also unclear how the quantities of BLAD listed in Figure 7.1 (e.g. 10-40 µg for 'reference' BLAD and 30 µg for grapevine leaf extract) relate to the amount applied in the test (0.4 mg/mL).

It should be noted that no other crop fractions were tested during this study and the study was not conducted in accordance with GLP nor the relevant OECD guidelines.

By comparing the apparent absence of response in column E to the samples of germinated lupin seeds, there is some supporting evidence that ‘low’ residues of aqueous extract from the germinated seeds of sweet *Lupinus albus* are expected in treated crops. It should be noted that the lack of residue here is contradictory to the positive residues reported in section 7.3, but this may be explained due to the under-dosing in this test; this is further considered in Volume 1.

<b>Reference:</b>	CA 6.2.1/02
<b>Report Title:</b>	Potential Allergenicity of Lupine Seeds ( <i>Lupinus</i> sp.) with Special Emphasis on BLAD, an Intermediate in the Breakdown Process of the Major Storage Protein during Germination of Lupine Seeds
<b>Author(s) &amp; Year:</b>	R.B Ferreira, 2011
<b>Document No, Authority registration No</b>	CEV110820
<b>Guideline(s):</b>	No
<b>Deviations:</b>	No, no guidelines available
<b>GLP or GEP:</b>	No
<b>Acceptability:</b>	Supplementary
<b>Study relied upon:</b>	Yes

The purpose of this study was to address the potential allergenicity of lupin seeds, with a specific consideration of BLAD. As part of this, comparison to an established allergen was made following criteria set out by Codex Alimentarius and FAO/WHO, considering amino acid composition. Resistance to proteolytic attack and ingestion of sufficient amounts was also considered. As part of the exposure aspect, information which may be used to support the residues and consumer risk assessment was presented.

### Study design



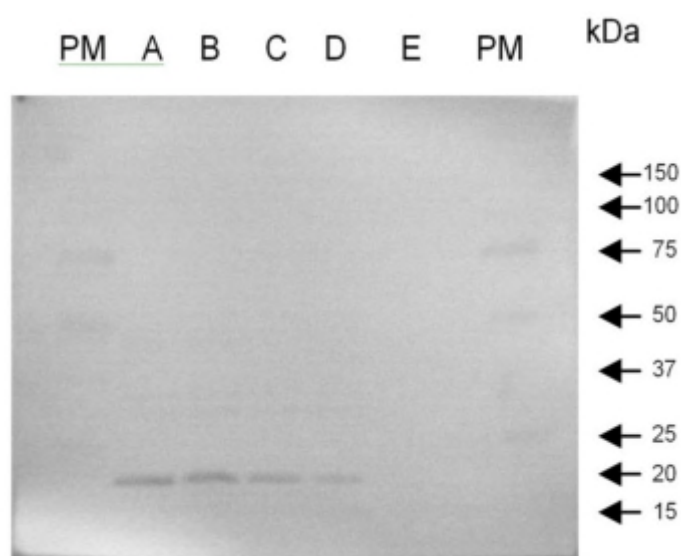
Cotyledons were detached from *Lupinus albus* plantlets 8 days after the onset of germination (8 DAG, days after germination) and used to extract and purify BLAD. *Lycopersicon esculentum* (tomato) plants containing ripe tomatoes were sprayed with a 400 mg/L pure BLAD solution (Note: 0.4 mg BLAD/L application solution equates to 0.1 N considering the critical GAP for tomato (4 g BLAD/L water) and 0.22 N considering the critical GAP for strawberry (1.78 g BLAD/L water), but is within  $\pm 25\%$  of the lowest spray concentration (0.5 g BLAD/L water for both representative GAPs). It appears that one application was made whereas the GAPs allow up to 6 applications). The tomatoes were harvested 18 h after the application.

The tomatoes were peeled and the total skin protein extracted and isolated. A similar procedure was followed for the extraction (5 mL water/g tissue fresh weight) of the total protein present in the 8-DAG lupin cotyledons. Total protein extracts were fractionated by denaturing electrophoresis (SDS-PAGE) and the polypeptides were electrotransferred onto a nitrocellulose membrane during 1 h 15 min at 15 V. The membrane was incubated in boiling water for 10 min, to fix the transferred polypeptides onto the membrane. Total polypeptides were stained with Ponceau S (stain) to assess the efficiency of the transfer process. The transferred polypeptides were finally probed with rabbit anti-BLAD polyclonal antibodies.

## Results

The study states that the immunoblotting methodology is capable of detecting BLAD at ng concentration levels. Since this level of sensitivity was considered as adequate, other variations of this technique, especially the Enzyme ChemiLuminescent (ECL) immunoassays, which allow greater sensitivities (pg or fg), were not required for further testing.

In this study, no significant BLAD residues were detected on treated tomatoes; the results are presented in Figure 7.2. It is unclear how the quantities of BLAD listed in Figure 7.2 relate to the amount applied in the test (0.4 g/L).



**Figure 7.2** Immunological detection of residual BLAD on tomato fruits following application of pure BLAD under field conditions. Lanes: PM- Molecular mass markers (kDa); A- 40  $\mu$ g pure BLAD loaded on gel (control); B- 30  $\mu$ g pure BLAD loaded on gel (control); C- 20  $\mu$ g pure BLAD loaded on gel (control); D- 10  $\mu$ g pure BLAD loaded on gel (control); E- Extract prepared from tomato skin 18 h after application to tomato fruit, under natural field conditions.

### Further information

This study report focussed mainly on the consideration of BLAD as a potential allergen. In addition to the data presented showing the analysis of treated tomato plants, further information is presented on the composition of the aqueous extract from the germinated seeds of sweet *Lupinus albus* in terms of plant products and amino acids. Additionally, data to demonstrate the vulnerability of the aqueous extract from the germinated seeds of sweet *Lupinus albus* to protease and a case regarding the levels found on specific crop surfaces were presented.

### Composition

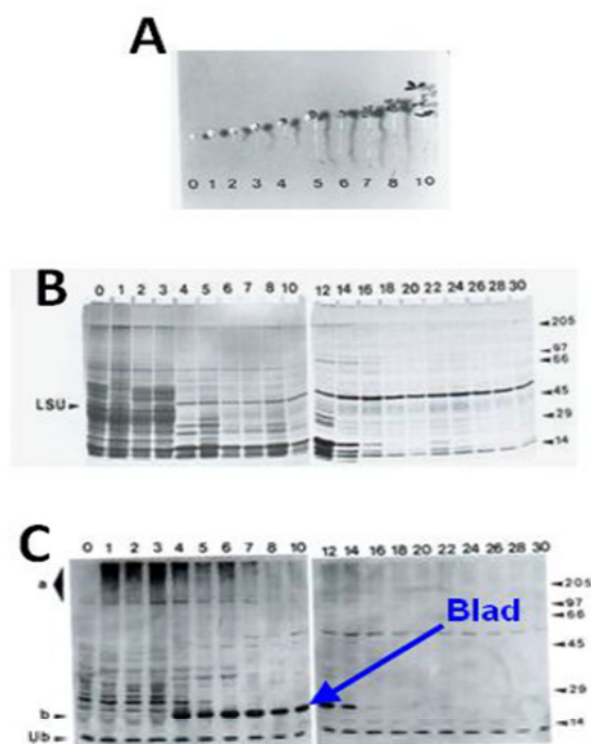
Useful excerpts from the study are presented below.

‘The cultivation of lupines (*Lupinus* sp.) is far behind those of other grain legumes, mostly due to their relatively high content of toxic alkaloids in the seed. Indeed, wild *Lupinus* species contain relatively high levels of toxic alkaloids in their seeds, typically in the range of 1.5–2.2% (w/w). More than 170 structures of quinolizidine alkaloids have been reported in lupine seeds (Wink, 1994). These secondary metabolites provide a good protection to the seeds against insect attack and fungal diseases, as well as stress tolerance.

They also have antinutritional properties and a bitter taste (Ciesiolka et al., 1988). For this reason, the seeds from wild lupine varieties are said to be 'bitter' to distinguish them from 'sweet', low-alkaloid cultivars (Luckett, 2004). These alkaloids did not prevent human consumption of lupine seeds, since previous soaking of bitter seeds results in removal of most of their alkaloids, which leach into the water.'

'White lupines (*Lupinus albus* L.), as the other *Lupinus* species, possess in their seeds three major storage proteins: the globulins  $\alpha$ -conglutin,  $\beta$ -conglutin and  $\gamma$ -conglutin.  $\beta$ -Conglutin is the main *Lupinus* globulin, and a lupine member of the vicilin-like or 7S family of storage proteins (Melo et al., 1994). The transcript encoding its precursor, pro- $\beta$ -conglutin, a 64 kDa polypeptide, is specifically translated during the period corresponding to seed formation and development. Before the storage in the dry seeds takes place, pro- $\beta$ -conglutin is subjected to very intense processing in a route which ends up with many tens, possibly hundreds of slightly different  $\beta$ -conglutin subunits.'

'Since native, mature  $\beta$ -conglutin is a trimer, the protein in dry *Lupinus* seeds exhibits a very high degree of micro-heterogeneity (Monteiro et al., 2010). In a subsequent cycle of growth, the seeds germinate during approximately one day and seedling growth ensues. Between days 3 and 5 after the onset of germination,  $\beta$ -conglutin suffers a dramatic change in its structure and concentration, involving the appearance of a new set of polypeptides, including a higher molecular mass group, whose concentration steadily declines until complete disappearance after 11 to 12 days, and a lighter molecular mass group, whose concentration surprisingly increases from day 5 to day 11 to 12. Particularly evident is the accumulation of a 20 kDa polypeptide, which is maintained in the cotyledons in high amounts between days 4 and 14 after the onset of germination (Figure 1). This polypeptide was termed BLAD, from the Portuguese expression "banda de *Lupinus albus* doce", meaning "Polypeptide band from sweet *Lupinus albus*".'



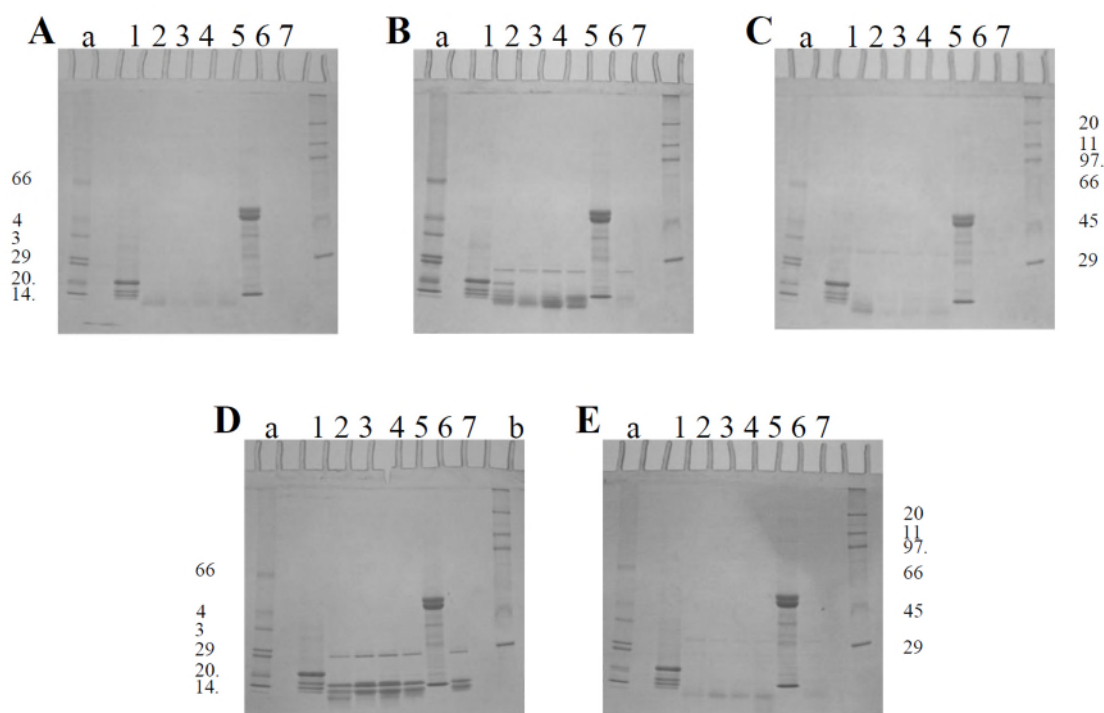
**Figure 1.** Experiment which lead to BLAD discovery back in 1991 (Ferreira *et al.*, 1995; Ramos *et al.*, 1997). (A) *Lupinus albus* from the dry seed (time zero) until 10-day old plantlets. (B) Electrophoretic (SDS-PAGE) analysis of total polypeptides present in cotyledons from the dry seed until their senescence (30 days after the onset of germination). (C) Immunoblotting analysis; the polypeptides present in the gels depicted in (B) were transferred onto a membrane and probed with polyclonal anti-ubiquitin antibodies. Days after the onset of germination are marked on top of gels. Molecular masses (kDa) of standards are shown on the right. LSU: large subunit of ribulose biphosphate carboxylase; Ub and a: free ubiquitin and large molecular mass ubiquitin-protein conjugates; b: BLAD.

The study also gives details on the consumption of lupine seeds. Lupine seeds have been consumed by humans for a long time. Classical breeding techniques allowed the development of sweet cultivars (lower alkaloid concentration), mostly from three lupine species: *Lupinus albus* (white lupine), *Lupinus angustifolius* (blue lupine) and *Lupinus luteus* (yellow lupine). The seeds from such cultivars are increasingly used as food. Lupine seeds are considered a source of protein, fibre and can be used as an emulsifier in food manufacturing.

### Vulnerability to protease

The literature states that the biological activity of BLAD exhibits is extremely resistant to inactivation, with the native oligomer exhibiting a very high stability against denaturation, withstanding boiling, treatment with organic solvents and detergents, and exposure to high concentrations of strong acids, as long as they don't cleave its peptide bonds. This has not been supported or demonstrated by any data. The

treatments which were found capable of abolishing the lectin activity of BLAD were those that induce cleavage of peptide bonds (e.g. use of proteases). This observation was confirmed by a study on the susceptibility of the BLAD-containing protein to proteolysis (Figure 7.3), performed using incubation at room temperatures (not extreme temperature/pH conditions described above). The oligomer was mixed with common proteolytic enzymes, incubated at room temperature for 1 h, 2 h or 3 h followed by addition of a marker protein (55 µg pure *Lemna minor* ribulose biphosphate carboxylase) and a further 1 h incubation. Pure ribulose biphosphate carboxylase was readily degraded by all proteases in all cases. The results obtained after SDS-PAGE analysis of the incubated reaction mixtures indicate that the BLAD-containing protein is readily hydrolysed by all proteases tested.



**Figure 7.3** Susceptibility to proteolysis of the *Lupinus albus* 210 kDa protein containing BLAD (20 kDa). Pure 210 kDa protein (lanes 1) was mixed with proteolytic enzymes [pronase in (A); trypsin in (B); proteinase K in (C);  $\alpha$ -chymotrypsin in (D); subtilisin in (E)] and incubated at room temperature for 1 h (lanes 2), 2 h (lanes 3) or 2 h followed by addition of pure ribulose biphosphate carboxylase (55  $\mu$ g) and a further 1 h incubation (lanes 4). In lanes 5, pure ribulose biphosphate carboxylase (55  $\mu$ g) was incubated for 1 h with the corresponding proteolytic enzyme. Lanes 6 and 7 contain pure ribulose biphosphate carboxylase (55  $\mu$ g) or the corresponding protease (20  $\mu$ g), respectively. Lanes a and b: molecular mass standards (kDa).

### Amounts found on treated crop surfaces

The further information provided in the report states the following:

‘Tests performed at CEV laboratories have indicated that on average, fruits like table grapes (typically around 6 g per berry), average sized strawberries (typically around 15 g per berry) and tomatoes/apples (typically around 150 g) will retain 35  $\mu$ L, 300  $\mu$ L and 580  $\mu$ L of the PROBLAD PLUS fungicide solution, respectively, when the corresponding crops are sprayed under field conditions at or near harvest time. Note that the amount of solution retained by each fruit does not correlate directly with its surface area. Thus, strawberries, with a highly irregular surface, retain proportionally a higher quantity of fungicide than the smooth-skinned grape, tomato or apple. In addition, only



part of each grape berry, corresponding to the external surface of the bunch, will become covered by a thin layer of fungicide solution. Therefore, it is possible to conclude that on average, after spraying a crop with PROBLAD PLUS, each individual fruit surface will retain 8.75 µg (i.e. 1.46 ppm; grape berry), 75.00 µg (i.e. 5.00 ppm; strawberry) or 145.00 µg (i.e. less than 1 ppm; tomato/apple) of BLAD. It is important to compare the average molecular mass (or size) of a typical chemical fungicide (a couple of hundred Da) with that of BLAD (20,408.95 Da), meaning that based on the same number of molecules, the ppm concentrations referred above for BLAD should be divided by a factor of about 60.'

It is unclear how these levels have been determined, other than by measuring the average sizes of various fruits and calculating the amount of PROBLAD PLUS applied on an area basis. An analytical method has not been used to determine these amounts. These calculations are not standard practice, however, they provide some reassurance that expected residue levels in treated crops will be comparatively low (i.e. < 1000 mg/kg as discussed in Volume 1). It should be noted that these estimates are based on the residue remaining after a single application, whereas the representative GAPs being considered are for multiple applications (up to 6), therefore the potential residue levels could be up to 6 times higher, considering no breakdown upon application. Additionally, it is unclear at what sampling timing the retained amounts relate to i.e. is this immediately after application or a number of days after the application was made.

## Conclusions

Large quantities of residues of BLAD were not found in treated tomatoes 18 hours after application (of either PROBLAD PLUS or purified BLAD), however the exact levels expected are unclear. It should be noted that the first test described in this study report was underdosed considering the proposed GAPs (application of 0.4 g/L with critical GAP of 6 applications at up to 4 g BLAD/L water). The analytical and crop testing aspect of the study was not conducted in accordance with GLP nor the relevant OECD guidelines. However, the study provides some supporting evidence that negligible residues of the aqueous extract from the germinated seeds of sweet *Lupinus albus* are expected.

From the additional information within the study report, there is confidence that when digested, protease enzymes will break down aqueous extract from the germinated seeds of sweet *Lupinus albus* into naturally occurring amino acid units. There is also some confidence based on observations of the amount of residue in physical terms (volume of spray solution) that the residues on treated crops will be comparatively low (i.e. < 1000mg/kg as discussed in Volume 1).

<b>Reference:</b>	6.2.1/03
<b>Report Title:</b>	The Unique Biosynthetic Route from Lupinus $\beta$ -Conglutin Gene to Blad
<b>Author(s) &amp; Year:</b>	S. Monteiro, R. Freitas, B.T. Rajasekhar, A. R. Teixeira, R. B. Ferreira, 2010
<b>Document No, Authority registration No</b>	n/a public literature study ref. PLoS ONE 5(1): e8542. doi:10.1371/journal.pone.0008542
<b>Guideline(s):</b>	n/a
<b>Deviations:</b>	n/a
<b>GLP or GEP:</b>	No, public literature study
<b>Acceptability:</b>	Supplementary
<b>Study relied upon:</b>	No, the study provides an additional information on BLAD formation

This literature study explains the structure and formation of BLAD in *Lupinus* plants. It is presented in Volume 3 CA B7 for completeness purposes; it is analysed in the literature review and it is a literature reference to study CEV110820.

This study provides insight into formation of BLAD from  $\beta$ -conglutin via  $\beta$ -conglutin catabolism.  $\beta$ -conglutin is a major seed storage protein in *Lupinus* species. In the case of *Lupinus albus*, the one dimensional SDS-PAGE analysis of  $\beta$ -conglutin revealed that the mature protein is composed of 10 to 12 major types of subunits with molecular masses ranging from 15 to 72 kDa.

In all investigated *Lupinus* species, during germination between days 3 and 5  $\beta$ -conglutin undergoes changes in its structure and concentration. Particularly evident is the accumulation of a 20 kDa polypeptide, termed BLAD, in the 4<sup>th</sup> day after imbibition (first step in seed germination, absorption of water, causes swelling of the seed which leads to the rupture of the seed coat). BLAD is maintained in high amounts during the following days until 12 to 14 days when its concentration rapidly declines. BLAD is composed of 173 amino acid residues with high proportion of nitrogen-rich amino acids (arginine, asparagine, glutamine, lysine).



Considering the precise and characteristic pattern of BLAD accumulation during *Lupinus* plantlet development, its abundance in cotyledons, its amino acid composition and its link to  $\beta$ -conglutin, it can be concluded that BLAD has an important role as a seed storage globulin. However, the atypical pattern of BLAD occurrence during the very limited period of time in the life cycle of *Lupinus* plants, suggests that this protein may display other physiological roles, which are currently under investigation.

#### **B.7.2.2. Poultry**

No livestock metabolism studies have been submitted or are required.

The proposed representative uses of PROBLAD PLUS are on strawberries and tomatoes which do not comprise a significant portion of livestock diets, therefore metabolism data is not required considering these proposed uses. The uses proposed to support MRLs beyond the representative uses also do not comprise a significant portion of livestock diets.

#### **B.7.2.3. Lactating ruminants**

See Section B.7.2.2.

#### **B.7.2.4. Pigs**

See Section B.7.2.2.

#### **B.7.2.5. Fish**

At present there is no agreed guidance on how to conduct fish metabolism studies to determine the residue definition for risk assessment and monitoring and there are no agreed guidance documents on how to conduct a fish feeding study. It is also the case that there is no agreed diet for farmed fish. The EU guidance: SANCO/10181/2013– rev. 5, 12 June 2019, Guidance Document For Applicants On Preparing Dossiers For The Approval Of A Chemical New Active Substance And For The Renewal Of Approval Of A Chemical Active Substance According To Regulation No 283/2013 and Regulation No 284/2013 states:

“In some cases, agreed test methods or guidance documents are not yet available for particular data requirements. In these cases, the non-submission of particular studies required by the EU legislation should be thoroughly justified and statements (often referred to as ‘position papers’) must be substantiated with data or information provided by the applicant in the dossier. Applicants should follow on a routine basis the current developments, e.g. activities of the European Food Safety Authority for guidance documents and

in particular publications in the Official Journal and the updates of the Commission Communications 2013/C 95/01 and 2013/C 95/02”

It is noted that the data requirements under assimilated Regulation No 283/2013 make it clear that bioaccumulation studies can be considered to address this data requirement. However, at the PRAS meeting in December 2017, for the expert discussion on spinosad, it was agreed by the experts and EFSA that the use of such a study could not be considered at this time as it was not clear how the study design was applicable to assessing residues for consumer exposure and agreement on the approach to the dietary assessment for fish was required.

Guidance on residues in fish (metabolism studies and feeding studies) has been under development in the EU. The OECD programme on residue guidelines has not yet considered guidelines applicable to fish.

Since no agreed guidance is available, and there is no agreed data on the diets of fish (to address fish dietary burden) at this time, it is considered that the above requirements do not need to be addressed in the current evaluation.

The applicant also stated that as the representative uses of PROBLAD PLUS are on tomatoes and strawberries, they are not part of commercial fish diet, therefore the potential for exposure to fish is minimal.

### B.7.3. Magnitude of residue trials in plants

The applicant has proposed uses of 'PROBLAD PLUS', outlined in Table 7.3-1.

**Table 7.3-1**      **Summary of applicant's proposed uses in GB**

Crop and/or situation (a)	Member State	Product Name	F G I (b)	Pests or group of pests controlled (c)	Formulation		Application				Application rate per treatment			PHI (days) (l)	Remarks (m)
					Type (d-f)	Conc of a.i. g/kg (i)	Method kind (f-h)	Growth stage and season (j)	Number min max (k)	Interval between applications (min)	L a.i./hl min max (g/ha)	Water L/ha min max	L a.i./ha min max (*) (kg/ha)		
Strawberry	GB	PROBLAD PLUS	F	Foliar fungi  BOTRCI  SPHRMA	Soluble concentrate (SL)	1000 g/kg (PROBLAD PLUS is a UVCB substance and is considered to be 100% pure with the lead component BLAD at 250 g/L)	Foliar overall	BBCH 61-89  Spring to Summer	1-6	8 days	0.2-0.71 L a.i./hL  (0.251-0.893 kg a.i./hL)	450-1000	Min 2.0 L/ha (2.51 kg/ha)  Max 3.2 L/ha (4.02 kg/ha)	1	Equivalent to min-max 502-803 g BLAD/ha  Note: (kg/ha) is based on a density of 1.255 g/mL.  Spray solution: 0.5-1.78 g BLAD/L water

**Aqueous extract from the germinated seeds of sweet *Lupinus albus***

**Volume 3 – B.7 (AS)**

Strawberry	GB	PROBLAD PLUS	G	Foliar fungi BOTRCI SPHRMA	Soluble concentrate (SL)	1000 g/kg (PROBLAD PLUS is a UVCB substance and is considered to be 100% pure with the lead component BLAD at 250 g/L)	Foliar overall	BBCH 61-89  All seasons	1-6	8 days	0.2-0.71 L a.i./hL  (0.251-0.893 kg a.i./hL)	450-1000	Min 2.0 L/ha (2.51 kg/ha)  Max 3.2 L/ha (4.02 kg/ha)	1	Equivalent to min-max 502-803 g BLAD/ha  Note: (kg/ha) is based on a density of 1.255 g/mL.  Spray solution: 0.5-1.78 g BLAD/L water
Tomatoes	GB	PROBLAD PLUS	F	Foliar fungi BOTRCI OIDINL	Soluble concentrate (SL)	1000 g/kg (PROBLAD PLUS is a UVCB substance and is considered to be 100% pure with the lead component BLAD at 250 g/L)	Foliar overall	BBCH 61-89  Spring to Summer	1-6	8 days	0.2-1.6 L a.i./hL  (0.251-2.01 kg a.i./hL)	200-1000	Min 2.0 L/ha (2.51 kg/ha)  Max 3.2 L/ha (4.02 kg/ha)	1	Equivalent to min-max 502-803 g BLAD/ha  Note: (kg/ha) is based on a density of 1.255 g/mL.  Spray solution: 0.5-4 g BLAD/L water

**Aqueous extract from the germinated seeds of sweet *Lupinus albus***

**Volume 3 – B.7 (AS)**

Tomatoes	GB	PROBLAD PLUS	G	Foliar fungi BOTRCI OIDINL	Soluble concentrate (SL)	1000 g/kg (PROBLAD PLUS is a UVCB substance and is considered to be 100% pure with the lead component BLAD at 250 g/L)	Foliar overall	BBCH 61-89  All seasons	1-6	8 days	0.2- 1.6 L a.i./hL  (0.251- 2.01 kg a.i./hL)	200- 1000	Min  2.0 L/ha (2.51 kg/ha)  Max  3.2 L/ha (4.02 kg/ha)	1	Equivalent to min-max 502-803 g BLAD/ha  Note: (kg/ha) is based on a density of 1.255 g/mL.  Spray solution: 0.5-4 g BLAD/L water
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F - field use, G – greenhouse application;

\* (m) based on density 1.255 g/mL and based on 20% w/w content of BLAD in PROBLAD PLUS, which is a lead component in PROBLAD PLUS and the aqueous extract from the germinated seeds of sweet *Lupinus albus*.

**B.7.3.1. Strawberries and Tomatoes**

<b>Reference:</b>	KCA 6.3.1/01
<b>Report Title:</b>	Magnitude and Decline of BLAD Residues Following Application of ProBLAD Plus to Grapes, Strawberries, and Tomatoes
<b>Author(s) &amp; Year:</b>	D. Vespestad, 2014
<b>Document No, Authority registration No</b>	Eurofins Agrosience Services, Inc. Study Number S13-04129
<b>Guideline(s):</b>	Yes  U.S. EPA Residue Chemistry Test Guidelines,  OPPTS 860.1500: Crop Field Trials
<b>Deviations:</b>	Yes – not in line with OECD 509 guideline on crop field trials
<b>GLP or GEP:</b>	Yes  Some minor deviations were noted, none of which were considered significant to impact the compliance with GLP
<b>Acceptability:</b>	Yes
<b>Study relied upon:</b>	Yes

In residue trials presented below the applicant included seven field trials, two for grape, two for strawberry, and three for tomatoes conducted in the 2013 growing season. Although, the representative GAPs do not include grapes, the data was presented as supporting information.

**Study design**

During the 2013 growing season 7 field decline trials in grapes (2 trials), strawberries (2 trials) and tomatoes (3 trials) were conducted outdoors in NAFTA Zone 10 (California, USA) to determine the residue level of BLAD in fruit samples. For the strawberry and tomato trials, hand-held boom spray equipment, with water as the

carrier, was used. For grapes, ground airblast spray equipment, with water as the carrier, was used. Weather data did not show any significant deviations except trial S13-04129-03 which was cancelled due to weather conditions (cold temperatures affecting harvest).

PROBLAD PLUS (SL formulation) containing 20% w/w BLAD was applied 5 times at 7 day intervals. This number of applications is within 25% of the representative GAPs (max. 6 applications). For strawberries, the first trial was conducted at 838 g BLAD/ha which is consistent with the representative GAP, the second trial was conducted with a higher application rate of 4110 g BLAD/ha which is overdosed (5N). For tomatoes, the first and second trials were conducted at 360 g BLAD/hL and 369 g BLAD/hL which is within 20% of the proposed GAP (in terms of spray concentration), the third trial was conducted at 1795 g BLAD/hL which is overdosed (4.5N). Overall the trials presented are either within 25% of the representative GAP in terms of application rate and number of applications, or are overdosed.

One untreated plot of each trial served as a control. Residues above 0.02 mg/kg were not determined in the untreated samples. Two treated plots were sampled at the strawberry field trial sites; these could be considered replicates. The design and conduct of these trial sites were different (significantly different application rate) therefore these can be considered independent, but only one trial considered supportive of any MRL calculations. The same situation was tested in two of the three tomato field trials. Similarly, a significantly different application rate was tested.

Both a small 'cherry' tomato variety and large variety (Quality 47) were tested in the tomato field trials.

The last application was performed at BBCH 87 for strawberries and BBCH 71 and 82 for tomatoes. Samples of fruit were collected on the day of the last application and 1, 3, 5, and 7 days after the final application. At each sampling event, one sample was collected from the untreated plot and two samples were collected from the treated plot(s). Each sample consisted of at least 1 kg fruit collected from at least 12 different areas of one plot.

Samples were stored deep-frozen for 19 to 117 days until analysis. Within each trial, samples taken at shorter PHIs were stored for longer time periods compared to those taken at later PHIs; the shorter PHI data is relevant to the representative uses. No data to address the stability of residues of the aqueous extract from the germinated seeds of sweet *Lupinus albus* upon frozen storage have been provided. In trials 01, 02, 04 and 05, samples were stored for > 30 days, whereas samples from trial 06 were analysed within 26 days. In accordance with OECD guideline 506, samples stored frozen (-18°C) and analysed within 30 days of sampling generally do not require supporting storage stability data. Therefore the data from trial 06 could be more reliable than the results from the other trials where samples were stored for a

longer time period. The results from this trial are generally similar to the results from trials where samples were stored for longer (all relatively low residues). However it is noted that samples from this trial showed more 'positive' residues (> 0.005mg/kg 0.1 mg/kg) compared to other trials dosed at similar application rates where the majority of results were reported as < 0.005mg/kg 0.1 mg/kg. The uncertainty with this aspect of the field trials data has been considered in the overall consideration of the aqueous extract from the germinated seeds of sweet *Lupinus albus*.

The samples of grapes and strawberries were analysed for BLAD residues using grape ELISA method (EASI Method No.: RA029). Tomato samples were analysed for BLAD residues using grape ELISA method (EASI Method No.: RA031). Validation data is presented in DAR Volume 3 CA B5. The method is not considered fully validated. There is some confidence that the method can determine the content of BLAD but is not fully validated. A reliable LOQ has not been established.

Procedural recoveries were determined during the analysis of test samples along with a range of QC samples. The recoveries are reported in Table 7.3.1-1.



**Table 7.3.1-1**  
**and grapes.**

**Procedural recoveries for BLAD in tomatoes, strawberries,**

Analyte	Matrix	Fortification level (mg/kg µg/mL)	Recoveries %		
			Individual recoveries	Mean	RSD
BLAD	Tomatoes	0.02 (~0.4 mg/kg)	60.5, 74.3, 89.7	74.8	19.5
		0.04 (~0.8 mg/kg)	49.0	-	
		0.05 (~1 mg/kg)	53.4, 61.6, 69.1	61.4	11.7
		0.08 (~1.6 mg/kg)	48.1, 61.7, 62.1	57.3	13.9
		0.1 (~2 mg/kg)	50.1	-	
		Overall:	-	61.8	20.1

## Volume 3 – B.7 (AS)

	Strawberries	0.02 (~0.4 mg/kg)	39.1, 39.7, 54.4	44.4	19.5
		0.04 (~0.8 mg/kg)	61.7	-	-
		0.05 (~1 mg/kg)	41.2, 48.0	-	-
		0.08 (~1.6 mg/kg)	33.4, 49.8	-	-
		Overall:	-	45.9	20.2
	Grapes	0.02 (~0.4 mg/kg)	44.8, 48.4	-	-
		0.04 (~0.8 mg/kg)	68.8	-	-
		0.05 (~1 mg/kg)	42.0, 46.1	-	-
		0.08 (~1.6 mg/kg)	44.1	-	-
		Overall:		49.0	20.2

The recoveries are mostly outside of the acceptable range of 70-110%. The recoveries are consistently around 40-70%. This may suggest that the method is either not validated at these levels but may be valid at a different range, or, the method was consistently under-reporting at the time of use.

The validation data for this method are presented in Volume 3 CA B5. Considering significant deficiencies of the study in terms of recoveries, repeatability and precision, the method is not validated according to SANTE/2020/12830 rev.1. No validation data were presented for the strawberry matrix (other than the procedural recoveries

reported above). There is some confidence that the method can determine the presence of BLAD in treated crops, but a reliable LOQ cannot be established. Therefore, the results as reported in the study have been reported below, but these values should be considered with caution as the analytical method is not validated. Where results are reported as not determined, the values determined are less than the LOD stated in the study, 0.005 µg/mL equivalent to 0.1 mg/kg. Where results are above this level, but lower than the LOQ stated in the study, these are reported as < 0.402 with the estimated value in brackets. However, the exact values determined at any level cannot be relied upon as a validated LOQ for the method has not been determined.

The results from the field trials are summarised in Table 7.3.1-2. As a validated LOQ for the analytical method has not been determined, the quantitative results are uncertain.

**Table 7.3.1-2      Residue data from crop field trials with PROBLAD PLUS, SL, 20% w/w BLAD (251.7 g/L)**

Trial No./ Location/ EU zone/ Year	Commodity/ Variety	Date of 1.Sowing or planting 2.Flowering 3. Harvest	Application rate per treatment			Dates of treatment or no. of treatments and last date	Growth stage at last treatment or date	Portion analysed	Residues (mg/kg) <sup>1</sup>	PHI (days)	Details on trial
			g BLAD/ ha	Water (L/ha)	g BLAD/hL				BLAD		
S13-04129 -01 Fresno, CA (outdoors) NAFTA Zone 10, CA 2013	Grape (Cabernet)	1. May 2008	861.9	479	180	28/08/2013	84	Grape bunches	ND (< 0.0051)	0	Samples stored for 56- 63 days
		2. –	878.2	487	180	04/09/2013			ND (< 0.0051)	1	
		3. 02/10/2013	803.4	445	181	11/09/2013			ND (< 0.0051)	3	
			827.4	459	180	18/09/2013			ND (< 0.0051)	5	
			832.6	462	180	25/09/2013			ND (< 0.0051)	7	
S13-04129-02 Madera, CA (outdoors) NAFTA Zone 10, CA 2013	Grape (Thompson seedless)	1. Feb 1999	848.8	476	178	15/08/2013	85-89	Grape bunches	ND (< 0.0051)	0	Samples stored for 69- 76 days
		2. –	844.7	474	178	22/08/2013			ND (< 0.0051)	1	
		3. 19/09/2013	841.6	472	178	29/08/2013			ND (< 0.0051)	3	
			845.6	474	178	05/09/2013			ND (< 0.0051)	5	
			841.7	472	178	12/09/2013			ND (< 0.0051)	7	

Aqueous extract from the germinated seeds of sweet *Lupinus albus*

Volume 3 – B.7 (AS)

Trial No./ Location/ EU zone/ Year	Commodity/ Variety	Date of 1.Sowing or planting 2.Flowering 3. Harvest	Application rate per treatment			Dates of treatment or no. of treatments and last date	Growth stage at last treatment or date	Portion analysed	Residues (mg/kg) <sup>1</sup>	PHI (days)	Details on trial
			g BLAD/ ha	Water (L/ha)	g BLAD/hL				BLAD		
S13-04129-04 Salinas, CA (outdoors) NAFTA Zone 10, CA 2013	Strawberry (San Andreas)	1. 27 Oct 2012 2. – 3. 28/10/2013	848.0	520	163	23/09/2013	87	Strawberry fruit	ND (< 0.0051)	0	Samples stored for 38- 45 days
			843.8	517	163	01/10/2013			ND (< 0.0051)	1	
			820.0	503	163	07/10/2013			ND (< 0.0051)	3	
			845.6	518	163	14/10/2013			ND (< 0.0051)	5	
			831.3	509	163	21/10/2013			ND (< 0.0051)	7	
	Strawberry (San Andreas)	1. 27 Oct 2012 2. – 3. 28/10/2013	4190.6	513	817	23/09/2013	87	Strawberry fruit	0.0273 0.546	0	Samples stored for 38- 45 days
			3971.0	486	817	01/10/2013			0.0279 0.558	0	
			4104.3	503	816	07/10/2013			0.0246 0.492	1	
			4094.8	501	817	14/10/2013			0.0247 0.494	1	
			4189.5	513	817	21/10/2013			< 0.024	3	
									(< 0.009519)	3	
									ND (< 0.0051)	5	
									ND (< 0.0051)	5	
									< 0.024	7	
									(< 0.0084168)		

Trial No./ Location/ EU zone/ Year	Commodity/ Variety	Date of 1.Sowing or planting 2.Flowering 3. Harvest	Application rate per treatment			Dates of treatment or no. of treatments and last date	Growth stage at last treatment or date	Portion analysed	Residues (mg/kg) <sup>1</sup>	PHI (days)	Details on trial
			g BLAD/ ha	Water (L/ha)	g BLAD/hL				BLAD		
S13-04129-05 Sanger, CA (outdoors) NAFTA Zone 10, CA 2013	Tomato (cherry tomato)	1.25/06/2013	869.5	242	359	26/08/2013	71	Tomato	ND (< 0.0051)	0	Samples stored for 110- 117 days
		2. –	860.2	238	361	02/09/2013			< 0.024	0	
		3. 30/09/2013	837.5	232	361	09/09/2013			(< 0.00612)	1	
			872.0	242	360	16/09/2013			ND (< 0.0051)	3	
			841.7	233	361	23/09/2013			ND (< 0.0051)	5	
									ND (< 0.0051)	7	
S13-04129-06 Madera, CA (outdoors) NAFTA Zone 10, CA 2013	Tomato (Quality 47)	1. 12/07/2013	873.1	237	368	07/10/2013	82	Tomato	ND (< 0.0051)	0	Samples stored for 19- 26 days
		2. –	866.8	235	369	14/10/2013			< 0.024	0	
		3. 11/11/2013	858.4	233	368	22/10/2013			(< 0.0062124)	1	
			866.8	235	369	28/10/2013			ND (< 0.0051)	3	
			871.2	236	369	04/11/2013			ND (< 0.0051)	5	
									ND (< 0.0051)	7	

Trial No./ Location/ EU zone/ Year	Commodity/ Variety	Date of 1.Sowing or planting 2.Flowering 3. Harvest	Application rate per treatment			Dates of treatment or no. of treatments and last date	Growth stage at last treatment or date	Portion analysed	Residues (mg/kg) <sup>1</sup>	PHI (days)	Details on trial		
			g BLAD/ ha	Water (L/ha)	g BLAD/hL				BLAD				
	Tomato  (Quality 47)	1. 12/07/2013	4159.7	232	1793	07/10/2013	82	Tomato	ND (< 0.0051)	0	Samples stored for 19- 26 days		
		2. –	4200.2	234	1795	14/10/2013			< 0.024	0			
		3. 11/11/2013	4206.0	234	1797	22/10/2013			(< 0.0056112)	1			
			4204.5	234	1797	28/10/2013			< 0.024 (< 0.0067134)	1			
			4178.3	233	1793	04/11/2013			< 0.024 (< 0.0053106)	3			
									< 0.024 (< 0.0053106)	5			
									ND (< 0.0051)	7			
									ND (< 0.0051)				
									ND (< 0.0051)				
									ND (< 0.0051)				

<sup>1</sup> Where results are reported as not determined (ND), the values determined are less than the LOD stated in the study, 0.005 µg/mL, 0.1 mg/kg. Where results are above this level, but lower than the LOQ stated in the study, these are reported as < 0.02 µg/mL, 0.4 mg/kg with the estimated value in brackets. However, the exact values determined at any level cannot be relied upon as a validated LOQ for the method has not been determined.



## Conclusions

A limited number of trials have been submitted. The number of trials is insufficient considering the requirements outlined in assimilated Regulation No 283/2013. However, given the unique situation for this substance, this is sufficient in this case as the trials are considered supporting information.

The trial which resulted in the highest residues tested an overdosed application rate (5N). The levels of BLAD were consistently low (< 0.03 0.6 mg/kg stated in the study), although it should be noted that there is uncertainty in the analytical method (no validated LOQ), the results reported should be treated with caution. Additionally, the procedural recoveries determined at the time of analysis were notably low. It is reasonable to assume that residues present could be higher (potentially double) what they were determined to be in the field trials data.

The field trials were performed outdoors in the USA, whereas the representative GAPs are for both indoor and outdoor uses in GB. Information to support the climatic relevance of these trials performed in the USA to the GB climate and GB agricultural practices has not been provided. This adds to the uncertainty regarding the trials being representative of the GAPs.

Freezer storage stability data is not available to support the storage of samples under frozen conditions for > 30 days. No data to address the stability of residues of the aqueous extract from the germinated seeds of sweet *Lupinus albus* upon frozen storage have been provided. The uncertainty with this aspect of the field trials data has been considered in the overall consideration of the aqueous extract from the germinated seeds of sweet *Lupinus albus*.

Residues were consistently not detected in samples from trials testing conditions within 25% of the representative GAPs. Although there is some uncertainty with the analytical method and stability of residues in samples upon storage, the data provide supporting evidence that relatively low residues are expected as a result of the representative uses.

### B.7.4. Feeding studies

No feeding studies have been submitted or are required.

The proposed representative uses of PROBLAD PLUS are on strawberries and tomatoes which do not comprise a significant portion of livestock diets, therefore feeding studies is not required considering these proposed uses.

## **B.7.5. Effects of processing**

### **B.7.5.1. Nature of the residue**

No data provided to address the nature or magnitude of residue upon processing. As described in the plant metabolism section, it is not feasible to test radiolabelled forms of the active substance, given its nature as a UVCB substance consisting of a range of proteins.

Assimilated Regulation No. 283/2013 outlines that if residues in products of plant origin are < 0.01 mg/kg, further consideration of processing is not required. In this case, there is confidence that the expected residues will be low, although there is uncertainty regarding the validated LOQ.

It is noted from the literature that BLAD is stated to have ‘high stability against denaturation, withstanding boiling, treatment with organic solvents and detergents, and exposure to high concentrations of strong acids, as long as they don’t cleave its peptide bonds’. This suggests that BLAD will remain stable during processing under these conditions. However, there is also confidence from the assessment that residues of the aqueous extract from the germinated seeds of sweet *Lupinus albus* will be readily biodegraded when present in/on treated plants, due to naturally occurring proteases/biodegradation. There is confidence that the aqueous extract from the germinated seeds of sweet *Lupinus albus* will break down into naturally occurring amino acids. Low levels of BLAD are expected to remain in the treated crops, which may be stable upon typical processing conditions.

Given the low levels expected in treated crops, and nature of the residue, there is sufficient information and confidence that there are no concerns with residues in processed commodities. No further information is required to address the nature or magnitude of residues of aqueous extract from the germinated seeds of sweet *Lupinus albus* in processed fractions.

### **B.7.5.2. Distribution of the residue in inedible peel and pulp**

Not required; the representative uses (strawberries and tomatoes) do not include inedible peel.

### **B.7.5.3. Magnitude of residues in processed commodities**

See section B.7.5.1. No further information is required to address the magnitude of residues of aqueous extract from the germinated seeds of sweet *Lupinus albus* in processed fractions.

### **B.7.6. Residues in succeeding or rotational crops**

No data provided. Although a moderate to high persistence in soil is suggested from the DT<sub>50</sub> estimates presented in B.8.1.4.1 (which is based on calculations from estimates of the chemical structure rather than data), in reality, as demonstrated in the biodegradation studies, aqueous extract from the germinated seeds of sweet *Lupinus albus* will be subject to biological degradation (likely via proteases) into natural components (amino acids) in the soil after application, and not persist in the natural environment. Therefore PROBLAD PLUS is considered to be readily biodegradable in soil (see Volume 3 CA B8.2.2.1). Therefore no further consideration of residues in rotational crops is required.

### **B.7.7. Other studies**

A consideration of aqueous extract from the germinated seeds of sweet *Lupinus albus* in the context of an allergen present in food is made in Volume 3 CA B6.

#### **B.7.7.1. Effect on the residue level in pollen and bee products**

No data provided. The applicant presented the following case regarding residues in pollen and bee products:

‘The data requirement objective of these studies is to determine the residue in pollen and bee products for human consumption resulting from residues taken up by honeybees from crops at blossom. PROBLAD PLUS is not a systemic substance and for the supported representative uses on fruit crops and fruiting vegetable crops, product is usually applied after flowering and exposure to honey bees by any route will be negligible. Therefore residues in pure blossom honey or other bee products will not occur from these uses. No data are required or available.’

The proposed GAP does permit application during flowering (BBCH 61-89). In accordance with SANTE/11956/2016 rev.9, tomatoes are not considered melliferous therefore no further consideration is required. Strawberries are considered melliferous therefore exposure to honey bees cannot be ruled out on this basis.

Based on the low levels of BLAD found in plants in the studies provided to address plant metabolism and magnitude of residues in plants, high residue levels are unlikely to be found in the flowering parts of strawberry plants. Hence, significant residues are not expected to be present in bee products. Additionally, the aqueous extract from the germinated seeds of sweet *Lupinus albus* is comprised of naturally occurring seed storage proteins found in lupin plants, which are themselves foraged by bees (however it is unclear if BLAD specifically is present in the crops when foraging takes place). Therefore the components that bees are potentially exposed to

are not a concern in terms of potential residues in honey for the assessment of consumer exposure.

Overall, no further consideration of residues in bee products is required.

### B.7.8. Literature review

Three literature searches were conducted by the applicant (1) K. Tucker, 2016, CEV/02/01-LRR1; 2) K. Tucker and L. Cartwright, 2018, CEV/02/01-LRR2; 3) M. Dinicica, 2019, CEV/02/01-LRR3; 4) ERM, 2024, CEV/02/01-LRR4). Some references were identified as relevant and have been included in the dossier. Some references are relevant to the residues assessment and others were relevant to other areas of the risk assessment; this has been detailed below.

#### Report: CEV/02/01-LRR1

In this report, the following databases were searched:

**STN databases:** Anabstr (analytical abstracts), Biosis, Caplus (chemical abstract plus), Chemlist, Embase (The Excerpta Medica database), Scisearch, Toxcenter, Medline, Rtecs (Registry of Toxic Effects of Chemical Substances)

**Other databases:** Pubmed, Science Direct, Wiley online library.

This search considers studies published since 2005 to evaluate any new information relevant to the toxicological and environmental risk assessment.

Search criteria:

- Within the STN databases, Pubmed, Wiley online library, Science Direct: names of the lead component and the product (BLAD, PROBLAD, PROBLAD PLUS)
- CAS number: 1219521-95-5
- The Science Direct and Wiley online library required **additional search terms** such as: tox OR hazard OR adverse OR health OR NOAEL OR NOEL OR LOAEL OR LOEL OR BMD OR "vivo" OR "vitro" OR 'storage stability' OR storage OR stability OR metabolic OR metabolism OR degradation OR breakdown OR 'residues' OR residue OR 'processing' OR hydrolysis OR rotation OR plant OR crop OR feed OR animal OR livestock OR hen OR cattle OR ruminant OR goat OR cow OR pig OR 'risk assessment' OR consume OR exposure OR 'soil' OR 'water' OR 'air' OR environment OR fate OR endocrine disrupt OR bioaccumulation OR biomagnification OR bioconcentration OR poison OR effect.  
An additional field was applied to the search to exclude references related to

“bladder”: NOT bladder NOT Urinary NOT incontinence NOT Pelvic floor NOT Urethral NOT urology NOT urological NOT urinary tract NOT renal

- Further search was conducted on lupinene,  $\beta$ -conglutin and vicilin to ensure any possible relevant data to BLAD was also searched
- As no relevant metabolites are known due to the rapid degradation, no potentially relevant metabolites could be searched
- The Wiley online library search produced too many results for “vicilin”, thus additional search terms were added (as above)

An initial assessment of studies for relevance was carried out by their titles and if necessary abstracts. Results for BLAD and PROBLAD PLUS showed 2556 publications. After initial assessment of the title and abstract, 2554 were excluded. Two full texts were assessed in detail and considered to be relevant and reliable, both of them will be included in the dossier.

Further search on lupinene,  $\beta$ -conglutin and vicilin resulted in 1303 publications. After initial assessment 1279 were excluded as not relevant. 24 full texts were analysed, from which 22 were excluded after detailed assessment. Two studies which were relevant were the same studies as in results for BLAD and PROBLAD PLUS.

## Conclusions

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. Following the search, two articles showed relevant results for BLAD and are included in the dossier:

1) S. Monteiro, R. Freitas, B.T. Rajasekhar, A. R. Teixeira, R. B. Ferreira, 2010, The unique biosynthetic route from lupinus beta-conglutin gene to blad.  
Included in the dossier as CA 6.2.1/03 (included in the residues assessment)

2) S. Monteiro, A. Carreira, R. Freitas, A. M. Pinheiro, R. B. Ferreira, 2015, A nontoxic polypeptide oligomer with a fungicide potency under agricultural conditions which is equal or greater than that of their chemical counterparts.  
Included in the dossier as CA 7.1.2.1.1/0004 (considered in the environmental fate and behaviour section CA B8)

## Report: CEV/02/01-LRR2

Additional literature review was conducted to consider the name of the active substance to comply with the botanical guidance (SANCO/11470/2012-rev. 8) “Sweet Lupin (seeds), *Lupinus albus* L., germ., ext.”. Therefore, additional search terms were

needed. Also, additional ecotoxicology filters were included in the search and some publications which were previously excluded were reviewed.

Searched databases and relevance assessment were the same as in the previous report (CEV/02/01-LRR1).

The search criteria were updated:

- Within the Pubmed, Wiley online library, Science Direct and STN databases, the search terms 'Lupinus albus', 'Lupinus albus (sweet lupin) seeds', 'Sweet lupine', 'Sweet lupin', 'Lupines', and 'Lupinus albus seeds' were searched. This was applicable to all fields within the articles.
- A search for the terms from CEV/02/01-LRR1 was conducted and where applicable additional search terms with regards to the ecotoxicology section were applied.
- Within the Pubmed, Wiley online library, Science Direct and STN databases, the search terms entered were 'BLAD', 'PROBLAD', 'PROBLAD PLUS', 'Lupinene', ' $\beta$ -conglutin', 'vicilin'. This was applicable to all fields within the articles.
- For the search terms 'BLAD', ' $\beta$ -conglutin' and 'vicilin', produced a large number of articles that would be impractical to review and therefore required further refinement with the additional ecotoxicology search terms were applied: 'AND birds OR mammals OR reptiles OR amphibians OR fish OR fishes OR daphnia OR algae OR bees OR arthropods OR soil organisms OR terrestrial plants OR effects on vertebrates birds, mammals OR feed OR diet OR seeds OR soil microorganisms OR vertebrates OR broiler chickens OR bacteria OR nitrogen transformation OR residues'.

Updated search criteria resulted in 4185 publications, 4171 were irrelevant based on the initial assessment. Detailed analysis was conducted for 14 and 2 were excluded, remaining 12 were recommended to be included in the dossier.

Re-review of the publications:

1) Salini M. et al. (2014): The study does not provide sufficient information with regard to sweet lupin. The fish diet consisted of only one amount of lupin and did not allow for comparison of effects with a range of lupin amounts. The study provided a comparison between different types of lupin as a suitable protein source and did not include sweet lupin.

2) Serrano E. et al. (2012): The study assessed the effect of the alkaloid sparteine rather than lupin in the diet of fish, thus it is not relevant to the assessment of sweet

lupin. Sweet lupin has a lower alkaloid content than other varieties, thus assessment of only alkaloids is not considered relevant in assessing the potential effects of lupin on fish.

3) Souza A.J. et al. (2012): This study addresses the seed coating of *Albizia lebbbeck* seeds and is not specific to lupin.

4) Souza A. J. et al. (2011): The study assesses seed coat toxicity to bruchid larvae but is not specific to sweet lupin seeds.

5) Zhang Y. et al. (2012): Klimisch score 3, the study is considered relevant for the weight of evidence assessment of the effect of lupin diet on fish and the applicant indicated that it has been included in the CA Section 8.

6) Uchoa A. F. et al. (2006): This study is not specific to lupin. The study was conducted using variant vicilins of cowpea seeds and so was not considered relevant for this assessment.

7) Serrano E. et al. (2011): The study assessed lupinine alkaloid from yellow lupin. The content and type of alkaloids found in different species of lupin will vary. Sweet lupins are known to have lower alkaloid content than other lupin varieties. As such with regard to the effect on fish it is more relevant to assess lupin as a whole rather than specific alkaloids.

8) Souza S.M. et al. (2010): The study is not specific for the effects of sweet lupin or BLAD and so is not considered relevant for further assessment.

## Conclusions

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. Additional publication included in the dossier:

Y. Zhang, M. Øverland, M. Sørensen, M. Penn, L. Mydland Torunn, K. D. Shearer; T. Storebakken, 2012, Optimal inclusion of lupin and pea protein concentrates in extruded diets for rainbow trout (*Oncorhynchus mykiss*) (considered in the ecotoxicology section CA B9)

## Report: CEV/02/01-LRR3

This literature review was conducted to address additional components detected in the formulation PROBLAD PLUS during the quantification of quinolizidine alkaloids.

In this report the following databases were searched:

**STN platform:** ANABSTR – Analytical abstracts, BIOSIS, CAPLUS - Chemical abstracts plus, EMBASE, Medline, RTECS, Scisearch, Toxcenter.

This search considers studies published since 2006.

The relevance assessment was the same as in the previous report (CEV/02/01-LRR1).

Search criteria:

**Search terms for the active substance Sweet Lupin and its potentially relevant metabolites**

Code number / name	Chemical name, other names, IUPAC name and CAS number
Lupanine	(1 <i>S</i> ,2 <i>R</i> ,9 <i>S</i> ,10 <i>S</i> )-7,15-diazatetracyclo[7.7.1.0 <sup>2,7</sup> .0 <sup>10,15</sup> ]heptadecan-6-one 550-90-3
13 $\alpha$ -OH-lupanine	4356-43-8
Lupinine	486-70-4
Sparteine	90-39-1

No endpoint specific search terms were used to refine the search. Patent literature was not considered to be relevant to the results of this search and it was excluded from results.

In total, 1418 records were retrieved from the search, only 1169 left after removing duplicates. After initial assessment 1163 records were excluded, and full texts of 6 publications were analysed. After detailed assessment, 3 publications were considered irrelevant or not sufficiently reliable. The remaining 3 publications are included in the dossier.

## Conclusions

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. Following the search, three articles showed relevant and reliable results:

- 1) D. Resta, G. Boschini, A. D'Agostina, A. Arnoldi, 2008, Evaluation of total quinolizidine alkaloids content in lupin flours, lupin-based ingredients, and foods. According to the applicant, included in the dossier in the section CA 5.(considered in the toxicology section Volume 4, in Section C.1.2.4)
- 2) E. Serrano, T. Storebakken, A. Borquez, M. Penn, K. D. Shearer, P. Dantagnan, L. T. Mydland, 2011, Histology and growth performance in rainbow trout (*Oncorhynchus mykiss*) in response to increasing dietary concentration of sparteine, a common alkaloid in lupins. (considered in the ecotoxicology section Volume 3 CA B9)



3) E. Serrano, T. Storebakken, M. Penn, T. Landsverk, J. O. Hansen, L. T. Mydland, Editor(s): J. A. Palta, J. D. Berger, 2008, Responses in rainbow trout (*Oncorhynchus mykiss*) to increasing dietary dose of lupinine alkaloid. (considered in the ecotoxicology section Volume 3 CA B9)

#### Report: CEV/02/01-LRR4

This literature review was conducted to capture any publications published during the time since the previous literature reviews, considering the date of submission of the dossier.

In this report the following databases were searched:

**STN platform:** AGRICOLA, BIOSIS, CABA, EMBASE, FSTA, HCAPLUS, Medline, NTIS, PQSCITECH, Scisearch, Toxcenter.

This search considers literature published between 2018 and 2022.

The relevance assessment was the same as in the previous report (CEV/02/01-LRR1).

#### Search criteria:

Literature search	Code number / name	Chemical name, other names, IUPAC name and CAS number
June 2018	Lupinus albus	Sweet lupin Sweet lupin# Lupines Lupinene
	BLAD	PROBLAD, PROBLAD PLUS 1219521-95-5
	$\beta$ -conglutin	-
	vicilin	9067-60-1
November 2019	Lupanine	(1S,2R,9S,10S)-7,15-diazatetracyclo[7.7.1.0 <sup>2,7</sup> .0 <sup>10,15</sup> ]heptadecan-6-one 550-90-3
	13 $\alpha$ -OH-lupanine	4356-43-8
	Lupinine	486-70-4
	Sparteine	90-39-1

No endpoint specific search terms were used to refine the search. Patent literature was not considered to be relevant to the results of this search and it was excluded from results.

In total, 839 records were retrieved from the search, only 594 left after removing duplicates. After initial assessment, all 594 records were excluded.

## Conclusions

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. Following the search, three articles showed relevant and reliable results:

- 1) D. Resta, G. Boschini, A. D'Agostina, A. Arnoldi, 2008, Evaluation of total quinolizidine alkaloids content in lupin flours, lupin-based ingredients, and foods. According to the applicant, included in the dossier in the section CA 5.(considered in the toxicology section Volume 4, in Section C.1.2.4)
- 2) E. Serrano, T. Storebakken, A. Borquez, M. Penn, K. D. Shearer, P. Dantagnan, L. T. Mydland, 2011, Histology and growth performance in rainbow trout (*Oncorhynchus mykiss*) in response to increasing dietary concentration of sparteine, a common alkaloid in lupins. (considered in the ecotoxicology section Volume 3 CA B9)
- 3) E. Serrano, T. Storebakken, M. Penn, T. Landsverk, J. O. Hansen, L. T. Mydland, Editor(s): J. A. Palta, J. D. Berger, 2008, Responses in rainbow trout (*Oncorhynchus mykiss*) to increasing dietary dose of lupinine alkaloid. (considered in the ecotoxicology section Volume 3 CA B9)

**B.7.9. References relied on**

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
CA 6.2.1/01	Monteiro, S.	2011	BLAD residue test Company Report No. Not stated  CEV110310 Not GLP, Unpublished	N	N	n/a	CEV	None
CA 6.2.1/02	Ferreira, R.B.	2011	Potential allergenicity of Lupine seeds ( <i>Lupinus</i> sp.) with special emphasis on BLAD, an intermediate in the breakdown process of the major storage protein during	N	Y	Data protection is claimed in accordance with Article 59 of assimilated Regulation No 1107/2009	CEV	None

			germination of lupine seeds Company Report No. CEV110820 Instituto de Tecnologia Química e Biológica Universidade Nova de Lisboa, Portugal Not GLP, Unpublished					
<b>CA 6.2.1/03</b>	Monteiro, S., Freitas, R., Rajasekhar, B.T., Teixeira, A.R., Ferreira, R.B.	2010	The unique biosynthetic route from <i>Lupinus</i> $\beta$ -Conglutin gene to BLAD Company Report No. Not applicable Not GLP, Published	N	N	n/a	PLoS one	None
<b>CA 6.2.1/04</b>	Vespestad, D.	2014	APPENDIX B: ELISA Analytical Methods for Determination of BLAD Protein in	N	Y	Data protection is claimed in accordance with Article 59 of assimilated	CEV	None

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			Grape and Tomato Residue  In Magnitude and Decline of BLAD Residues Following Application of ProBLAD Plus to Grapes, Strawberries, and Tomatoes  Report No. S13-04129  CEV, S.A, Portugal  GLP, Unpublished			Regulation No 1107/2009		
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