



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.8 (AS)

Environmental Fate & Behaviour

Great Britain

February 2025

Version History

When	What
June 2024	Initial DAR
February 2025	Updates made after ECP
February 2025	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.8. ENVIRONMENTAL FATE AND BEHAVIOUR	5
B.8.1. FATE AND BEHAVIOUR IN SOIL 6	
B.8.1.1. Laboratory route and rate of degradation in soil	6
B.8.1.1.1. Route of degradation in soil.....	6
B.8.1.1.1.1. Aerobic degradation in soil	6
B.8.1.1.1.2. Anaerobic degradation in soil	7
B.8.1.1.1.3. Soil photolysis	7
B.8.1.1.2. Rate of degradation in soil.....	7
B.8.1.1.2.1. Aerobic degradation in soil	17
B.8.1.1.2.2. Anaerobic degradation in soil	18
B.8.1.2. Field studies	18
B.8.1.2.1. Soil dissipation studies	18
B.8.1.2.2. Soil accumulation studies.....	18
B.8.1.3. Selection of laboratory and field endpoints for modelling purposes	19
B.8.1.4. Adsorption and desorption in soil	22
B.8.1.4.1. Adsorption and desorption in soil	22
B.8.1.4.2. Aged sorption	26
B.8.1.5. Mobility in soil.....	26
B.8.1.5.1. Column leaching.....	26
B.8.1.5.2. Lysimeter studies	26
B.8.1.6. Persistence in soil	27
B.8.2. FATE AND BEHAVIOUR IN WATER AND SEDIMENT 27	

B.8.2.1. Route and rate of degradation in aquatic systems (chemical and photochemical degradation).....	27
B.8.2.1.1. Hydrolytic degradation.....	27
B.8.2.1.2. Direct photochemical degradation	27
B.8.2.1.3. Indirect photochemical degradation.....	28
B.8.2.2. Route and rate of biological degradation in aquatic systems	28
B.8.2.2.1. Ready biodegradability	28
B.8.2.2.2. Aerobic mineralisation in surface water	45
B.8.2.2.3. Water / sediment studies	45
B.8.2.3. Degradation in the saturated zone	46
B.8.2.3.1. Water treatment procedures.....	46
B.8.2.4. Persistence in water	47
B.8.3. FATE AND BEHAVIOUR IN AIR	47
B.8.3.1. Route and rate of degradation in air	47
B.8.3.2. Transport via air	49
B.8.3.3. Local and global effects.....	49
B.8.4. DEFINITION OF THE RESIDUE	49
B.8.4.1. Definition of the residue for risk assessment (Data Requirement 7.4.1)	49
B.8.4.2. Definition of the residue for monitoring.....	50
B.8.5. MONITORING DATA CONCERNING FATE AND BEHAVIOUR OF THE ACTIVE SUBSTANCE, METABOLITES, DEGRADATION AND REACTION PRODUCTS	50
B.8.6. REFERENCES RELIED ON	50
B.8.6.1. Literature review.....	50
B.8.6.2. References relied upon	59
B.8.7. APPENDIX 1: EPI SUITE RESULTS FOR LUPANINE	65

B.8. Environmental fate and behaviour

The active substance aqueous extract from the germinated seeds of sweet *Lupinus albus*, is a plant extract with fungicidal properties that can be used on food and non-food crops. It is extracted from the germinated seeds of sweet *Lupinus albus* and formulated into PROBLAD PLUS. In general, botanical active substances are complex mixtures comprising of numerous components, therefore, the whole technical grade is regarded as the active substance which is described as a UVCB substance (Substance of Unknown or Variable composition, Complex reaction product or Biological material).

A major component of the aqueous extract from the germinated seeds of sweet *Lupinus albus* is the BLAD protein. BLAD is a naturally occurring seed storage protein in germinated sweet lupins. It is a 20 kDa¹ polypeptide that is comprised of 173 amino acid residues and is a stable intermediate of the catabolism of β -conglutin², or characterised as a fragment of the amino acid sequence of β -conglutin, therefore, BLAD has no specific molecular or structural formula. BLAD is present in the aqueous extract from the germinated seeds of sweet *Lupinus albus* as a 210 kDa glyco-oligomer which is mainly composed of the 20 kDa polypeptide. The published literature (Monteiro et al. 2015) indicates that the 210 kDa protein present in PROBLAD PLUS is a glyco-oligomer composed of several polypeptides, the major ones exhibiting molecular masses of 14, 17, 20 (BLAD) 32, 36, 48 and 50kDa. It is noted that BLAD contains a high proportion of the nitrogen-rich amino acids, which is consistent with its role as a seed storage protein.

BLAD forms 20% w/w of the PROBLAD PLUS formulation and is not isolated during the preparation of the product. The majority of the testing summarised in the dossier has therefore been conducted with PROBLAD PLUS. The applicant notes that very small amounts of BLAD have been isolated and purified using biochemical procedures described in the published study at section B 8.1.1.2. In addition, the applicant states that BLAD is difficult and costly to generate, and is impractical to prepare on a large scale. Nevertheless, it has been used as a test material in a small number of studies summarised in this section.

¹ Unit of molecular mass, 1 Dalton (Da) = 1g/mol

² major *Lupinus* seed storage protein

B.8.1. Fate and behaviour in soil

B.8.1.1. Laboratory route and rate of degradation in soil

B.8.1.1.1. Route of degradation in soil

B.8.1.1.1.1. Aerobic degradation in soil

Standard laboratory studies to determine the route of degradation in soil have not been provided. A case has been made by the applicant that the production of ^{14}C radiolabelled aqueous extract from the germinated seeds of sweet *Lupinus albus* or BLAD to use in such studies would be very difficult. BLAD is formed by the natural process within the germinating lupin seeds, and the labelling of a specific part of the BLAD protein is not possible. Furthermore, if specific labelling was possible, the protein structure would require many different labelling positions to determine the fate of all portions of this very large molecule (210 kDa). Tritium labelling may be possible, as it is sometimes possible to exchange hydroxyl groups, but the applicant considered this type of labelling is not considered as being suitable for environmental fate studies. It is suggested by the applicant that it may be possible to produce ^{14}C BLAD by growing lupins on media containing a ^{14}C substrate, so that the lupin plants become uniformly labelled with ^{14}C . It would follow that BLAD protein isolated from these plants would also be uniformly labelled with ^{14}C . The applicant stated that production would require large amounts of radiochemical and produce a vast amount radiochemical waste. The applicant also considered it unrealistic to assume that sufficient amount of radiolabelled BLAD could be produced to perform environmental fate studies, using such a synthesis route. If such material was produced, it would be useful to measure mineralisation in the fate studies from the production of radiolabelled CO_2 . However, the very large number of minor radiolabelled metabolites that would be produced due to the uniformly labelled material would not allow the route of degradation to be determined. HSE acknowledges the challenges and agrees that it would be difficult to gain any meaningful results using the proposed approach to attempt to generate radiolabelled BLAD.

The applicant has also proposed that BLAD can be regarded as a naturally occurring component, as it will be present in the soil wherever lupin seedlings are growing. It is noted that lupins are widely grown as green manure crops all over the world and sweet varieties are extensively grown in Mediterranean region and Australia for food and animal feed. However, no information on the naturally occurring levels of BLAD in seeds or soils during germination has been provided to give an understanding of the natural occurrence of BLAD in the environment.

Aqueous extract from the germinated seeds of sweet *Lupinus albus* along with its lead component, BLAD, have been demonstrated to be readily biodegradable according to the conditions of OECD test guideline 301 D (closed bottle test) and

OECD test guideline 301 B (CO₂-evolution test) respectively. Supporting information has also been presented in an OECD test guideline 301 D (closed bottle test) to further support the ready biodegradability of BLAD, see section B.8.2.2.1. According to Section 3 of the OECD guidelines, a positive result in a test for ready biodegradability (e.g., $\geq 60\%$ of the theoretical oxygen demand (ThOD) in a 10 day window for test 301 B, and 60% removal of the chemical oxygen demand (COD) in a 14 day window in the OECD 301 D test) can be considered as indicative of rapid and ultimate degradation in most environments because the remaining fraction of 30-40% of the test substance is assumed to be assimilated by the biomass or present as products of biosynthesis. Thus, further studies to investigate the route of degradation in soil are not considered necessary, see Section B.8.2.2.1.

In summary, it is considered that aqueous extract from the germinated seeds of sweet *Lupinus albus* along with its lead component, BLAD, will degrade readily and completely within natural soil systems, without the formation of any significant soil metabolites, see Section B.8.2.2.1.

B.8.1.1.1.2. Anaerobic degradation in soil

Aqueous extract from the germinated seeds of sweet *Lupinus albus* along with its lead component, BLAD, have been demonstrated to be readily biodegradable (see Section B.8.2.2.1). A positive result in a test for ready biodegradability can be considered as indicative of rapid and ultimate degradation in most environments. Therefore, exposure of aqueous extract from the germinated seeds of sweet *Lupinus albus* and, BLAD to anaerobic soil conditions is not anticipated based on rapid degradation in aerobic soil and the proposed use of the product.

B.8.1.1.1.3. Soil photolysis

Aqueous extract from the germinated seeds of sweet *Lupinus albus* along with its lead component, BLAD, have been demonstrated to be readily biodegradable (see Section B.8.2.2.1). A positive result in a test for ready biodegradability can be considered as indicative of rapid and ultimate degradation in most environments. Therefore, biological degradation is considered to be the most significant route of dissipation for aqueous extract from the germinated seeds of sweet *Lupinus albus* in soil, and soil photolysis studies are not considered necessary.

B.8.1.1.2. Rate of degradation in soil

Reference:	KCA 7.1.2.1/04
-------------------	----------------

Report Title:	A nontoxic polypeptide oligomer with a fungicide potency under agricultural conditions which is equal or greater than that of their chemical counterparts.
Author(s) & Year:	S. Monteiro, A. Carreira, R. Freitas, A. Margarida Pinheiro, R. Boavida Ferreira (2015)
Document No, Authority registration No	Published paper, PLoS ONE 10(4): e0122095. Doi:1371/journal.pone.0122095
Substance used:	BLAD, Isolated protein from an extract from plants of the genus <i>Lupinus</i> . Lot/Batch No. and purity: not provided
Guideline(s):	None
Deviations:	n/a
GLP or GEP:	No
Acceptability:	Supplementary
Study relied upon:	Yes. However as this is a published paper data protection would not be applicable

Introduction

The susceptibility of BLAD protein to proteolysis and hence degradation in soils was investigated by mixing the protein with common proteolytic enzymes, namely pronase, trypsin, proteinase K, α -chymotrypsin and subtilisin.

Materials and methods

Preparation and isolation of BLAD protein

Dry seeds of white lupin (*Lupinus albus* L.) cv. Leblanc were obtained from a local market and the seeds were germinated for periods up to 10 days. In all cases, the seed coats were removed and the intact cotyledons dissected from the axes and stored frozen at -80°C until needed. The cotyledons from germinated seedlings, were ground and homogenised with a mortar and pestle in water (pH adjusted to 8.0) containing 10 mM CaCl_2 and 10mM MgCl_2 (2 mL/g fresh weight) incubated at 4°C for 30 min with agitation, filtered through cheesecloth and centrifuged at 30,000g for 1 h

at 4°C. The precipitate was suspended in the globulin solubilising buffer (2 mL/g fresh weight; 100 mM Tris-HCl buffer, pH 7.5, containing 10% (w/v) NaCl, 10 mM ethylenediaminetetraacetic acid (EDTA) and 10 mM ethyleneglycol-bis(β -aminoethylether)-N,N,N',N'-tetraacetic acid (EGTA) agitated during 30 min at 4°C and followed by a centrifugation step for 1 h at 30,000g and 4°C and desalted on PD-10 columns. For individual globulins purification, the total fraction was fractionated and purified by AKTA anion exchange chromatography on a Q-Sepharose column (\emptyset = 1 cm; h = 8 cm; flow rate = 1.5 mL/min). The bound proteins were eluted with a gradient of NaCl (0 to 1M) and desalted into 50 mM Tris-HCl buffer, pH 7.5. α - and γ -conglutins were purified from *L. albus* cotyledons. BLAD protein was extracted and isolated from the cotyledons of eight day old seedlings. The protein corresponding to β -conglutin was purified by AKTA anion exchange chromatography as explained above and subsequently subjected to AKTA gel filtration on the Superose 12 HR 10/30 column previously equilibrated in 50 mM Tris-HCl buffer, pH 7.5. This last purification step does not affect the polypeptide pattern of the protein, but removes unidentified low molecular mass compounds.

Protein content measurements

Protein content was measured using a modification of the Lowry method.

Analysis of proteins by SDS-PAGE

One-dimensional, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to a modification of the methods described by Weber and Osborn and Laemmli. The modification included the use of m-cresol purple as the tracking dye and the inclusion of 0.1 M sodium acetate in the anode buffer to allow the resolution of polypeptides with molecular masses ranging from 2.5 to > 200 kDa. The molecular mass polypeptide standards used ranged from the 205 kDa subunit of rabbit muscle myosin to the 14.2 kDa of bovine milk R-lactalbumin. Western blotting was performed using a semi-dry electrophoretic transfer cell (Trans-Blot SD, Bio-Rad, Richmond, CA). After protein transfer, the proteins were immobilized on nitrocellulose membranes, visualized by incubation in an amido black 10B/methanol/acetic acid solution and de-stained in 2-propanol/acetic acid.

Study Design

The isolated BLAD protein was mixed with common commercially available proteolytic enzymes (pronase, trypsin, proteinase K, α -chymotrypsin and subtilisin) and incubated at room temperature for 1 hour, 2 hours, or 2 hours followed by the addition of marker protein (55 μ g pure ribulose biphosphate carboxylase) and a further 1 hour incubation. The marker protein was also separately incubated for 1 hour with the proteolytic enzymes as a positive control. The protein in each incubate

was analysed using SDS-PAGE along with reference solutions of the isolated BLAD protein, the proteolytic enzyme and the pure ribulose biphosphate carboxylase. The gels also contained molecular mass standards to enable the molecular mass of the polypeptides to be estimated.

Table 8.1.1-1: Identification of lanes on the polyacrylamide gels

Lane	Description
Lane 1	Pure 210 kDa BLAD protein (composed mainly of a 20 kDa polypeptide)
Lane 2	Pure 210 kDa BLAD protein mixed with proteolytic enzymes and incubated at room temperature for 1 hour
Lane 3	Pure 210 kDa BLAD protein mixed with proteolytic enzymes and incubated at room temperature for 2 hours
Lane 4	Pure 210 kDa BLAD protein mixed with proteolytic enzymes and incubated at room temperature for 2 hours followed by addition of pure ribulose biphosphate carboxylase (55 µg) and a further 1 hour of incubation.
Lane 5	Pure ribulose biphosphate carboxylase (55 µg) incubated for 1 hour with the proteolytic enzyme
Lane 6	Pure ribulose biphosphate carboxylase (55 µg)
Lane 7	Protease enzyme (20 µg)
a and b	Molecular mass standards (kDa)

Results and Discussion

The gels from the SDS-PAGE analysis of the incubation with each proteolytic enzyme are shown in Figures 8.1.1.2./01 to 8.1.1.2./05. The analysis show that the BLAD protein is degraded by all five proteolytic enzymes, with the BLAD protein completely degraded after 2 hours. The marker protein (ribulose biphosphate carboxylase) was also completely degraded by all the proteolytic enzymes. The marker protein was also readily degraded by all proteases tested when added to the reaction medium after the enzymatic digestion of the BLAD protein, demonstrating the absence of proteolytic fragments, which could act as proteinase inhibitors or anti-nutritional factors.

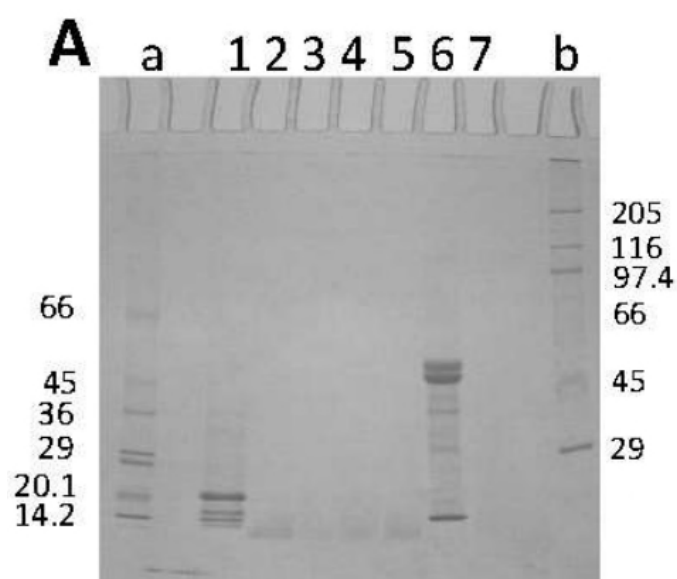


Figure 8.1.1.2./01. SDS-PAGE analysis showing susceptibility of BLAD protein to pronase. Image of SDS-PAGE gel with marker compounds in column a and b. See table 8.1.1-1 for details of the samples in each lane.

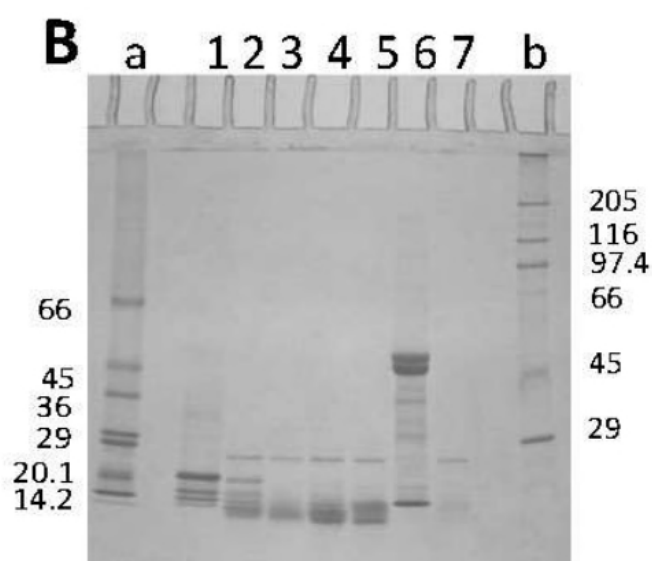


Figure 8.1.1.2/02. SDS-PAGE analysis showing susceptibility of BLAD protein to trypsin. Image of SDS-PAGE gel with marker compounds in column a and b. See table 8.1.1-1 for details of the samples in each lane.

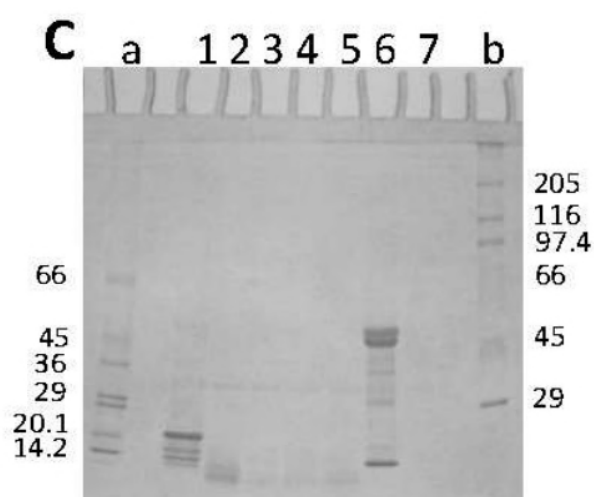


Figure 8.1.1.2./03. SDS-PAGE analysis showing susceptibility of BLAD protein to proteinase K. Image of SDS-PAGE gel with marker compounds in column a and b. See table 8.1.1-1 for details of the samples in each lane.

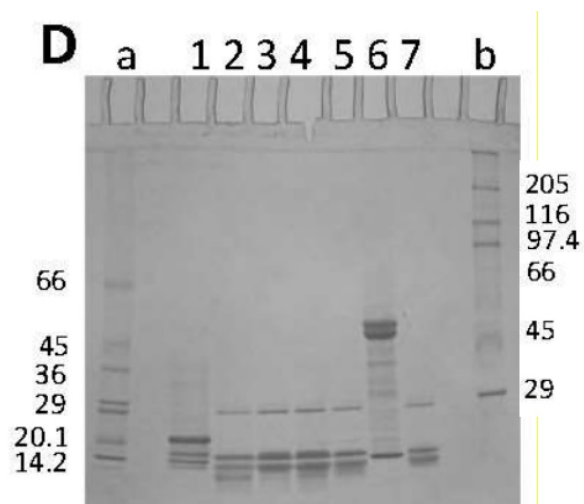


Figure 8.1.1.2./04. SDS-PAGE analysis showing susceptibility of BLAD protein to α -chymotrypsin. Image of SDS-PAGE gel with marker compounds in column a and b. See table 8.1.1-1 for details of the samples in each lane.

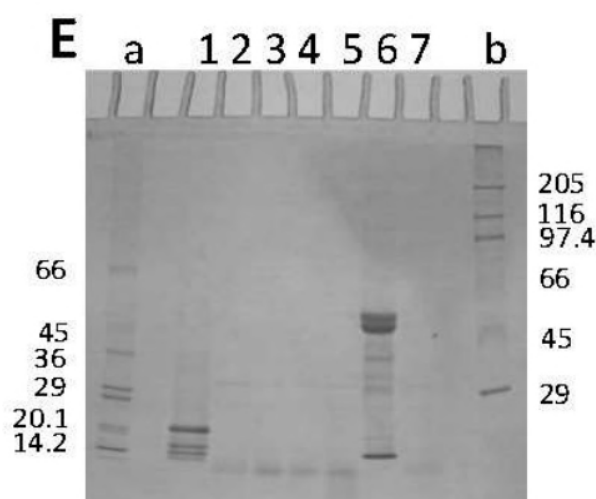


Figure 8.1.1.2./05. SDS-PAGE analysis showing susceptibility of BLAD protein to subtilisin. Image of SDS-PAGE gel with marker compounds in column a and b. See table 8.1.1-1 for details of the samples in each lane.

Relevance to rate of degradation in soil

The relevance of the above experiments to the rate of degradation of the BLAD protein in soil depends on the extent of occurrence of the tested proteolytic enzymes in soil. The following summary information was provided for each protease and its sources:

Pronase

Pronase is a commercially available mixture of proteases isolated from the extracellular fluid of *Streptomyces griseus* – a species of bacteria in the genus *Streptomyces*. *Streptomyces* are found predominantly in soil and decaying vegetation. Most streptomycetes produce spores, and in soil they behave much like fungi, helping to decompose the organic matter of dead organisms. They are also noted for their distinct "earthy" odour.

Trypsin

Trypsin is protease from the PA clan superfamily. It is found in the digestive system of many vertebrates, where it hydrolyses proteins. Trypsin is formed in the small intestine when its proenzyme form, the trypsinogen produced by the pancreas, is activated.

Proteinase K

Proteinase K is a broad-spectrum serine protease able to digest hair (keratin). It was originally found in extracts of the fungus *Engyodontium album*, the most common

species of the *Engyodontium* genera of fungi, which is common in soil and plant debris.

α -Chymotrypsin

α -Chymotrypsin is a protease from the PA clan superfamily. It is found in the pancreatic juice acting in the duodenum, where it performs proteolysis. α -Chymotrypsin is synthesized in the pancreas by protein biosynthesis as a precursor called chymotrypsinogen that is enzymatically inactive.

Subtilisin

Subtilisin is a non-specific protease initially obtained from *Bacillus subtilis*. Subtilisins belong to subtilases, a group of serine proteases. Subtilisins can be obtained from many types of soil bacteria, for example, *Bacillus amyloliquefaciens*, a root-colonizing bacteria, from which they are secreted in large amounts.

Of the enzymes tested pronase, proteinase K and subtilisin are found in commonly occurring soil bacteria or fungi.

Conclusion

The study indicates rapid proteolytic degradation of the major constituent BLAD by the tested enzymes, three of which are found in naturally occurring soil micro-organisms.

The applicant has stated that 'although it is difficult to provide endogenic concentrations of these enzymes in soil, the enormous concentrations of bacteria and fungi in agricultural soil would indicate that concentrations of these enzymes would be significant. For example, the approximate range of biomass in a typical temperate grassland soil is 1-2t ha⁻¹ for bacteria (1-4t ha⁻¹ if actinomycetes are included) and 2-5t ha⁻¹ for fungi. Therefore, the above experiments indicate that fast degradation of BLAD in soil is expected.' The source of this information was not provided by the applicant. Expert ECP advice was sought on the relevance of this information to the risk assessment. Members noted that this value could be variable and the levels presented were not unreasonable. However the use of this value was not critical to the risk assessment and did not provide any benefit to the fate and behaviour assessment. The ECP suggested that it would be more appropriate to use knowledge on likely enzyme levels in the soil and the fate and behaviour of similar size peptides within the environment. Based upon the literature more general principles could then be applied to this peptide and its fate and behaviour in the environment.

The applicant has considered this further and noted that the methodologies adopted for soil enzyme measurement are not universal and this often creates difficulties

when making comparisons.

“However, the nature of enzymes means that the activity, i.e. the function of breaking down the proteins, is the crucial aspect, and therefore is considered an acceptable substitute when discussing the degradation of proteins in soil. A number of papers are available to support the general statement on the importance of microbial decomposition of proteins but as examples Watanabe K & Hayano K (1994) state ‘soil protease is an important enzyme in the nitrogen cycle,’ and Greenfield et al. (2021) states that ‘proteases play a crucial role in the soil nitrogen (N) cycle by converting protein to oligopeptides and amino acids’.

Furthermore, within Greenfield et al. (2021), a meta-analysis of studies on protease activity across the globe using the substrates leucine-7-amino-4-methylcoumarin (AMC), N-benzoyl L-arginine amide (BAA), casein, p-nitroaniline (pNA) and Z-phe-leu was conducted. As stated above, these assays measured soil protease activity, but did not relate this measured activity to specific environmental activity, for example per hectare or per cubic metre of soil. In the assays, mean soil protease activity ranged between 0 and 15 million nmol product /g/h . Across all substrates, around 60% of the data lay between 0 and 1000 nmol product /g/h and 80% lay between 0 and 5000 nmol product /g/h.

Additionally, Adamczyk (2021) explores the proteases produced by plant roots (e.g. *Allium porrum*, *Allium cepa*, *Zea mays*, *Cucurbita pepo*, *Cucumis sativus*, *Hippophae rhamnoides*, *Geranium pusillum*, *Lactuca sativa*, *Ruta graveolens*, *Raphanus sativus*), which was quantified as contributing up to 15-19% of rhizosphere proteolysis in maize and wheat plants (Greenfield et al. 2020).

This demonstrates that protease activity in soil generally, but especially in agricultural soil, is a constitutively active process with multiple contributors. Therefore, when exposed to the soil, BLAD protein will be subject to enzymatic degradation within this system.”

To support additional consideration of the stability of similar sized peptides in soil the applicant has referenced further information in the published paper by Jan et al. (2009) that assessed the proteolytic breakdown of a radiolabelled protein produced in Atlantic salmon. This salmon serum protein was dominated by three abundant serum proteins which had molecular weights of 65, 75 and 120 kDa with labelling found in all the amino acid residues. Soil was sampled from the top 20 cm of a lowland agricultural site in Gwynedd, North Wales described as freely-draining grassland.

To assess the rate of protein and amino acid mineralization, the ^{14}C -labelled protein or protein hydrolysate containing free ^{14}C -labelled amino acids were added to 5 g soil at a rate of 10 mg kg^{-1} in a 50 ml polypropylene tube. Before sealing the tubes, a 1 M NaOH trap (1 ml) was added to each tube to collect the $^{14}\text{CO}_2$ evolved (Jones, 1999). The soils were then kept in the dark at either 10°C or 20°C and the NaOH traps

replaced periodically over a 30-d period. The amount of $^{14}\text{CO}_2$ captured was determined using LSC. To determine the amount of ^{14}C -label remaining in soil after 30 d, the soil was extracted with 0.5 M K_2SO_4 (1:5 w/v soil-to-solution ratio) for 20 min by shaking at 200 rpm. After shaking, the soil suspension was centrifuged (16000g, 5 min) and the supernatant recovered for liquid scintillation counting. At the end of the 30-day incubation period, < 5% of the applied radioactivity could be recovered from the soil by 0.5 M K_2SO_4 , indicating extensive degradation of the proteins within the soil at 10°C or 20°C. It was noted that the presence of plants and increased temperatures resulted in reduced protein half-life in soil. For example proteins in planted soil at 10°C had an indicated half-life at 224 hours compared to 59 hours in soil at 20°C.

It is recognised that the quantitative assessment of soil protease levels is challenging and there is inconsistency in the analytical approaches taken. The applicant has referred to an estimate of extracellular leucine aminopeptidase activity using 7-amido-4-methylcoumarin (AMC) as reference standard from Greenfield et al (2020) and related this to root biomass. They note that the Wheat Growth Guide (2008) (HGCA) gives wheat root biomass of 0.9 t/ha to 1.05 t/ha i.e. approximately 1 ton or 1,000 kg ha⁻¹. Greenfield et al. (2020) gives leucine aminopeptidase activity of around 5-6 µmol AMC /mg root/h. This is equivalent to 5-6 mmol AMC /g root/h or 5-6 mol AMC /kg root /h. Therefore, this is equivalent to around 5,000 to 6,000 mol AMC /kg root /h/ha for this specific enzyme and protein combination. Given AMC's molecular weight of 175.2, this suggests 5,000 to 6,000 x 175.2 AMC /g/h/ha (i.e. 876 to 1050 kg AMC) can be broken down by this one enzyme per hour per hectare. The applicant notes that the quantity of protein that can potentially be broken down per hour per hectare by this enzyme is approximately 200 times the annual application rate of BLAD.

It is noted that the understanding of the role of the extracellular root proteases and protease levels in the rhizosphere is a complex area and this example from a published paper considering the levels of extracellular leucine aminopeptidase in wheat is just one example but it does indicate that the protease breakdown of the seed storage protein BLAD (which contains 9% Leucine) by this or other proteases would be relatively rapid in the natural environment.

In summary the additional information provided by the applicant highlights the role that protease enzymes play in peptide degradation in soil. It is noted that the extracellular protease activity can contribute approx. 15% of the protease activity in soil with additional protease activity in the rhizosphere. In addition the soil temperature and presence of planted material can all impact on the protease activity levels.

The proposed DT50 values used in the environmental risk assessment are default values based upon a ready biodegradability study indicating that the substance is ready biodegradable and account has been taken of the higher predicted Koc value for the lead component BLAD within the aqueous extract from the germinated seeds of sweet *Lupinus albus* and the impact this could have on the proposed DT50 for this active substance in the absence of the additional supporting information. Hence a value of 300 days has been used. The submitted information further supports the understanding that the DT50 values used in the risk assessment for soil and water are likely to be highly conservative and are sufficient to address the environmental risks from the proposed use of the aqueous extract from germinated seed of sweet *Lupinus albus*.

Overall conclusion

This substance is a plant storage protein that is intended to be broken down and degraded as an energy source in the natural environment. The results of a ready biodegradability screening study indicate that the substance would be ready biodegradable. However, the high Kp values for this large protein could indicate some dependence on degradation such that this would be extended beyond the 30 days default value. The proposed DT50 value in soil of 300 days is used in the risk assessment and recognised as likely conservative. The additional supporting information provided illustrating the protease levels in the soil, and likely rapid degradation of similar sized peptides in the soil environment, would support the reduction of the soil DT50 for this peptide substance.

Therefore, based upon a weight of evidence approach using the submitted ready biodegradability data, the additional supporting information from the literature and the in vitro testing submitted by the applicant a proposal of 30 days DT50 can be supported for future assessments of BLAD peptide and the aqueous extract from germinated seed of sweet *Lupinus albus*.

Conclusion

The study indicates rapid proteolytic degradation of the major constituent BLAD by the tested enzymes, three of which are found in naturally occurring soil micro-organisms.

B.8.1.1.2.1. Aerobic degradation in soil

Aqueous extract from the germinated seeds of sweet *Lupinus albus* along with its lead component, BLAD, have been demonstrated to be readily biodegradable (see Section B.8.2.2.1). The applicant has used the ready biodegradability studies

assessed in section B.8.2.2.1 to further support the degradation in soil. It is noted that the additional supplementary information detailed in section B.8.1.1.2 indicates further that the aqueous extract of sweet *Lupinus albus* would likely be subject to biological degradation in soil and not persist in the natural environment.

B.8.1.1.2.2. Anaerobic degradation in soil

Aqueous extract from the germinated seeds of sweet *Lupinus albus* along with its lead component, BLAD, have been demonstrated to be readily biodegradable (see Section B.8.2.2.1). The applicant has used the ready biodegradability studies assessed in section B.8.2.2.1 to further support the degradation in soil.

B.8.1.2. Field studies

B.8.1.2.1. Soil dissipation studies

Aqueous extract from the germinated seeds of sweet *Lupinus albus*, along with its lead component, BLAD, have been demonstrated to be readily biodegradable (see Section B.8.2.2.1). The applicant has therefore, stated that field soil dissipation studies are not considered necessary. The implication is that the dissipation of BLAD would not meet the DT50 and DT90 triggers in the data requirements for conduct of field dissipation studies. HSE notes that due to the higher predicted Koc value for the lead component BLAD within the aqueous extract from the germinated seeds of sweet *Lupinus albus* the proposed DT50 for this active substance would be greater than the minimal default of 30 days and a value of 300 days is proposed (see Section B.8.1.4). Whilst this DT50 would trigger the need for field studies it is noted that there is additional qualitative evidence to indicate that the aqueous extract from the germinated seeds of sweet *Lupinus albus* would likely be subject to biological degradation in soil and not persist in the natural environment. HSE agrees that the generation of field dissipation studies would not add further to the exposure assessment.

B.8.1.2.2. Soil accumulation studies

Aqueous extract from the germinated seeds of sweet *Lupinus albus*, along with its lead component, BLAD, have been demonstrated to be readily biodegradable (see Section B.8.2.2.1). The applicant has stated therefore that field soil accumulation studies are not considered necessary. HSE notes that due to the higher predicted Kp value for the aqueous extract from the germinated seeds of sweet *Lupinus albus* the proposed DT50 for this active substance is greater than the minimal default of 30 days and a value of 300 days is proposed. Whilst this DT50 would trigger the need for accumulation assessment it is noted that there is additional qualitative evidence to indicate that the aqueous extract from the germinated seeds of sweet *Lupinus albus* would likely be subject to biological degradation in soil and not persist in the natural

environment. HSE agrees that the generation of soil accumulation studies would not add further to the exposure assessment.

B.8.1.3. Selection of laboratory and field endpoints for modelling purposes

Aqueous extract from the germinated seeds of sweet *Lupinus albus* along with its lead component, BLAD, have been demonstrated to be readily biodegradable according to the conditions of OECD test guideline 301 D (closed bottle test) and OECD test guideline 301 B (CO₂-evolution test) respectively. According to ECHA guidance (ECHA, 2017), when results from degradation tests simulating the conditions in soil are not available (e.g., OECD test guidelines 307), the use of results from various screening tests may be considered. ECHA guidance gives a proposal for a first order half-life of readily biodegradable substances in soil for use in exposure models as a range from 30 days to more than 3000 days depending on the substance's sorption potential.

Table 8.1.3-1: Copy of the Table 6 taken from the ECHA Guidance on the Biocidal Products Regulation Volume IV Environment, Assessment & Evaluation (Parts B+C) version 2.0 that details half-lives (days) for (bulk) soil based on results from standardised biodegradation test results.

K_p, soil [l.kg⁻¹]	Readily biodegradable	Readily biodegradable, failing 10-d window	Inherently biodegradable
≤ 100	30	90	300
> 100, ≤ 1000	300	900	3,000
> 1000, ≤ 10,000	3,000	9,000	30,000
etc.	etc.	etc.	etc.

It is recognised in this screening test that conditions are different to natural environmental compartments. The concentration of the test substance is greater than generally occurring in the environment and the microbial biomass is normally lower under environmental conditions than those occurring in these tests. These factors are taken into account in the proposed half-lives. The general experience is that a substance passing a test for ready biodegradability may under most environmental conditions be rapidly degraded and the estimated half-lives for such substances

should therefore be regarded as being in accordance with “the realistic worst-case concept”.

The ECHA guidance also notes that “The guidance for use of such data is based on the general recognition that for substances with low K_p values at present not enough empirical data is available to assume some sort of dependence of the soil biodegradation half-life on the solids/water partition coefficient. Nevertheless, for substances with high K_p values there is evidence that some sort of K_p dependence exists.” Therefore, degradation half-life classes for (bulk) soil, partly based on K_p^3 , are also proposed. The ECHA guidance determines the $K_{p\text{soil}}$ value as the K_{oc} soil value multiplied by the fraction of organic carbon in the compartment i.e.

$$K_{p\text{soil}} [\text{L/kg}] = F_{oc\text{soil}} \cdot K_{oc}$$

In addition a default assumption for $F_{oc, \text{soil}}$ is made at of $0.02 \text{ kg}_{oc} \cdot \text{kg}_{solid}^{-1}$. Therefore a direct correlation exists such that a high K_p value would also relate to a high K_{oc} value.

PROBLAD PLUS consists of many different constituents and components (see Volume 4). The major component in the aqueous extract from the germinated seeds of sweet *Lupinus albus* is the protein BLAD which represents 20% w/w in the final product. The applicant has provided information to indicate that the different constituents of PROBLAD PLUS have a Log K_{oc} ranging from 3.11-347 (See section 8.1.4 and Volume 4). This is equivalent to a log K_p range of 1.4-345 and according to the guidance a higher K_p results has the consequence of a longer DT50 value being attributed to a readily biodegradable compounds (ECHA, 2017). The log K_{oc} estimate for the glyco-oligomer in the aqueous extract of sweet *Lupinus albus* extract is indicated as 347 based upon the applicants quantitative structure property relationships (QSPR) approach. Based upon this, a DT50 of 300 or 3000 days for the product PROBLAD PLUS and its major constituent BLAD could be proposed.

The applicant has noted that the additional published report at section B.8.1.1.2 details SDS-PAGE analysis of incubated reaction mixtures that indicate rapid proteolytic degradation of the major constituent BLAD by enzymes, found in naturally occurring soil micro-organisms (section B.8.1.1.2) should be considered further here. They propose a DT90 of 200 hours for the BLAD protein based upon completion degradation in the in vitro testing after two hours and a safety factor of 100. This supplementary study does indicate further support for the likely rapid degradation of

³ Solids-water partition coefficient [$\text{L} \cdot \text{kg}^{-1}$]

BLAD by soil dwelling organisms but these data have not been used directly in the risk assessment.

In considering the selection of endpoints in soil, reference is made to the outcome of the ready biodegradability tests (section B.8.2.2.1) which are aimed at measuring the ultimate biodegradability of a substance. These studies are screening tests for identifying substances that, based on general experience, are assumed to undergo rapid and ultimate biodegradation in the aerobic environment and they assess complete degradation to CO₂. Based upon these tests PROBLAD PLUS and the lead component BLAD are classified as readily biodegradable. However, based upon the high molecular size and likely high K_{oc} value for the substance (Section B.8.1.4.1) following the ECHA (2017) guidance, it is not appropriate to select the lower 30 days default value for degradation in soil for the most strongly sorbed components of the mixture. The ECHA guidance notes that for substances with high K_p values there is evidence that some sort of K_p dependence of the soil biodegradation half-life on the solids/water partition coefficient exists.

The PROBLAD PLUS extract contains a range of compounds with a range of log K_{oc} values from 3.11 (lupanine) to 347 > 1000 (the 210 kDa glyco-oligomer BLAD). Therefore, based on this a DT₅₀ value from 30- > 3000 days could be selected for the individual components, with the lead component BLAD attracting a higher DT₅₀ in soil.

However, the supporting information provided does indicate likely biological degradation in the field and it is understood that this naturally occurring active substance is a plant storage protein consisting of nitrogen rich amino acid residues. Furthermore, the bioavailability of the substance when sorbed to the soil is uncertain and the ECHA guidance assumptions on K_p dependence of soil degradation may be less relevant for such large molecules. In the absence of any further data beyond a screening study it is proposed that to try and keep alignment with the ECHA guidance a DT₅₀ in soil value of 30 days is not appropriate because the mixture includes components with much higher predicted K_p values which may degrade more slowly in soil. But equally the supporting evidence suggests that DT₅₀ values ≥ 3000 days are also likely to be unrealistic. HSE proposes that the soil DT₅₀ is set at 300 days for the purposes of the exposure assessment. With the additional supporting information giving an indication of the likely biological degradation following use and further information provided post ECP to give additional support to the likely degradation in the soil environment, it is considered that a DT₅₀ in soil of 30 days can be considered for future assessments of this active substance. No consideration of accumulation in soil is required. HSE also proposes that this biological active substance does not need to be assessed for long term accumulation in soil although strictly classed as persistent in soil.

B.8.1.4. Adsorption and desorption in soil

B.8.1.4.1. Adsorption and desorption in soil

BLAD is a naturally occurring seed storage protein in germinated sweet lupins. It is present in the aqueous extract from the germinated seeds of sweet *Lupinus albus* as a 210 kDa glyco-oligomer which is mainly comprised of a 20 kDa polypeptide comprised of 173 amino acid residues. Therefore, there is no specific molecular or structural formula. In the absence of structural formula, the soil adsorption cannot be estimated with standard Molecular connectivity index (MCI) or quantitative structure property relationships (QSPR). There are other parameters that can be used but these require reliable experimental data to support them. The applicant has referred to a QSPR model for predicting log K_{OC} from molecular weight (MW), number of benzene rings (N_{\emptyset}), and number of N (N_N), O (N_O) and S (N_S) atoms, of which MW and N_{\emptyset} were the most important descriptors considered to be positively correlated with sorption, developed by Delgado et al. (2003)⁴.

The applicant notes that this QSPR model was not developed for proteins, but states that the data set used to develop the model was made up of 82 structurally diverse compounds and contained; polar, non-polar, saturated, unsaturated, aliphatic, aromatic and polycyclic compounds covering a log K_{OC} range from about 1 to 6 log units. The experimental data set of the sorption coefficients for these compounds was collected from several literature sources and the model has been validated against predicted log K_{OC} with a correlation coefficient of 0.94. In addition, the model requires little information on structural formula, and the major inputs can be derived from just molecular formula. Although there is no specific molecular formula for BLAD, a reasonable estimate of the number of benzene rings, and the number of N, S and O atoms, can be made by considering its molecular weight (210 kDa) and the natural occurrence of the amino acid constituents of proteins. The table below elucidates this calculation. These values of N_{\emptyset} , N_N , N_O and N_S were then used in the equation of Delgado et al. to calculate an estimate of log K_{OC} for BLAD.

$$\log K_{OC} = 0.51 + 0.60 \cdot N_{\emptyset} + 1.02 \times 10^{-2} \cdot MW - 0.48 \cdot N_N - 0.25 \cdot N_O + 0.61 \cdot N_S$$

⁴ Delgado et al. (2003) A simple QSPR model for predicting soil sorption coefficients of polar and non-polar organic compounds from molecular formula. J. Chem. Inf. Comput. Sci., 43, 1928-1932

Table 8.1.4-1: Estimation of number of benzene rings (N_{\emptyset}), and number of N (N_N), O (N_O) and S (N_S) atoms in BLAD

Amino acid	MW	Occurrence in proteins ¹	In protein structure				
			MW ²	N_{\emptyset}	N_N	N_O	N_S
Alanine	89	9.0%	71	0	1	1	0
Arginine	174	4.7%	156	0	4	1	0
Asparagine	132	4.4%	114	0	2	2	0
Aspartate	133	5.5%	115	0	1	3	0
Cysteine	121	2.8%	103	0	1	1	1
Glutamine	146	3.9%	128	0	2	2	0
Glutamate	147	6.3%	129	0	1	3	0
Glycine	75	6.2%	57	0	1	1	0
Histidine	155	2.1%	137	0	3	1	0
Isoleucine	131	4.6%	113	0	1	1	0
Leucine	131	7.5%	113	0	1	1	0
Lysine	146	7.0%	128	0	2	1	0
Methionine	149	1.7%	131	0	1	1	1
Phenylalanine	165	3.5%	147	1	1	1	0
Proline	115	4.6%	97	0	1	1	0
Serine	105	7.1%	87	0	1	2	0
Threonine	119	6.0%	101	0	1	2	0
Tryptophan	204	1.1%	186	1	2	1	0
Tyrosine	181	3.5%	163	1	1	2	0

Volume 3 – B.8 (AS)

Valine	117	6.9%	99	0	1	1	0
Sum			2375	3	29	29	2
Number per kDa				1.3	12.2	12.2	0.8
Number per kDa corrected for occurrence in proteins				0.7	12.2	13.5	0.4
Approximate number in BLAD protein (210 kDa)				156	2570	2836	87

¹ Average occurrence in over 200 proteins. From Klapper (1977) Biochem. Biophys. Res. Commun. 78, 1018-1024. ² Molecular weight taking account of loss of H₂O when forming peptide bonds

Using QSPR approach detailed above, the applicant has added a correction step where each component is corrected for natural abundance using the following equation:

$$\text{component number} * (\text{occurrence in protein} / (1/\text{number of amino acids})).$$

Based on the number of amino acids at 20 and $1/20 = 0.05$, this can be simplified to give the following equation:

$$\text{component number} * (\text{occurrence in protein}/0.05).$$

Using this approach the applicant has estimated a log K_{OC} of BLAD at 347 suggesting that BLAD is an immobile compound and based upon this, a K_{OC} of 10,000 L/kg is proposed for use in the modelling. This value is used as it would support the determination as an immobile compound but can also be accepted by the modelling software.

HSE notes that the QSPR model approach used here was not developed for proteins, and whilst it covers a wide range of compounds with differing properties; polar, non-polar, saturated, unsaturated, aliphatic, aromatic and polycyclic compounds the log K_{OC} range tested, from about 1 to 6 log units, is very different to that determined for BLAD. In addition, the paper indicates that the most important descriptors are mw and N_Ø and it is noted that there are fewer N_Ø components in amino acids compared to synthetic chemicals. However, it is clear that the BLAD compound will have a high molecular weight and if the same approach is applied to

the 20 kDa polypeptide BLAD then the outcome is an estimated log K_{oc} of 27.9 which suggests that the BLAD component is likely to be an immobile compound. Therefore the use of a K_{oc} value at 10,000 is accepted by HSE as the substance will likely have low mobility in soil. Using this value will also avoid any potential issues with upper limit values in the modelling software. ECP independent expert advice was sought on this methodology. Members agreed that this approach is not appropriate for the determination of K_{oc} for a large polypeptide such as BLAD. Based on the substance properties and high molecular weight it was agreed that the BLAD is likely an immobile substance. However, the members raised concern regarding the use of the value of 10,000 L/kg and felt that the use of this value was not fully justified. In addition they felt it was inconsistent that this value was used in the groundwater modelling with a high aqueous solubility (Volume 3 CP B.8.). HSE accepts the members views on the inconsistency of the use of these parameters from a chemical perspective but notes that the use of the aqueous solubility parameter in the modelling is to inform the likely removal of the compound from the soil via volatility and does not impact on the mobility of the substance. HSE considers the default K_{oc} of 10,000 to still be acceptable for such a large molecular weight compound and is accepted as a maximum implementable value in the model.

Based upon the taxonomy and current knowledge the legume plant family is understood to contain quinolizidine alkaloids (QAs) which are components that contain the quinolizidine structure. There are many quinolizidine alkaloids present at very low levels within the active substance and lupanine has been indicated as a marker compound for these QAs. The QAs have been identified as relevant impurities (SANCO/10597/2003 –rev. 10.1) and SANCO/11470/2012 rev. 8, indicates these alkaloids as components of possible concern for humans, animals and/or the environment and hence the aqueous extract from the germinated seeds of sweet *Lupinus albus* is considered a 'group 2' botanical active substance.

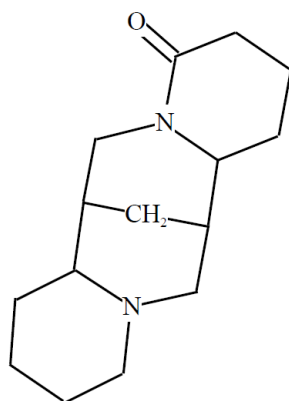


Figure B.8.1.4./01 Chemical structure of lupanine

The soil adsorption of lupanine has been estimated by the MCI and QSPR methods in the model KOCWIN (version 2.00), part of the US EPA 'EPI Suite' QSAR suite of models for estimating physical, chemical and biological properties of substances; version 4.11 was used. The full output file is given in Appendix 1. Within the model documentation, the MCI method is stated as being the most accurate approach. Using QSPR, the log K_{oc} is estimated to be 1.758 ($K_{oc} = 57.3$ mL/g). Using MCI, the log K_{oc} is estimated to be 3.11 ($K_{oc} = 1287$ L/kg). The calculated adsorption characteristics for this compound have been used to give an understanding of the potential range of components within the aqueous mixture.

B.8.1.4.2. Aged sorption

No data have been provided. This is acceptable as studies to address the data requirement for aged sorption are not considered necessary to support the risk assessment.

B.8.1.5. Mobility in soil

Based upon the ~~QSPR analysis done in section B.8.1.4.1.~~ high molecular weight of the major component BLAD it is considered that BLAD and PROBLAD PLUS will have low mobility. This will be addressed in the CP document (Volume 3 CP B.8.).

B.8.1.5.1. Column leaching

No data required.

B.8.1.5.2. Lysimeter studies

No data required.

B.8.1.6. Persistence in soil

Aqueous extract from the germinated seeds of sweet *Lupinus albus*, along with its lead component, BLAD, have been demonstrated to be readily biodegradable (see Section B.8.2.2.1). The applicant has therefore stated that PROBLAD PLUS and its individual constituents are not considered to be persistent in soil. HSE notes that due to the higher predicted K_p value for the aqueous extract of sweet *Lupinus albus* the proposed DT50 for this active substance is greater than the minimal default of 30 days and a value of 300 days is proposed. This would require the substance to be classified as ‘persistent in soil’.

B.8.2. Fate and behaviour in water and sediment

B.8.2.1. Route and rate of degradation in aquatic systems (chemical and photochemical degradation)

B.8.2.1.1. Hydrolytic degradation

Aqueous extract from the germinated seeds of sweet *Lupinus albus* along with its lead component, BLAD, have been demonstrated to be readily biodegradable (see Section B.8.2.2.1). A positive result in a test for ready biodegradability can be considered as indicative of rapid and ultimate degradation in most environments. The applicant has proposed that biological degradation is considered to be the most significant route of dissipation for aqueous extract from the germinated seeds of sweet *Lupinus albus* and hydrolytic degradation studies are not considered necessary. HSE consider that biological degradation will be a major route of dissipation for the aqueous extract from the germinated seeds of sweet *Lupinus albus*, but it is also noted that hydrolysis could be an additional route of degradation. The determination of ready biodegradability and associated assumptions on the degradation enables an environmental exposure assessment to be conducted and the generation of additional hydrolysis data may not add to the overall assessment of environmental exposures for this active substance. Therefore, it is agreed that these data are not required.

B.8.2.1.2. Direct photochemical degradation

Aqueous extract from the germinated seeds of sweet *Lupinus albus* along with its lead component, BLAD, have been demonstrated to be readily biodegradable (see Section B.8.2.2.1). A positive result in a test for ready biodegradability can be considered as indicative of rapid and ultimate degradation in most environments. Therefore, biological degradation is considered to be the most significant route of dissipation for aqueous extract from the germinated seeds of sweet *Lupinus albus* and aqueous photolysis studies are not considered necessary.

B.8.2.1.3. Indirect photochemical degradation

See Section B.8.2.1.2.

B.8.2.2. Route and rate of biological degradation in aquatic systems

Aqueous extract from the germinated seeds of sweet *Lupinus albus* along with its lead component, BLAD, have been demonstrated to be readily biodegradable (see Section B.8.2.2.1). The applicant has used the ready biodegradability studies assessed in section B.8.2.2.1 to further support the degradation in aquatic systems.

B.8.2.2.1. Ready biodegradability

Reference:	KCA 7.2.2.1/01
Report Title:	Biodegradability, CO ₂ -evolution test according to OECD 301 B
Author(s) & Year:	A. Brunswik-Titze (2015)
Document No, Authority registration No	Unpublished report No.: 1035
Substance used:	BLAD, Isolated protein from an extract from plants of the genus <i>Lupinus</i> . Lot/Batch No.: No. 06.2015 Purity: > 99% (w/w) BLAD protein
Guideline(s):	Yes OECD Guideline for Testing of Chemicals No. 301 B (July 1992)
Deviations:	No
GLP or GEP:	Yes
Acceptability:	Yes
Study relied upon:	Yes

Introduction

The ready biodegradability of BLAD was assessed by measurement of the formed carbon dioxide (OECD 301 B: CO₂ evolution test). The Applicant used sodium benzoate as the reference item.

Materials and methods

Table 8.2.2-1: Test material information for the study of the ready biodegradability of BLAD

Compound	Test item	Reference material
Description	BLAD, Isolated protein from an extract from plants of the genus <i>Lupinus</i> .	Sodium benzoate
Lot/Batch No.	No. 06.2015	BCBH0034V
Purity	> 99% (w/w) BLAD protein	Not stated
Carbon content	43.9% (0.439 mg/mg)	58.3% (0.583 mg/mg)
ThCO ₂	1.61 mg/mg (based on carbon content)	2.137 mg/mg (based on carbon content)
CAS No.	1219521-95-5	532-32-1
Stability	Two years at ambient temp.	Not stated

The test item is described as > 99% BLAD protein and the purity certification references the approach taken in published literature (Monteiro et al. 2015) in which the protein corresponding to β -conglutin was purified by AKTA anion exchange chromatography and subsequently subjected to AKTA gel filtration on the Superose 12 HR 10/30 column previously equilibrated in 50 mM Tris-HCl buffer, pH 7.5. The test was conducted with activated sludge obtained from the municipal wastewater treatment plant at Breisgauer Bucht/Germany. The sampling date of the activated sludge was on 07 July 2015 and the dry solid content of the activated sludge was determined as 4.4 g/L by weight measurements after drying at 105°C (mean of triplicate measurements). The activated sludge was washed twice with tap water and one time with mineral medium by settling the sludge, decanting the supernatant and re-suspending the sludge. The mineral medium was prepared from four stock solutions using demineralised water, in compliance with the OECD 301 B guidelines, and described in Table 8.2.2-2:

Table 8.2.2-2: Composition of mineral medium

Prepared from	Compound	Final concentration
Stock solution A	KH_2PO_4	8.5 g/L
	K_2HPO_4	21.75 g/L
	$\text{Na}_2\text{HPO}_4 \cdot 2 \text{H}_2\text{O}$	33.40 g/L
	NH_4Cl	0.5 g/L
Stock solution B	$\text{CaCl}_2 \cdot 2 \text{H}_2\text{O}$	36.4 g/L
Stock solution C	$\text{MgSO}_4 \cdot 7 \text{H}_2\text{O}$	22.5 g/L
Stock solution D	$\text{FeCl}_3 \cdot 6 \text{H}_2\text{O}$	0.25 g/L

The medium was prepared mixing 10 mL of solution A with 900 mL of demineralised water before adding 1 mL of solutions B, C and D. The final solution was made up to 1 L.

Each reaction vessel was filled with 10.2 mL of the prepared activated sludge and made up to 1500 mL volume with the mineral medium. The final solution contained 30 mg/L of dry solids. The reaction vessels were sealed, strongly aerated with CO_2 free air under diffuse light and kept mixed with magnetic stirrers throughout the study. The vessels were left overnight before the addition of the test and reference materials.

Test systems were divided into four study groups, with three replicates of each system. These were:

1. inoculum blanks to which nothing was added;
2. the test group to which 68.3 mg of the BLAD protein was added directly to the reaction vessels;

3. the reference group to which 5.15 mL of 10 g/L stock solution of sodium benzoate was added.
4. the toxicity control to which both BLAD and the reference material sodium benzoate was added at the same rate as for the test and the reference groups.

Three replicates of the blank, test and reference systems were conducted. The temperature was 20.7-21.5°C throughout the whole study.

Each reaction vessel was equipped with two gas wash bottles containing 0.2 M NaOH in series to collect the evolved CO₂. Sampling of the traps was made by syringe through butyl rubber septum to prevent loss of CO₂. Inorganic carbon content of the inoculum in the reactors was made at the start and at the end of the incubation period (28 days) using a total carbon analyser. The inorganic carbon in the NaOH traps was measured at 0, 4, 7, 11, 14, 21 and 28 days using a total carbon analyser. The mean CO₂ production from the blank incubate was subtracted from the test and reference reaction vessels and the toxic control for each time point. The amount of CO₂ was then expressed as a % of the maximum theoretical CO₂ production for the test or reference systems. For the 28 day time point the results were also calculated including the inorganic carbon remaining in the reaction vessels.

Results

The degradation results are summarised in Table 8.2.2-3. The validity of the test was confirmed by the following criteria:

- Inorganic carbon in the blank inoculum was less than 5% of the total organic carbon added to the test systems.
- CO₂ in the blank inoculum did not exceed 40 mg/L at the end of the incubation.
- The difference between the replicates at the end of the 10 day window was < 20%.
- Degradation of the reference exceeded 60%ThCO₂ by day 14.
- More than 25% degradation occurred in the toxicity test and no inhibition observed.

Table 8.2.2-3: Summary of biodegradation results as % of maximum theoretical CO₂ production from degradation

Sample	Rep.	Days after treatment							
		0	4	7	11	14	21	28	28*
Test flasks (BLAD)	1	0	47.9	61.3	73.9	70.6	87.4	93.7	96.3
	2	0	48.8	66.1	75.3	71.4	86.0	97.7	94.1
	3	0	50.8	66.8	69.9	75.6	83.3	88.0	83.1
Reference flask (Sodium Benzoate)	1	0	59.2	77.6	81.0	80.2	86.7	76.2	75.3
	2	0	62.2	78.1	86.3	87.0	86.7	98.4	99.2
	3	0	64.2	69.6	82.0	78.7	96.0	90.7	88.7
Toxicity control BLAD + Sodium benzoate	-	0	51.3	69.8	78.2	77.3	87.8	89.9	88.6

* Final measurement including inorganic carbon measurements from the reaction vessel

Conclusion

The ready biodegradability of BLAD was determined with the closed bottle test. BLAD was tested at a nominal concentration of 45.53 mg/L. At the end of the 28 day period the following biodegradation was determined: BLAD 83.1-96.3%; Sodium-benzoate 75.3-99.2%; Toxicity control 88.6%. Biodegradation of BLAD was greater than 60% in a 10 day window and is therefore considered as readily biodegradable.

Reference:	KCA 7.2.2.1/02
Report Title:	Assessment of the ready biodegradability of BLAD with the closed bottle test and SDS-PAGE
Author(s) & Year:	A. Carreira (2014)
Document No, Authority registration No	Unpublished report No.:CEV-ABB-0914

Substance used:	BLAD, Isolated protein from an extract from plants of the genus <i>Lupinus</i> . Lot/Batch No. and purity not provided
Guideline(s):	Yes OECD Guideline for Testing of Chemicals No. 301 D (July 1992)
Deviations:	Yes: Degradation was not determined by oxygen consumption. A specific method was used to measure the degradation of the protein. Also, higher concentration of the test substance was used to ensure reliable detection in the analytical system
GLP or GEP:	No – internal study
Acceptability:	Supplementary
Study relied upon:	No, non-GLP study which does not meet requirements of uniform principles

Introduction

The ready biodegradability of BLAD was assessed by the closed bottle test (OECD 301 D). The applicant has stated that “the evaluation of BLAD degradation was not assessed by oxygen consumption (as a measure of microbial activity), because the amount of BLAD generally used in this type of test (limit of 2 mg/L) is not enough to promote significant changes in that parameter. The present test was adjusted to determine biodegradability as measured by the disappearance of the protein throughout time assessed in a SDS-PAGE gel. For this purpose, BLAD was tested in two different concentrations: 100 mg/L and 200 mg/L”.

Materials and methods

Details of the purity of the BLAD test material are not provided, the applicant refers to purified BLAD protein being isolated using biochemical procedures as described in Report number:1035, this in turn refers to the approach taken in published literature (Monteiro et al. 2015) in which the protein corresponding to β -conglutin was purified by AKTA anion exchange chromatography and subsequently subjected to AKTA gel filtration on the Superose 12 HR 10/30 column. The mineral medium was prepared from four stock solutions using demineralised water, and described in Table 8.2.2-4 the composition is not in compliance with the OECD 301 D guidelines. One mL of each stock solution was mixed with 800 mL ultrapure water and made up to one litre. The medium was then sterilized by filtration (0.22 μ m pore size) and divided into one

litre Erlenmeyer flasks (no more than 250 mL per flask). The flasks were aerated for 20 minutes (200 rpm in an orbital shaker) and allowed to stand for 20 h at the test temperature.

Table 8.2.2-4: Composition of mineral medium

Prepared from	Compound	Final concentration
Stock solution A	KH_2PO_4	8.5 g/L
	K_2HPO_4	21.75 g/L
	$\text{Na}_2\text{HPO}_4 \cdot 2 \text{H}_2\text{O}$	33.40 g/L
	NH_4Cl	0.5 g/L
Stock solution B	$\text{CaCl}_2 \cdot 2 \text{H}_2\text{O}$	36.4 g/L
Stock solution C	$\text{CaCl}_2 \cdot 2 \text{H}_2\text{O}$	36.4 g/L
Stock solution D	$\text{FeCl}_3 \cdot 6 \text{H}_2\text{O}$	36.4 g/L

The test item was dissolved in mineral medium at 2 mg/mL, on the day of the test. Two different inocula were used that were sourced from the effluent of a settling tank of two distinct treatment plants, the location of these was not specified. Inoculum 1 was considered to be more active than inoculum 2. The inocula were prepared by filtration and pre-conditioned by aeration for 5 days at the test temperature.

Two sets of bottles were prepared with two different concentrations of BLAD: 100 mg/L and 200 mg/L. These solutions were then inoculated in order to achieve two different concentrations of each inoculum in the final volume of the bottle: 0.5 mL/L and 5.0 mL/L. The bottles were then completely filled with aerated mineral medium. A control test was conducted of mineral media with no inocula or BLAD added. The differing test variants are described in table 8.2.2-5. Each variant comprised 5 bottles, each for each sampling point (0, 7, 14, 18, and 21 days). The bottles were incubated in the dark at $22^\circ\text{C} \pm 2^\circ\text{C}$.

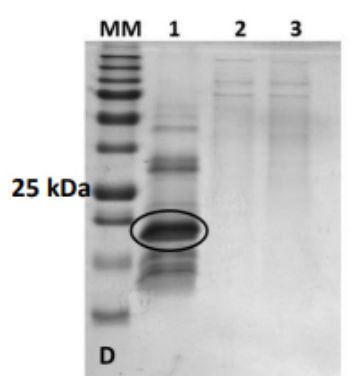
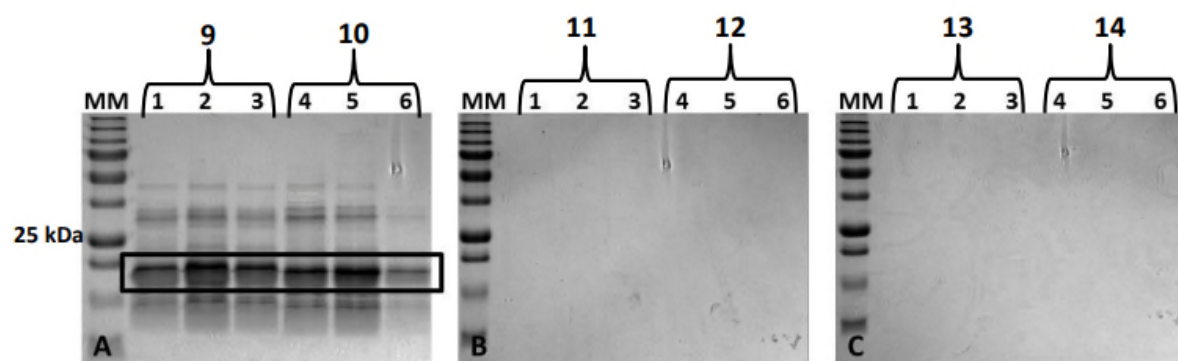
Table 8.2.2-5: Test situations

Sample	BLAD	Inoculum 1	Inoculum 2
1	100 mg/L	0.5 mL/L	-
2		5 mL/L	-
3		-	0.5 mL/L
4		-	5 mL/L
5	200 mg/L	0.5 mL/L	-
6		5 mL/L	-
7		-	0.5 mL/L
8		-	5 mL/L
9	100 mg/L	-	-
10	200 mg/L	-	-
11	-	0.5 mL/L	-
12		5 mL/L	-
13		-	0.5 mL/L
14		-	5 mL/L

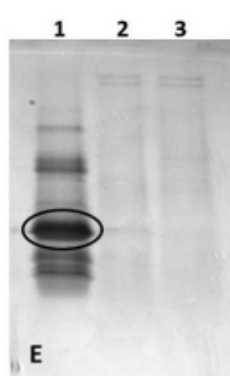
SDS-PAGE analysis

For the Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis, either 125 µL or 250 µL of each sample to correspond to 25 µg of added BLAD protein, was collected from each of the test systems at 3 sampling points. Samples 11, 12, 13 and 14 were used as negative controls of the study. All the samples were precipitated with cold acetone at -20°C for 30 minutes. After centrifugation at 11,000g, the pellets obtained were dissolved in a 3 sample buffer containing 2% (w/v) SDS and 2-β-mercaptoethanol (0.1 M), and then boiled for 3 minutes. Proteins were then separated by means of SDS-PAGE in pre-cast 4% to 20% Tris-HCl Ready Gels (Bio-Rad Laboratories, Inc.) based on the method of Laemmli by using the manufacturer's suggested protocol. Each well was loaded with

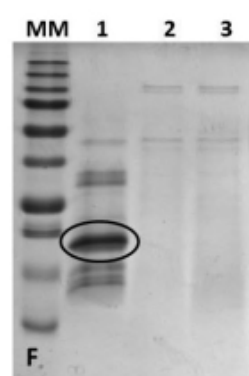
25 µg of protein. Precision Plus molecular weight markers (Bio-Rad Laboratories, Inc.) were used as control molecular markers. The gels were stained with Coomassie Brilliant Blue R250 (CBB R-250). Polypeptides were fixed in TCA 10% (w/v) for 15 minutes. After that, the mini gels were stained for a period longer than 3 hours with a solution containing 0.25% (w/v) CBB R-250, 25% (v/v) 2- propanol and 10% (v/v) glacial acetic acid. The destaining solution composed of 25% (v/v) 2-propanol and 10% (v/v) glacial acetic acid was kept until the polypeptides could be visualised. After Coomassie staining procedure, all polyacrylamide gels were submitted to densitometric analysis followed by BLAD's relative quantitation using ImageLab software version 4.1 (Bio-Rad). The software integrates the raw data in a three dimension format, namely, the width and length of the band as x and y respectively, and the colour intensity of the Coomassie protein staining. Therefore, the density of each band is measured as the total area under the three dimensional peak. For relative quantitation, BLAD "0 days" sample was used as the reference band and the density peak area value was set with a nominal value of 100%. All samples in the same polyacrylamide gel (days 7 and 14) were presented as a relative value in comparison with the reference sample. The applicant has indicated that the protein detection technique used in the study has a detection limit of 0.05 µg of protein, therefore relative to the 25 µg sample, a non-detection represents 0.2% of the initial concentration or > 99% degradation.



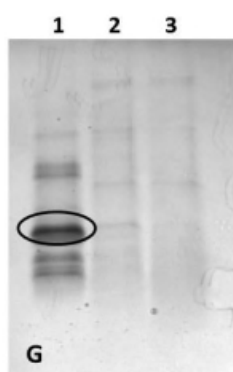
Relative amount	
Lane 1	1
Lane 2	0
Lane 3	0



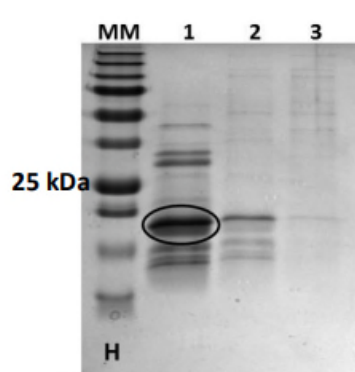
Relative amount	
Lane 1	1
Lane 2	0.03
Lane 3	0



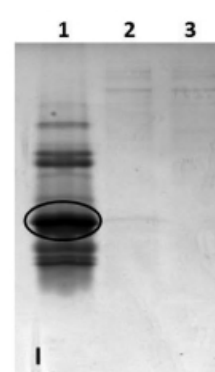
Relative amount	
Lane 1	1
Lane 2	0
Lane 3	0



Relative amount	
Lane 1	1
Lane 2	0.07
Lane 3	0



Relative amount	
Lane 1	1
Lane 2	0.27
Lane 3	0.03



Relative amount	
Lane 1	1
Lane 2	0.06
Lane 3	0

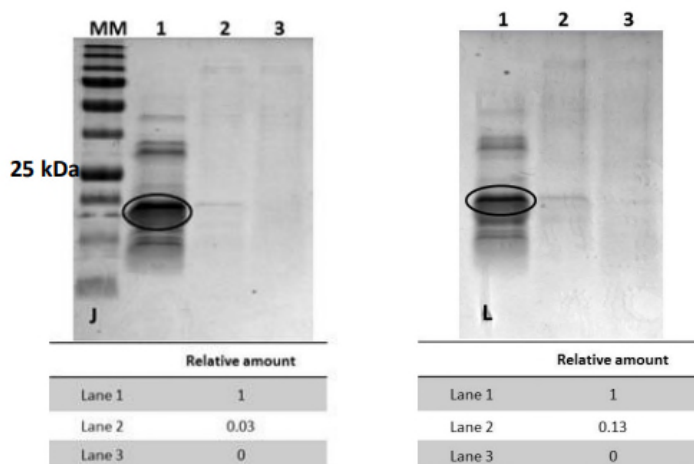


Figure B.8.2.2.1/01: Run outputs from the SDS-PAGE analysis. Image of SDS-PAGE gel with marker compounds in column MM. The sample numbering relates to the sample situations identified in Table 8.2.2-5. Samples 9-14 are the control and blank tests. D= Sample 1 (250 μ L); E = Sample 2 (250 μ L); F = Sample 3 (250 μ L); G = Sample 4; H = Sample 5 (125 μ L); I = Sample 6 (125 μ L); J = Sample 7 (125 μ L); L = Sample 8 (125 μ L). In all cases (lane 1 and 4 –0 days, Lane 2 and 5 – 7 days, lane 3 and 6 – 14 days). MM = molecular marker. The 25 kDa marker is detailed. The polypeptide BLAD, is highlighted by a black circle which has 20 kDa of molecular mass.

Results and discussion

Graphic representation of the gels were presented. The control samples nine and ten indicate that the BLAD protein is stable in the sterile mineral medium without the presence of the inoculum over the 14 days. The control samples 11 to 14 indicate that neither of the inoculums contain anything that interferes with the SDS-PAGE analysis.

The results show that degradation was rapid with very little or no BLAD remaining after 14 days. With the lower concentration of BLAD (100 mg/L) greater than 90% was degraded after 7 days. Test samples with higher concentrations of BLAD (200 mg/mL) indicate complete degradation was achieved after 14 days (> 98%) in all tests except the test with lower concentration of inoculum one, where 3% was remaining after 14 days.

Table 8.2.2-6. Amount of BLAD remaining in the samples after the incubation for 7 and 14 days (by comparison with day 0)

Sample No	BLAD (mg/L)	inoculum	Inoculum concentration (mL/L)	BLAD remaining after 7 days (%)	BLAD remaining after 14 days (%)
1	100	1	0.5	0	0
2	100	1	5.0	3	0
3	100	2	0.5	0	0
4	100	2	5.0	7	0
5	200	1	0.5	27	3
6	200	1	5.0	6	0
7	200	2	0.5	3	0
8	200	2	5.0	13	0

Conclusion

This study is not conducted to GLP and lacks information on the source and purity of the BLAD protein and the source of the inoculum used. The stock solution of mineral medium deviates from the OECD 301 guidelines. The study uses SDS-PAGE electrophoresis as a means of analysing relative BLAD levels in the samples tested. The data provide a relative assessment of the biodegradability in control and treated samples and indicates that the absolute levels of BLAD proteins degrade under the conditions of the ready biodegradability test with on average less than 10% remaining after 10 days across the conditions tested. The testing of samples between day 0 and day 7 would have been a useful addition to this study. This study can be used as supporting information that BLAD is ready biodegradable under the conditions of the ready biodegradability test with no major metabolites being formed.

Reference:	KCA 7.2.2.1/03
Report Title:	PROBLAD PLUS: Assessment of ready biodegradability with the closed bottle test.
Author(s) & Year:	D. Dengler (2010)
Document No, Authority registration No	CEV, SA Unpublished report No.: S10-02624
Substance used:	PROBLAD, extract Lot/Batch No. 201009 Purity 20% (w/w) BLAD protein
Guideline(s):	Yes OECD Guideline for Testing of Chemicals No. 301 D EC method C.4-E (92/69/EEC), biological degradability, determination of the ready degradability, part VI, closed bottle test.
Deviations:	Yes/No
GLP or GEP:	Yes
Acceptability:	Yes
Study relied upon:	Yes

Introduction

The ready biodegradability of the product formulation PROBLAD (also named PROBLAD PLUS) was assessed by the closed bottle test (OECD 301 D).

Materials and methods

Table 8.2.2-7: Test material information for the study of the ready biodegradability of BLAD

Compound	Test item	Reference material
Description	PROBLAD, extract from plants of the genus <i>Lupinus</i> .	Sodium benzoate
Lot/Batch No.	No. 201009	6U006469
Purity	20% (w/w) BLAD protein	99% w/w
COD (determined)	0.676 mgO ₂ /mg	-
ThOD	-	1.67 mg O ₂ /mg
CAS No.	n/a	532-32-1
Stability	Not stated	Not stated

The test was conducted with activated sludge obtained from the municipal wastewater treatment plant at Pforzheim/Germany. The effluent was kept under aerobic conditions in the period between sampling and application. The inoculum was filtered through a coarse filter, the first 200 mL being discarded, and was aerated by shaking for one week in an Erlenmeyer flask. The initial number of micro-organisms was determined by enumerating the bacterial colonies in 1 mL inoculated test medium after incubation with plant count agar in Petri dishes for 4 days. The mineral medium was prepared from four stock solutions (1000 fold concentrated) using ultra pure grade water, in compliance with the OECD 301 D guidelines, following dilution the final concentrations are described in Table 8.2.2-8:

Table 8.2.2-8: Composition of mineral medium

Prepared from	Compound	Final concentration
Stock solution A	KH ₂ PO ₄	8.5 mg/L
	K ₂ HPO ₄	21.75 mg/L
	Na ₂ HPO ₄	26.65 mg/L
	NH ₄ Cl	0.5 mg/L
Stock solution B	CaCl ₂ 2 H ₂ O	36.4 mg/L
Stock solution C	MgSO ₄ 7 H ₂ O	22.5 mg/L
Stock solution D	FeCl ₃ 6 H ₂ O	0.25 mg/L

Four test assays were assessed as follows:

Study group	PROBLAD PLUS	Na-Benzate
Inoculum blank	0 mg/L	0 mg/L
Test item	2 mg/L	0 mg/L
Reference item	0 mg/L	2 mg/L
Toxicity control	2 mg/L	2 mg/L

The test was performed in Biochemical oxygen demand (BOD) flasks with ground-in-glass stoppers in single, 2 and 3-fold test assays for each of the 6 measurement dates (0, 4, 7, 11, 14, 21 and 28 days). The 5 L volumetric flasks were filled ultra-pure grade water at first to about three quarters of their volume. The water was

strongly aerated for ca 10 minutes to achieve oxygen saturation and allowed to stand for about 24 h without aeration at test temperature. The O₂ content was measured at the start of the test.

Initially each study group was prepared in 5 L volumetric flasks. The individual salt stock solutions were added (5 mL of each to < 5000 mL), and either the respective test or reference items were added in the final concentrations of 2 mg/L.

Subsequently each flask was inoculated with 5mL of inoculums and the volume made up to 5 L with oxygen saturated water. The contents of the flasks were distributed into the test vessels and the oxygen contents measured. The bottles were incubated in the dark between 19.1 and 22.0°C. 3 or 2 bottles of each treatment group were removed at each timepoint for analysis of O₂ content. Oxygen concentrations were measured with a WTW Microprocessor Oximeter OXI340 and a calibrated electrode.

Evaluation of degradability

For each date of measurement, the exerted BOD was calculated by subtracting the oxygen concentration (mg O₂/L) of the mean initial inoculums blank from that of the other study groups. Then, for each test assay the measured values of the controls were subtracted. This corrected depletion was divided by the concentration (mg/L) of the test item, to obtain BOD as mg oxygen per mg test item. The percentage biodegradation was calculated by dividing the specific BOD by the specific oxygen demand, calculated from the molecular formula in accordance with the OECD guideline.

The chemical oxygen demand (COD) of PROBLAD PLUS was determined to be 0.676 mg O₂/mg using the method DIN 38409-H41. The method uses potassium hydrogen phthalate solution as the reference item, samples were incubated at 148°C for two hours and final determination was done by titration using ammonia iron (II) sulphate solution and a ferroin indicator (Keifer, 2010). The theoretical oxygen demand (ThOD) of the reference item, sodium benzoate, was calculated to be 1.67 mg O₂/mg, and the ThOD of the mixture of sodium benzoate and test item was 1.173 mg O₂/mg.

Table 8.2.2-9: Percent degradation of PROBLAD PLUS, sodium benzoate reference item and toxicity control

Time (d)	% degradation		
	PROBLAD PLUS	Sodium-benzoate	Toxicity control
4	65.9	54.5	45.6
7	82.4	77.8	68.2
11	79.2	81.7	69.9
14	81.4	85.4	71.6
21	93.7	85.8	75.9
28	91.7	84.6	77.6

The results of the test are considered valid due to the following reasons:

- The percentage degradation of the reference item reached 85.4% by day 14
 - The residual O₂ content did not fall below 0.5 mg/L
 - The degradation of the toxicity control was > 25% (71.6%) after 14 days.
- Therefore, toxic effects of PROBLAD PLUS can be excluded.

Conclusions

The ready biodegradability of PROBLAD PLUS was determined with the closed bottle test. PROBLAD PLUS was tested at a nominal concentration of 2 mg/L. At the end of the 28 day period the following biodegradation was determined: PROBLAD PLUS 91.7%; Na-benzoate 84.6%; Toxicity control 77.6%. At the end of the 14 day period the criteria for classification of a substance as being readily biodegradable of 60% removal of the chemical oxygen demand (COD) was met. The study deviates from the guidelines in that the mineral medium should be prepared as a whole mixed solution and aerated prior to addition of the inoculum and test substances. The applicant added the stock solutions directly to the sample bottles together with the inoculum and test substances and diluted these up to the required amount of oxygen saturated water. It is considered that these deviations will not have an impact on the obtained results and the study is acceptable. This study concludes that PROBLAD PLUS, an aqueous extract from the germinated seeds of sweet *Lupinus albus* is readily biodegradable.

B.8.2.2.2. Aerobic mineralisation in surface water

Aqueous extract from the germinated seeds of sweet *Lupinus albus* along with its lead component, BLAD, have been demonstrated to be readily biodegradable (see Section B.8.2.2.1). A positive result in a test for ready biodegradability can be considered as indicative of rapid and ultimate degradation in most environments (ECHA, 2017). The applicant has proposed therefore that biological degradation is considered to be the most significant route of dissipation for aqueous extract from the germinated seeds of sweet *Lupinus albus* in water, and additional aquatic mineralisation studies are not considered necessary. HSE notes that the ready biodegradability test is a high level screening test but does agree that biological degradation will be a significant route of degradation for this active substance.

B.8.2.2.3. Water / sediment studies

Aqueous extract from the germinated seeds of sweet *Lupinus albus* along with its lead component, BLAD, have been demonstrated to be readily biodegradable (see Section B.8.2.2.1). A positive result in a test for ready biodegradability can be considered as indicative of rapid and ultimate degradation in most environments. The applicant has therefore proposed that, biological degradation is considered to be the most significant route of dissipation for aqueous extract from the germinated seeds of sweet *Lupinus albus* in water, and additional aquatic degradation studies are not considered necessary.

According to ECHA guidance (ECHA, 2017), when results from degradation tests simulating the conditions in surface waters are not available (e.g. OECD test guidelines 308), the use of results from various screening tests may be considered. ECHA guidance gives a proposal for a first order half-life of readily biodegradable substances in water for use in exposure models as 15 days and sediment as 30 days.

The guidance indicates that “The general experience is that a substance passing a test for ready biodegradability may under most environmental conditions be rapidly degraded and the estimated half-lives for such substances should therefore be regarded as being in accordance with “the realistic worst-case concept”.” It is further noted that “for use in exposure models these half-lives do not need to be corrected for different environmental temperatures.” Therefore a DT50 in surface waters of 15 days is to be used.

For assessment of sediment the guidance indicates that “it is noted that the conditions in laboratory screening tests are very different from the conditions in various environmental compartments. The concentration of the test substance is several orders of magnitude greater in these screening tests than the concentrations of xenobiotic substances generally occurring in the environment and thus the kinetic

regimes are significantly different. The temperature is also higher in screening tests than those generally occurring in the environment. Furthermore the microbial biomass is normally lower under environmental conditions than those occurring in these screening tests, especially in the tests for inherent biodegradability.”

As noted in section B.8.1.3 PROBLAD PLUS consists of many different constituents and components (see Volume 4). The major component in the aqueous extract from the germinated seeds of sweet *Lupinus albus* is the protein BLAD which represents 20% w/w in the final product. The different constituents of PROBLAD PLUS have a Log K_{oc} ranging from 3.11 – 347 to an estimated value of >1000 this means that the K_p is likely to be > 100 (See Volume 4). A higher K_p results in a DT₅₀ of 300 or even higher 3000 days for readily biodegradable compounds, see table 8.1.3-1 (ECHA, 2017). As detailed in section B.8.1.3, based upon the information provided in support of the approval of PROBLAD PLUS a DT₅₀ of 300 days for the product PROBLAD PLUS and it's major constituent BLAD is proposed for sediment.

B.8.2.3. Degradation in the saturated zone

Data not required.

B.8.2.3.1. Water treatment procedures

The applicant has presented an assessment of the likelihood of water treatment by-products of PROBLAD PLUS being present in drinking water. It is noted that the levels in groundwater are predicted to be below the maximum drinking water limit of 0.1 µg/L. The applicant has noted that the substance with the highest PEC_{GW} (BLAD) has been shown to be readily biodegradable. Such substances are assumed to undergo rapid and ultimate degradation in the aerobic environment. Therefore the levels of the components of PROBLAD PLUS are predicted to be negligible in drinking water prior to disinfection processes. It is therefore very unlikely that disinfection by-products of PROBLAD PLUS will be present in drinking water. The applicant has presented some suggested dilution factors to support their case which are inappropriate to use in a quantitative way.

HSE note that the major component of PROBLAD PLUS considered in the exposure assessment (BLAD, making up 20% of PROBLAD PLUS) has been shown to be readily biodegradable under the conditions of the OECD 301 B test (Brunswick-Titze, 2015). There is also further supporting information in the literature (Monteiro, S. 2015) and an additional OECD 301 D study (Carreira, 2014) that would support the ready biodegradability of the BLAD in the environment.

The predicted environmental exposure values following use of the product are detailed in Volume 3 CP B.8. Levels in groundwater are expected to be less than the parametric drinking water limit of 0.1 µg/L which would indicate that levels in drinking

water from this source would be below that of concern with regards to the consideration of the impact of water treatment processes and the formation of harmful transformation products.

Predicted levels in surface water of the BLAD component following use of PROBLAD PLUS were 2.873 µg/L from drainflow and 12.85 µg/L from spray drift. It is considered that the predicted levels of BLAD in surface waters will decrease prior to reaching the water treatment plant due to a number of factors. PROBLAD PLUS and its lead component BLAD are readily biodegradable and have a default DT50 in surface water of 15 days, which is recognised as being a realistic worst-case assumption. Due to the likely high Koc value for PROBLAD PLUS and its lead component BLAD, partitioning to sediment is likely to occur, contributing to removal of BLAD from surface waters. It is also recognised that there will be some dilution effects as water moves from the small water body in the edge of field scenario through to water treatment works which are usually located further down the catchment and tend to be associated with larger water bodies. It is therefore considered that levels in drinking water sourced from surface water would likely be below the drinking water limit of 0.1 µg/L and further consideration of the impact of water treatment processes and the formation of harmful transformation products is not required. In addition whilst the structural formula and composition of PROBLAD PLUS is uncertain it is considered that there is unlikely to be any components present in the aqueous plant extract, the BLAD polypeptide or used in the manufacturing process that would cause the formation of potentially harmful transformation products.

B.8.2.4. Persistence in water

Aqueous extract from the germinated seeds of sweet *Lupinus albus*, along with its lead component, BLAD, have been demonstrated to be readily biodegradable (see Section B.8.2.2.1). The applicant has proposed that PROBLAD PLUS and its individual constituents are not considered to be persistent in water. In light of the default degradation values of 15 days in water HSE would agree with this statement.

B.8.3. Fate and behaviour in air

B.8.3.1. Route and rate of degradation in air

The applicant has proposed that the aqueous extract from the germinated seeds of sweet *Lupinus albus*, is a protein based aqueous solution, therefore, it is not technically feasible to conduct vapour pressure studies on this substance. They have added that studies conducted with single components may provide more reliable information on physico-chemical properties – such as (partial) vapour pressures. However, because there are numerous components, and none of these are isolated during the preparation of the product, experimental studies on individual components

are generally not possible. In order to provide some information on the potential of the active substance to volatilise to air, the applicant has presented consideration of quantitative structure property relationships (QSPR).

They have stated that the vapour pressure of a substance depends upon its boiling point (T_b) and its molar enthalpy of vaporisation (ΔH_{vap}), with vapour pressure decreasing as boiling point increases. In turn, the boiling point is associated with a number of molecular properties and features. Most important is molecular weight (MW); the boiling point generally increases with this parameter. Next is the strength of the intermolecular bonding. A final set of parameters are related to molecular structure (molecular rigidity, symmetry and branching). The strong correlation of boiling points to molecular weights and structural features has made it relatively easy to derive structure based estimation methods. For an estimation of boiling point for a compound with unusual elements, functional groups, and/or structural features, the Banks' equation⁵ is recommended⁶. This equation has the advantage that only molecular weight is required as an input.

$$\text{Log}Tb = 2.98 - 4/\sqrt{MW}$$

From this equation it can be estimated that compounds with a molecular weight greater than 1000 g/mol (1 kDa) have boiling points greater than approximately 714K (441°C). The applicant notes that they consider this to include the vast majority of components in aqueous extract of the germinated seeds of sweet *Lupinus albus*.

Conclusion

HSE notes the applicant's proposal and the challenges with the conduct of a standard Atkinson calculation for this UVCB substance. It is considered that reference to the Banks equation as supporting information is a legitimate approach. However, the recommended method is the 'modified Watson Correlation' using the 'Handbook of Chemical Property Estimation Methods'. In considering this HSE has concluded that this high molecular weight compound will likely have a low vapour pressure and there is additional information in the published literature indicating that the lead component BLAD can withstand prolonged boiling which would support the indicated low volatility of this substance (Monteiro et al. 2015). In addition, PROBLAD

⁵ Banks (1939) Consideration of a vapor pressure-temperature equation, and their relationship to Burnop's boiling point function. J. Chem. Soc., 292-295

⁶ Boethling & Mackay (2000) Handbook of property estimation methods for chemicals: environmental and health sciences. Lewis publishers, Boca Raton, Florida, U.S.A

PLUS has a boiling point at 100°C (Volume 3 CA B.2), which would indicate a low vapour pressure for this aqueous extract. Volatilisation to air following application to soil and plant surfaces is not anticipated, and route and rate of degradation in air studies are not considered necessary.

B.8.3.2. Transport via air

The applicant has stated that aqueous extract from the germinated seeds of sweet *Lupinus albus*, is a protein based aqueous solution, therefore, it is not technically feasible to conduct vapour pressure studies on this substance. It is considered that whilst the determination of vapour pressure data is technically feasible HSE notes that based upon **QSPP estimate and indicated the** high molecular weight of the substance **and high boiling point** this would indicate that volatilisation to air is not anticipated, and transport via air studies are not considered necessary.

B.8.3.3. Local and global effects

Local and global effects must be considered for substances that are to be applied in high amounts. This data requirement was not triggered by PROBLAD PLUS.

B.8.4. Definition of the residue

B.8.4.1. Definition of the residue for risk assessment (Data Requirement 7.4.1)

The following compounds are to be considered for the environmental risk assessment.

Soil:

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

Groundwater:

BLAD

Lupanine

Surface Water:

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

Sediment

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

Air:

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

B.8.4.2. Definition of the residue for monitoring

Setting a residue definition for aqueous extract from the germinated seeds of sweet *Lupinus albus*, is not considered to be appropriate. As the main components in this extract are seed storage proteins and quinolizidine alkaloids that are naturally occurring and not specific to the product.

B.8.5. Monitoring data concerning fate and behaviour of the active substance, metabolites, degradation and reaction products

No monitoring data are available as this is a new active substance.

B.8.6. References relied on

B.8.6.1. Literature review

A literature review has been carried out for the active substance BLAD. The literature review has been conducted in accordance with Article 8(5) of assimilated Regulation No 1107/2009 and is based on the EFSA guidance document as published in EFSA Journal 2011; 9(2):2092.

BLAD is a protein, which occurs naturally in the seeds of *Lupinus albus*, and is readily biodegradable. Degradation of BLAD is expected to be rapid and complete, without the formation of any potentially relevant soil metabolites, therefore no potentially relevant metabolites could be searched. A further search was therefore performed on lupinene, β -conglutin and vicilin to ensure any possible relevant data to BLAD was also searched. The additional search terms were chosen as they are closely related proteins to BLAD.

The key objective of the submitted literature reviews was to establish whether any scientific peer-reviewed open literature published within the last ten years before the date of submission of the dossier would be relevant for the risk assessment of BLAD and the product PROBLAD PLUS. In this section the conduct of the literature search methods in relation to fate and behaviour studies has been evaluated; the conclusions of which are presented here. Key information from the reports has been summarised below.

Reference:	KCA 9.01/01
Report Title:	PROBLAD PLUS; Literature Review Report; Submission of Scientific Peer-Reviewed Open Literature under Regulation (EC) No 1107/2009
Author(s) & Year:	K.Tucker (2016)
Document No, Authority registration No	Unpublished report No.: CEV/02/01-LRR1
Guideline(s):	EFSA Journal 2011; 9(2):2092
Deviations:	None specified
GLP:	No

Reference:	KCA 9.01/02
Report Title:	Sweet Lupin (seeds), <i>Lupinus albus</i> L., germ.,ext. and PROBLAD PLUS; Literature Review Report; Submission of Scientific Peer-Reviewed Open Literature under Regulation (EC) No 1107/2009
Author(s) & Year:	K.Tucker and L Cartwright (2018)
Document No, Authority registration No	Unpublished report No.: CEV/02/01-LRR2
Guideline(s):	EFSA Journal 2011; 9(2):2092
Deviations:	None specified
GLP:	No

Reference:	KCA 9.01/03
Report Title:	Sweet Lupin (seeds), <i>Lupinus albus</i> L., germ.,ext. and PROBLAD PLUS; Literature Review Report; Submission of Scientific Peer-Reviewed Open Literature under Regulation (EC) No 1107/2009
Author(s) & Year:	Dinicica (2019)
Document No, Authority registration No	Unpublished report No.: CEV/02/01-LRR3
Guideline(s):	EFSA Journal 2011; 9(2):2092
Deviations:	None specified
GLP:	No

The reliability assessment for relevant studies was done according to Klimisch et al. (1997)⁹. A full list of the references retrieved by this literature search after rapid assessment of title is provided in the original report. The search strategy was based on a single concept search and considered studies published since 2005. The selection process resulted in three categories of publication:

- Publications which meet the relevance criteria are assessed to be reliable and where the endpoints will have an impact on the risk assessment.
- Publications which meet the relevance criteria but are assessed to be non-reliable are referenced and a justification for not meeting the reliability criteria.
- Publications not meeting the relevance criteria.

Table 8.6-1: Specific criteria used for the assessment of relevance for environmental fate and behaviour

Data requirement	Criteria for relevance
Environmental fate and behaviour studies	<ol style="list-style-type: none"> 1. Contains information on active substance 2. Paper includes sufficient detail eg. information on test system characteristics 3. Paper includes new or useful information. If it confirms GLP and guideline study results, paper would be considered to provide no new information and regulatory studies would be used instead 4. Information provided that would satisfy data requirements, answer specific point under EC regulation 1107/2009 (eg. information on isomers) or could be used for the risk assessment

The databases searched were:

STN-Databases:

Anabstr- Analytical abstracts
 Biosis
 Caplus - chemical abstracts 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

Following an initial search and assessment of the literature dated 2016 an updated search was conducted in 2018 to include a search for the botanical active substance name and also additional ecotoxicology filters were applied.

The applicant has provided the following summary with regard to the search terms used:

BLAD and PROBLAD PLUS (Report number: CEV/02/01-LRR1)

Within the Pubmed, Wiley online library and Science Direct search, the search terms 'BLAD', 'PROBLAD', 'PROBLAD PLUS' and the CAS number '1219521-95-5' was used. This was applicable to all fields within the articles. The Science Direct and

Wiley online library search of BLAD produced a large number of articles that would be impractical to review and therefore required further refinement. A search was performed as before with additional search terms:

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. It was noted that a large number of articles present from the search term 'BLAD' were actually from the word 'bladder' and had no relation to the active substance 'BLAD'. An additional field was therefore applied to the search.

NOT bladder NOT Urinary NOT incontinence NOT Pelvic floor NOT Urethral NOT urology NOT urological NOT urinary tract NOT renal.

Within the search of the STN databases, the search terms 'BLAD', 'PROBLAD' and 'PROBLAD PLUS' was used as was the CAS number '1219521-95-5'.

It was considered that as BLAD is the common name for this active substance and PROBLAD PLUS is the common name for this formulation, any relevant article from the search period would make reference to these terms somewhere within the text of the article, or that the article will be referenced with this CAS number.

Further search terms (Report Number: CEV/02/01-LRR1)

BLAD is a protein, which occurs naturally in the seeds of *Lupinus albus*, and is readily biodegradable. Degradation of BLAD is expected to be rapid and complete, without the formation of any potentially relevant soil metabolites, therefore no potentially relevant metabolites could be searched. A further search was therefore performed on lupinene, β -conglutin and vicilin to ensure any possible relevant data to BLAD was also searched.

The additional search terms were chosen as they are closely related proteins to BLAD. BLAD is a 20 kDa polypeptide of β -conglutin and vicilin is a storage protein commonly found in lupines.

Within the Pubmed, Wiley online library, Science Direct and STN search, the search terms 'lupinene', ' β -conglutin' and 'vicilin' was used. This was applicable to all fields within the articles.

The Wiley online search of 'vicilin' produced a large number of articles that would be impractical to review and therefore required further refinement. A search was performed as before with additional search terms:

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

Sweet Lupin (seeds), *Lupinus albus* L., germ., ext, (Report number: CEV/02/01-LRR2)

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

Additional search for ecotoxicology section (Report number: CEV/02/01-LRR2)

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', 'β-conglutin', 'vicilin'. This was applicable to all fields within the articles.

For the search terms 'BLAD', 'β-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'.

Search potentially relevant metabolites (Report number: CEV/02/01-LRR3)

For each substance identified, the search terms were chemical name, other names, IUPAC name and CAS number, if available. CAS numbers were also used to search registry fields. If CAS numbers were not already known, a chemical structure search in the STN Registry file was used to determine registry numbers for this search.

The search terms were lupanine and its chemical name (1S,2R,9S,10S)-7,15-diazatetracyclo [7.7.1.0^{2,7}.1^{0,15}]heptadecan-6-one (CAS number 550-90-3). 13α-OH-lupanine (CAS 4356-43-8), lupinine (CAS number 486-70-4) and sparteine (CAS number 90-39-1).

An initial assessment of studies for relevance was carried out by reference to their titles and if necessary abstracts. Those studies that were felt to meet the relevance

criteria or unclear relevance following review of their abstracts were obtained and the full text of the document was assessed further to determine if the information contained in the study could impact on the endpoints and risk assessment parameters related to the active substance. Reviews of the relevance and reliability of the articles brought up in the literature search were carried out by experts in the particular fields

Following assessment of the output from both searches performed in 2016 and 2018, 15 results were considered to be relevant to the risk assessment of human health, animal health or the environment and were included in the dossier. Two published literature studies were included within the environmental fate and behaviour assessment which are detailed below :

Table 8.6-2: Results to be included in the dossier for fate and behaviour.

Author(s)	Year	Title	Source	Reason(s) for including this study in the dossier
Monteiro S, Carreira A, Freitas R, Pinheiro AM, Ferreira RB.	2015	A nontoxic polypeptide oligomer with a fungicide potency under agricultural conditions which is equal or greater than that of their chemical counterparts.	PLoS One. 2015;10(4):e0122095. doi: 10.1371/journal.pone.0122095.	Relates to efficacy and not relevant for residues data requirements. Publication demonstrates the susceptibility of BLAD to proteolytic enzymes and is summarised in CA 7.1.1.1 for environmental fate. Paper also refers to a study of the acute toxicity of BLAD-oligomers to bees. This study is already included in the dossier (the test item is referred to as PROBLAD PLUS in the study report but reports the same results for the oligomer in this paper) see point M-CA 8.3.1 for full study summary and so this paper is not required further for ecotox.
Monteiro S, Freitas R, Rajasekhar BT, Teixeira AR, Ferreira RB.	2010	The unique biosynthetic route from lupinus beta-conglutin gene to blad.	PLoS One. 2010 Jan 6;5(1):e8542. doi: 10.1371/journal.pone.0008542	This paper is referenced from CA 6.2.1/02 in relation to residues data and supports conclusions that no consumer exposure is expected.

Following assessment of the output from the search performed in 2019, three results were considered to be relevant to the ecotoxicology risk assessment and were included in the dossier. These studies were not relevant as supporting information for the fate and behaviour assessment.

In conclusion, the HSE evaluator agrees that the studies identified in the literature review from 2016 and 2018 are considered relevant as supporting information for the Environmental Fate and behaviour dossier.

A further literature review was conducted to cover the period from the last search and up to six months of submission

Reference:	KCA 9.01/04
Report Title:	Aqueous extract from the germinated seeds of sweet <i>Lupinus albus</i> ; Literature Review Report; Submission of Scientific Peer-Reviewed Open Literature under Regulation (EC) No 1107/2009
Author(s) & Year:	ERM (2024)
Document No, Authority registration No	Unpublished report No.: CEV/02/01-LRR4
Guideline(s):	EFSA Journal 2011; 9(2):2092
Deviations:	None specified
GLP:	No

A single concept search was carried out for the search terms used in the June 2018 literature search and November 2019 literature search. No endpoint specific search terms were used. Patent literature was not considered to be relevant to the results of the search. The search was therefore filtered to non-patent document types.

The following relevance criteria were employed:

1. Well defined test material (including purity/content).
2. Relevant test species (Mammalian related studies are evaluated by toxicology. For the risk assessment of birds, fish, aquatic invertebrates or macrophytes, sediment dwelling organisms, algal growth, arthropods, soil meso- and macrofauna and non-target plants – all species are evaluated).
3. Well defined application method and rates, and exposure route is clearly defined and environmentally relevant.
4. Effects are related to single test item, and a quantitative relationship exists between the reported endpoint and risk assessment endpoints.
5. Study conditions should not differ significantly from recommended protocols.
6. Relevant endpoints for ecotoxicological risk assessment.
7. The article contains adequate information about the persistency or bioconcentration of the substance of concern.

8. The article contains adequate information about the toxicity of the substance of concern to non-target organisms living in the environment.
9. The publication represents field observations on individual mortalities of terrestrial vertebrates related to the use of the substance of concern.
10. The article contains adequate information on the effect of the substance of concern on biological activity used for sewage clearance.
11. The article reports on residues of the substance of concern in plant moieties adequate for the assessment of the exposure of bees.
12. The article provides information that elucidates the mechanism of effects or explores on the environmental behaviour of the substance of concern.
13. The article's relevance for the hazard assessment or risk characterization clear after full-text analysis.

The databases searched were:

AGRICOLA - Agriculture Online Access
Anabstr- Analytical abstracts
BIOSIS - BIOSIS Previews
CAB Abstracts
HCaplus - chemical abstracts plus
Chemlist
Embase - The Excerpta Medica database
FSTA – Food Science and Technology Abstracts
Scisearch - Science Citation Index
Toxcenter - Toxicology Center Database
Medline - MEDlars onLINE
NTIS – National Technical Information Service
PQSCITECH - ProQuest Science & Technology
Rtecs- Registry of Toxic Effects of Chemical Substances

The search resulted in 839 summary records retrieved from search(after automatic STN duplicates removal) and 594 retrieved after manual removal of duplicates. No further records were removed after rapid assessment for relevance (by title/abstract). However, after detailed assessment of full-texts (i.e. not relevant or not sufficiently reliable) it was concluded that no publications were deemed relevant based on their titles or abstracts and thus no detailed assessment was conducted or required following this further literature review. The conclusion therefore remains unaffected and the two studies identified in the literature review from 2016 and 2018 and detailed in Table 8.6-2 are considered relevant as supporting information for the Environmental Fate and behaviour dossier.

B.8.6.2. References relied upon

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
7.1.1.1/01 7.1.2.1.1/01 7.2.2.1/01	Brunswik-Titze, A.	2015	Biodegradability, CO ₂ -evolution test according to OECD 301 B (July 1992) Hydrotox .- Labor für, Ökotoxikologie und, Gewässerschutz GmbH Report No. 1035 GLP, Unpublished	N	Y	Data protection is claimed in accordance with article 59 of assimilated Regulation No 1107/2009	CEV	N
7.1.1.2/01	ERM	2024	Position paper: Fate request for additional	N	N	n/a	ERM	N

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
			information following ECP advice Report No: 0728696 Non GLP. Unpublished					
7.1.1.2/02	Adamczyk, B.	2021	Root-derived proteases as a plant tool to access soil organic Nitrogen; current state of knowledge and controversies. Plants (Basel) 10(4): 731 Non GLP Published	N	N	-	n/a	N

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
7.1.1.2/03	Greenfield, L.M., Hill, P.W., Paterson, E., Briggs, E.M., Jones, D.L.	2020	Do plants use root-derived proteases to promote the uptake of soil organic nitrogen? Plant Soil, 456(1): 355-367. Non GLP Published	N	N	-	n/a	N
7.1.1.2/04	Greenfield, L.M., Puissant, J., Jones, D.L.	2021	Synthesis of methods used to assess soil protease activity. Soil Biology and Biochemistry Volume 158 Non-GLP	N	N	-	n/a	N

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
			Unpublished					
7.1.1.2/05	Jan, M.T., Roberts, P., Tonheim, S.K., Jones, S.L.	2009	Protein breakdown represents a major bottleneck in nitrogen cycling in grassland soils. Soil Biology & Biochemistry 41: 2272-2282. Non-GLP Unpublished	N	N		n/a	N
7.1.1.1/03 7.1.2.1.1/03 7.2.2.1/03	Dengler, D.	2010	PROBLAD PLUS: Assessment of ready biodegradability with the closed bottle test. Company report no. S10-02624 Eurofins	N	Y	Data protection is claimed in accordance with article 59 of assimilated	CEV	N

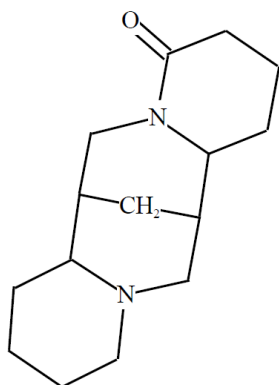
Data Point	Author(s)	Year	Title Company Report No. Source (where different from company) GLP or GEP status Published or not	Vertebrate Study Y/N	Data Protection Claimed Y/N	Justification if Data Protection is claimed	Owner	Previous evaluation
			Agroscience Services GmbH GLP, unpublished			Regulation No 1107/2009		
7.2.2.1/03	Keifer, R.	2010	PROBLAD PLUS: Assessment of ready biodegradability with the closed bottle test. Analytical phase Determination of COD. Company report no. S10-02624-L2 Eurofins Agroscience Services GmbH GLP, unpublished.	N	Y	Data protection is claimed in accordance with article 59 of assimilated Regulation No 1107/2009	CEV	N
7.1.2.1.1/04	Monteiro, S., Carreira, R., Freitas, R.,	2015	A nontoxic polypeptide oligomer with a fungicide potency under agricultural conditions which is equal	N	N	-	Published	N

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

Volume 3 – B.8 (AS)

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
	Margarida Pinheiro, A., Boavida Ferreira, R.		or greater than that of chemical counterparts. PLoS ONE 10(4): e0122095. Doi 1371/journal.pone.0122095 Not GLP,					

B.8.7. Appendix 1: EPI Suite Results For Lupanine



SMILES : C12N(CC3CC2CN4C3CCCC4)C(=O)(CCC1)

CHEM :

MOL FOR: C15 H24 N2 O1

MOL WT : 248.37

----- EPI SUMMARY (v4.11) -----

--

Physical Property Inputs:

Log Kow (octanol-water): -----

Boiling Point (deg C) : -----

Melting Point (deg C) : -----

Vapor Pressure (mm Hg) : -----

Water Solubility (mg/L): -----

Henry LC (atm-m³/mole) : -----

Log Octanol-Water Partition Coef (SRC):

Log Kow (KOWWIN v1.68 estimate) = 1.71

Boiling Pt, Melting Pt, Vapor Pressure Estimations (MPBPVP v1.43):

Boiling Pt (deg C): 374.71 (Adapted Stein & Brown method)

Melting Pt (deg C): 138.38 (Mean or Weighted MP)

VP(mm Hg,25 deg C): 3.39E-005 (Modified Grain method)

VP (Pa, 25 deg C) : 0.00452 (Modified Grain method)

MP (exp database): < 25 deg C

BP (exp database): 187 @ 1 mm Hg deg C

Water Solubility Estimate from Log Kow (WSKOW v1.42):

Water Solubility at 25 deg C (mg/L): 831.5

log Kow used: 1.71 (estimated)

no-melting pt equation used

Water Sol Estimate from Fragments:

Wat Sol (v1.01 est) = 8089.1 mg/L

ECOSAR Class Program (ECOSAR v1.11):

Class(es) found:

Aliphatic Amines

Amides

Henrys Law Constant (25 deg C) [HENRYWIN v3.20]:

Bond Method : 1.29E-011 atm-m³/mole (1.31E-006 Pa-m³/mole)

Group Method: Incomplete

For Henry LC Comparison Purposes:

User-Entered Henry LC: not entered

Henry's LC [via VP/WSol estimate using User-Entered or Estimated values]:

HLC: 1.332E-008 atm-m³/mole (1.350E-003 Pa-m³/mole)

VP: 3.39E-005 mm Hg (source: MPBPVP)

WS: 832 mg/L (source: WSKOWWIN)

Log Octanol-Air Partition Coefficient (25 deg C) [KOAWIN v1.10]:

Log Kow used: 1.71 (KowWin est)

Log Kaw used: -9.278 (HenryWin est)

Log Koa (KOAWIN v1.10 estimate): 10.988

Log Koa (experimental database): None

Probability of Rapid Biodegradation (BIOWIN v4.10):

Biowin1 (Linear Model) : 0.6342

Biowin2 (Non-Linear Model) : 0.4876

Expert Survey Biodegradation Results:

Biowin3 (Ultimate Survey Model): 2.3413 (weeks-months)

Biowin4 (Primary Survey Model) : 3.4068 (days-weeks)

MITI Biodegradation Probability:

Biowin5 (MITI Linear Model) : 0.2621

Biowin6 (MITI Non-Linear Model): 0.0600

Anaerobic Biodegradation Probability:

Biowin7 (Anaerobic Linear Model): -1.8490

Ready Biodegradability Prediction: NO

Hydrocarbon Biodegradation (BioHCwin v1.01):

Structure incompatible with current estimation method!

Sorption to aerosols (25 Dec C)[AEROWIN v1.00]:

Vapor pressure (liquid/subcooled): 0.00452Pa (3.39E-005 mm Hg)

Log Koa (Koawin est): 10.988

Kp (particle/gas partition coef. (m³/ug)):

Mackay model : 0.000664

Octanol/air (Koa) model: 0.0239

Fraction sorbed to airborne particulates (phi):

Junge-Pankow model : 0.0234

Mackay model : 0.0504

Octanol/air (Koa) model: 0.656

Atmospheric Oxidation (25 deg C) [AopWin v1.92]:

Hydroxyl Radicals Reaction:

OVERALL OH Rate Constant = 97.0350 E-12 cm³/molecule-sec

Half-Life = 0.110 Days (12-hr day; 1.5E6 OH/cm³)

Half-Life = 1.323 Hrs

Ozone Reaction:

No Ozone Reaction Estimation

Fraction sorbed to airborne particulates (ϕ):

0.0369 (Junge-Pankow, Mackay avg)

0.656 (Koa method)

Note: the sorbed fraction may be resistant to atmospheric oxidation

Soil Adsorption Coefficient (KOCWIN v2.00):

Koc : 1287L/kg (MCI method)

Log Koc: 3.110 (MCI method)

Koc : 57.31 L/kg (Kow method)

Log Koc: 1.758 (Kow method)

Aqueous Base/Acid-Catalyzed Hydrolysis (25 deg C) [HYDROWIN v2.00]:

Rate constants can NOT be estimated for this structure!

Bioaccumulation Estimates (BCFBAF v3.01):

Log BCF from regression-based method = 0.797 (BCF = 6.271L/kg wet-wt)

Log Biotransformation Half-life (HL) = -1.1499 days (HL = 0.07082 days)

Log BCF Arnot-Gobas method (upper trophic) = 0.652 (BCF = 4.492)

Log BAF Arnot-Gobas method (upper trophic) = 0.652 (BAF = 4.492)

log Kow used: 1.71 (estimated)

Volatilization from Water:

Henry LC: 1.29E-011 atm-m³/mole (estimated by Bond SAR Method)

Half-Life from Model River: 7.153E+007 hours (2.98E+006 days)

Half-Life from Model Lake : 7.803E+008 hours (3.251E+007 days)

Removal In Wastewater Treatment:

Total removal: 2.05 percent

Total biodegradation: 0.09 percent

Total sludge adsorption: 1.96 percent

Total to Air: 0.00 percent

(using 10000 hr Bio P,A,S)

Level III Fugacity Model:

Mass Amount Half-Life Emissions

(percent) (hr) (kg/hr)

Air 9.58e-005 2.64 1000

Water 11.6 900 1000

Soil 87.5 1.8e+003 1000

Sediment 0.835 8.1e+003 0

Persistence Time: 1.84e+003 hr