



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.5 (AS)

Methods of Analysis

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]

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B.5. Methods of analysis

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

The active substance and plant protection product are identical; the methods for the determination of the lead component and any relevant impurities in the plant protection product are reported in this section of the DAR. For more information please refer to Volume 4.

The following methods are summarised and their purpose in the evaluation are outlined in Table B.5.1-1.

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

Table B.5.1-1 Summary of methods presented and their purpose

Section of Volume 3 CA B5	Method reference	Method principle	Purpose	Use of the method in the evaluation	Reference to the relevant section of the evaluation	Overall conclusion on the validity of the method
B.5.1.1 Methods for the analysis of the active substance as manufactured Lead component BLAD	Study 32342	HPLC-UV	Determination of BLAD content in PROBLAD PLUS	Determination BLAD content in pilot and commercial scale batches of PROBLAD PLUS	Volume 4	Sufficient for regulatory purposes.
	'Modified Lowry method' or Study: CEV-QCLR-18.06-01 or 'IM02'	Colourimetric assay	Determination of BLAD content in PROBLAD PLUS	Comparison of the HPLC-UV method and modified Lowry method Determination of content of BLAD in PROBLAD PLUS before and after accelerated and ambient storage.	Volume 4 (comparison of methods) Volume 3 CP B2 (storage stability and spray application test)	Sufficient for regulatory purposes.

Volume 3 – B.5 (AS)

Section of Volume 3 CA B5	Method reference	Method principle	Purpose	Use of the method in the evaluation	Reference to the relevant section of the evaluation	Overall conclusion on the validity of the method
Relevant impurities QAs (with marker)				<p>Determination of BLAD in spray solution samples in spray application test.</p> <p>Determination of BLAD in PROBLAD PLUS in the 13 week oral toxicity study in the rat.</p>	Volume 3 CA B6 (toxicology)	
	<p>Study 32342</p> <p>or</p> <p>Study 35987</p>	GC-FID	Determination of QA content (lupanine) in PROBLAD PLUS	<p>Determination QA content (lupanine) in pilot scale batches of PROBLAD PLUS</p> <p>Determination of QA content (lupanine) in PROBLAD PLUS before</p>	Volume 4	Sufficient for regulatory purposes, considering the context of use and the lack of reliance on the data generated using this method.

Volume 3 – B.5 (AS)

Section of Volume 3 CA B5	Method reference	Method principle	Purpose	Use of the method in the evaluation	Reference to the relevant section of the evaluation	Overall conclusion on the validity of the method
component lupanine)				and after accelerated and ambient storage.	Volume 3 CP B2 (storage stability)	
	Study MVR 3.2.40_01 & study CEV-QCLR-19.11-01	GC-MS/MS	Determination of QA content in PROBLAD PLUS	Determination QA content in commercial scale batches of PROBLAD PLUS	Volume 4	Validated in accordance with SANCO/3030/99 rev. 5.
B.5.1.2 Methods for risk assessment	Study 'S13-04129'	ELISA	Determination of BLAD content in crops	Determination of BLAD content in grape, strawberry and tomato	Volume 3 CA B7 (residues)	Not validated in accordance with SANTE/2020/12830 rev. 1. There is some confidence that the method can

Volume 3 – B.5 (AS)

Section of Volume 3 CA B5	Method reference	Method principle	Purpose	Use of the method in the evaluation	Reference to the relevant section of the evaluation	Overall conclusion on the validity of the method
						determine the presence of BLAD in treated crops, but a reliable LOQ cannot be established.
	Method 'S18-08251'	ELISA	Determination of BLAD and PROBLAD PLUS content in water solutions	Determination of BLAD (in PROBLAD PLUS) in water test media W2 and W4	Volume 3 CP B9 (ecotoxicology)	Validated in accordance with SANTE/2020/12830 rev. 1. for matrix W4. Sufficient for regulatory purposes for matrix W2.
	Method 'S19-21256'	ELISA	Determination of BLAD and PROBLAD PLUS content in water and	Determination of BLAD (in PROBLAD PLUS) in deionised water and 50% w/v sucrose solution	Volume 3 CP B9 (ecotoxicology)	Validated in accordance with SANTE/2020/12830 rev. 1.

Section of Volume 3 CA B5	Method reference	Method principle	Purpose	Use of the method in the evaluation	Reference to the relevant section of the evaluation	Overall conclusion on the validity of the method
			sugar solutions			

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

The active substance and plant protection product are identical; the methods for the determination of the lead component and any relevant impurities in the plant protection product are reported in this section of the DAR.

Lead component: BLAD

Reference:	
Report Title:	1) PROBLAD PLUS - Preliminary Analysis. Converde SA 2) Addendum to the study report Zehr, P.S. (2013) – PROBLAD PLUS: Preliminary analysis (PSL Study Number #32342) - containing the validation data of the HPLC method for the quantification of BLAD lead component and GC method for the quantification of Lupanine in PROBLAD PLUS Fungicide
Author(s) & Year:	1) P. S. Zehr, 2013 2) A. Carreira, 2018a
Document No, Authority registration No	1) 32342 2) CEV-QCLR-18.07-02
Guideline(s):	Yes The applicant used SANCO 3030/99 rev. 4, the current version is SANCO 3030/99 rev. 5
Deviations:	No
GLP or GEP:	1) Yes 2) No
Acceptability:	Yes
Study relied upon:	Yes

Principle of the method:

Determination of BLAD by HPLC-UV under the following analytical conditions:

System	Agilent 1100/MSD		
Column	Agilent Zorbax 300RB – C18, 4.6 x 250 nm, 5 µm		
Column temperature	40°C		
Injection Volume (µL)	10-50		
Detector and wavelength	UV, 214 nm		
Flow rate (mL/min)	1.0		
Mobile phase	Mobile phase A: 0.1% TFA in water Mobile phase B: 0.1% TFA in acetonitrile		
Gradient	Time (min)	A (%)	B (%)
	0.0	70	30
	14.0	55	45
	14.1	0	100
	19.0	0	100
	19.1	70	30
	23.0	70	30
Run time (min)	23		
Retention time (min)	9-14 (8 peaks)		

Sample preparation

0.25 g triplicate aliquots of each lot of test substance were weighed into separate 100 mL volumetric flasks and diluted to volume with HPLC grade water. The solutions were sonicated for 10min and mixed well. Final sample concentration: 2.5 mg/mL PROBLAD PLUS in water. This is equivalent to 0.5 mg BLAD/mL solvent, hence an expected amount in 30 µL (the injection volume) of 0.015 mg.

A summary of the validation data is presented in Table B.5.1.1-1.

Table B.5.1.1-1. Summary of validation data for the determination of BLAD in PROBLAD PLUS

Matrix	Analyte	Recovery fortification level	% Recovery	Repeatability % RSD (n)	Linearity	Specificity
PROBLAD PLUS	BLAD	0.015 mg BLAD equivalent to 20% w/w (Total BLAD in spiked solutions 2.45 mg)	100.3 103.9	Reference standard: % RSD 0.93 at 0.0135 mg BLAD, equivalent to 18% w/w BLAD in PROBLAD PLUS, five replicate injections of the same sample of mid point linearity sample made using reference standard.	0.0045-0.0225 mg BLAD (n = 5) $R^2 = 0.9991$ $y = 246379x + 47.616$ $221.74x + 47.616$	Retention time and peak match to reference standard. No significant interfering peaks observed.

Matrix	Analyte	Recovery fortification level	% Recovery	Repeatability % RSD (n)	Linearity	Specificity
				<p>Modified Horwitz = 1.74 @ 18% w/w therefore $H_r = 0.53$</p> <p>Test samples:</p> <p>Five batches, each sampled 3 times and analysed in duplicate, at ~20% w/w</p> <p>Three samples < 0.33% RSD</p> <p>Overall %RSD 2.41</p>		

Matrix	Analyte	Recovery fortification level	% Recovery	Repeatability % RSD (n)	Linearity	Specificity
				Modified Horwitz = 1.70 Therefore $H_r = 1.42$		

Specificity

A UV/Vis chromatogram of BLAD protein reference standard and BLAD in PROBLAD PLUS were provided. No significant interference with the solvent system is observed. As the 'active substance' is not isolated, it is directly formulated into the product, a 'blank' formulation is not available. The method specificity is sufficiently addressed considering the nature of the test substance.

A range of peaks are observed in the chromatograms of the BLAD reference standard (90% pure) and the test sample (BLAD in PROBLAD PLUS). BLAD is a sub-unit of a 210 KDa oligomer, that is fragmented in its individual units (polypeptides) under the denaturing conditions of the HPLC method (organic solvent in mobile phase and high pressure). This results in a HPLC profile not characterized by a single peak, but by a multiple cluster of peaks, each one corresponding to a specific polypeptide of the oligomer. Consequently, the entire total peak area is used to quantify the BLAD content.

Linearity

Linearity was demonstrated by analysing 5 standard solutions with increasing concentrations in the range of 0.0045-0.0225 mg BLAD. The coefficient of determination (R^2) was 0.9991 and the linear regression equation was $y = 246379x + 47.616$ $221.74x + 47.616$. The linear range is sufficient to accommodate the content of BLAD in PROBLAD PLUS.

Precision (Repeatability)

The applicant provided data for five replicate injections of 0.0135 mg standard solution which is not an acceptable approach to address precision. The test samples consisted of three individual weighings, analysed in duplicate, therefore these have been considered in the context of precision data.

Given the complexity of the active substance and formulation being the same, it is not possible to generate a blank formulation or technical material to fortify with BLAD. Therefore the precision data generated using test samples of batches of the 'active substance' can be relied upon in the absence of a blank formulation.

The Horrat ratio is above 1 for the test samples, therefore further justification is necessary. Considering the UVCB nature of the substance, this variation is justified in this case. Additionally, these are the test samples rather than fortified validation samples so more variation may be expected. The values reported are also the overall precision of five batches of material, each prepared and analysed three times, rather than one single batch sample or validation sample prepared and analysed five times, further justifying the higher variation observed.

Overall the information provided shows that the method is capable of determining the content of BLAD, but is not fully validated in accordance with SANCO/3030/99 rev. 5.

Recovery (Accuracy)

Recovery is not required for the determination of the active substance in the technical material, but in this case the technical material and product are identical and it is the 'lead component' being analysed in the samples. Hence, it seems appropriate that two independent recovery determinations should be provided. Recovery data was provided in the study addendum. Recovery samples were prepared by spiking solution of PROBLAD PLUS (040511 Batch 1) with stock solution of BLAD protein reference standard. Two spiked solutions were prepared at one fortification level (0.015 mg BLAD). The samples were analysed in duplicate. As only two independent samples are provided %RSD was not calculated. The average recovery was 102.1 which is within the acceptable range (97-103%). One individual recovery was outside of this range (103.9%) but as the mean recovery was within this range, this is considered acceptable.

Conclusion

The method is not fully validated in accordance with SANCO/3030/99 rev. 5 due to the precision data not being in line with the requirements outlined. However the method is considered sufficient for regulatory purposes considering the available validation data, the purpose of the method (determination of the 'lead component') and the nature of the substance (UVCB substance of which the active substance and formulation are the same).

Reference:	
Report Title:	<p>a) Addendum to the study report Gravelle, W.D. (2016) – PROBLAD PLUS: Storage Stability and Corrosion Characteristics Study (PSL Study Number #35987) - containing the validation data of the modified Lowry method for the quantification of BLAD lead component in PROBLAD PLUS Fungicide</p> <p>b) PROBLAD PLUS ; Physical and Chemical Characteristics : UV/Visible Absorption</p>
Author(s) & Year:	<p>a) A. Carreira, 2018b</p> <p>b) C. Wo, 2018</p>

Document No, Authority registration No	a) CEV-QCLR-18.06-01 b) 48182
Guideline(s):	Yes AOAC Guidelines for Single Laboratory Validation of Chemical Methods for Dietary Supplements and Botanicals (19-12-2002).
Deviations:	No
GLP or GEP:	Yes
Acceptability:	Yes
Study relied upon:	Yes

Method description:

The samples are analysed by the modified Lowry method, which is a colorimetric assay widely used for protein quantification. The procedure involves the reaction of proteins with cupric sulfate and tartrate salt in an alkaline solution. The reaction forms tetradentate copper-protein complexes which can be oxidised by Folin-Ciocalteu reagent, producing a water-soluble product with a characteristic blue colour. The intensity of the blue colour was measured by UV/Vis spectrophotometry at 750 nm.

Sample preparation:

Prepare reagent C by dilution of 1 part of Reagent B with 99 parts of Reagent A.

Reagent A: 4.0 g sodium hydroxide, 1.6 g di-Sodium tartrate dihydrate, 20.0 g sodium carbonate, 10.0 g dodecyl sulfate sodium salt in 1000 mL water

Reagent B: 4% w/v Copper (II) sulfate pentahydrate in water

Weigh 0.5 g PROBLAD PLUS sample into a 50 mL volumetric flask and fill with water to volume. Dilute this solution 10 times (200 µL solution in 1800 µL MilliQ-water). Take 75 µL sample of the diluted PROBLAD PLUS solution and put it in 1.5 mL Eppendorf tube, add 175 µL water. Add 50 µL sodium deoxycholate 0.1% (w/v) fresh solution. Add 1 mL trichloroacetic acid 10% (w/v) solution. Vortex the tube and afterwards keep still for 10 minutes. Centrifuge the tube for 5 minutes at 15000g and discard the supernatant. Add 1 mL of Reagent C fresh solution. Vortex the tube and wait for 10 minutes (after adding Reagent C the colour should have changed to light

blue). Add 100 μL of Folin-Ciocalteu (1N) fresh solution and vortex again. Keep the tube at 25°C in a dark cabinet for 30 min, measure the absorbance at 750 nm in a UV/vis spectrophotometer.

The final sample concentration is: 0.075 mg of PROBLAD PLUS (approximately 0.015 mg BLAD) in approximately 1.5 mL solvent.

Specificity and non-analyte interference:

The method is specific to proteins and no interactions of other organic compounds are expected. With different proteins slightly different spectrophotometric signal responses could be obtained, it depends on the specific amino acid composition of the protein. In order to calibrate blue colour intensity to BLAD, a calibration curve was prepared using pure BLAD polypeptide.

This method is specific for protein analysis. It does not account for the presence of small molecules; only proteins react with the reagents used. A specific precipitation step avoids the interference of other compounds present in the sample matrix.

Matrix effects and non-analyte interference were not investigated. The applicant stated that it is impossible to separate BLAD from samples of PROBLAD PLUS, therefore it is impossible to obtain a blank sample.

The use of wavelength 750 nm was justified in an additional study (Wo, C., 2018) where wavelength scans of the test sample, analytical standard and reagent blank were presented.

Further justification for the specific nature of this method for the determination of BLAD is provided by the analysis of the same samples by the HPLC-UV method provided above, giving sufficiently similar results.

Linearity:

Calibration samples were prepared by pipetting appropriate amounts of the reference standard of pure BLAD and deionized water. Linearity was demonstrated by analysing 5 samples with increasing BLAD content in the range of 2.48–34.65–2.38 – 33.33 μg . The samples were prepared in triplicate, the coefficient of determination (R^2) was 0.993567 and calibration equation was $y = 0.0113x + 0.0125 - 0.0043x + 0.0123$. It is noted that this calibration curve included the origin. Residual analysis also supported the linear relationship.

The linear range covers a sufficient range considering the content of BLAD in PROBLAD PLUS (approximately 15 μg , in both active substance and plant protection product samples).

Precision (repeatability)

Data were presented for replicate measurements of standard solutions. This does not demonstrate precision in line with SANCO/3030/99 rev. 5. The analysis of the test samples at the initial time point in the storage stability study (see Volume 3 CP B2) could be considered precision data as these were replicate weighings of representative samples (n = 6). These samples all contained ~20% w/w of BLAD and gave an overall %RSD of 4.56. This gives a Horrat ratio of 2.67 which is not acceptable. None of the results are considered significant outliers when analysed using the Grubbs test. Considering the uncertainty with quantitation using this method (colorimetric determination) and complexity of the sample preparation, this variation in results could be considered justified.

Recovery (accuracy)

Recovery samples were prepared by spiking samples of PROBLAD PLUS with standard solution at two fortification levels: 2.38 and 11.90 µg, three samples at each fortification level were prepared. Mean recoveries are 96.28% for 2.38 µg fortification level and 102.1% for 11.90 µg fortification level. The mean recoveries are within acceptable range and %RSD is below 20% for both fortification levels.

Table B.5.1.1-2 Summary of recovery data

Sample no	Calculated amount (µg)	Expected amount in spiking solution (µg)	Expected in PROBLAD PLUS solution (µg)	Total BLAD expected (µg)	Recovery (%)
Spike1-1	4.63	2.38	2.42	4.8	96.28
Spike1-2	4.63	2.38	2.42	4.8	96.28
Spike1-3	4.63	2.38	2.42	4.8	96.28
Spike2-1	24.56	11.90	12.23	24.13	101.76
Spike2-2	24.56	11.90	12.23	24.13	101.76
Spike2-3	24.79	11.90	12.23	24.13	102.73

Within the validation report, several determinations of the BLAD content of samples of PROBLAD PLUS were reported. Although these are not fortified samples, these

are replicate samples analysed using the above method, and may be indicative of the method accuracy and precision.

Table B.5.1.1-3: Summary of precision data

Sample no	Sample weight (g)	Expected content of BLAD (µg)	Determined content of BLAD (µg)	% BLAD (w/w)¹	Recovery (%)
A1	0.5164	15.0	15.41	19.9	102.7
A1	0.5164	15.0	15.50	20.0	103.3
A1	0.5164	15.0	15.50	20.0	103.3
A2	0.5190	15.0	15.77	20.3	105.1
A2	0.5190	15.0	15.77	20.3	105.1
A3	0.5190	15.0	15.59	20.0	103.9
A4	0.5298	15.0	15.68	19.7	104.5
A4	0.5298	15.0	15.59	19.6	103.9
A4	0.5298	15.0	15.95	20.1	106.3
				Overall mean	104.2%
				%RSD	1.06

¹ Note the study report stated the units as w/v in error.

In accordance with SANCO/3030/99 rev. 5, for a method determining the active substance content in the technical material, at least 5 determinations should be made. For a method determining the active substance content in the plant protection product, at least two independent recovery determinations should be made. As the active substance and formulation are identical, the reduced validation data set described above is considered sufficient to demonstrate method accuracy.

Conclusions

The method is not fully validated according to SANCO/3030/99 rev. 5 or SANTE/2020/12830 for detection of BLAD protein in PROBLAD PLUS samples and the determination of BLAD in the assessment of physical and chemical properties due to a lack of repeatability data. However, there is some confidence with the data provided that the method is capable of determining the content of BLAD in PROBLAD PLUS at the levels considered (~20% w/w). Therefore the method is considered sufficient for regulatory purposes.

This method (modified Lowry method) was used to determine the content of BLAD in the spray solution tested in the physical and chemical property studies (spray application equipment test). The content of BLAD in the samples analysed (~ 0.016-0.019 mg) is broadly the same as the content of BLAD in the test samples of PROBLAD PLUS analysed by the sample method and considered sufficient for regulatory purposes above. The matrix in these tests was water which is already present in the PROBLAD PLUS samples, therefore no additional interference is expected. Full details of the sample preparation have not been provided but there is confidence from the available information that the samples are prepared to give appropriate content in the injection samples.

Impurities: quinolizidine alkaloids including the lead component lupanine

Total quinolizidine alkaloids (QAs) are a relevant impurity in aqueous extract from the germinated seeds of sweet *Lupinus albus*. As the plant protection product and active substance are identical, the method for determination in the active substance is suitable for the determination of QAs in the plant protection product.

Reference:	
Report Title:	<p>1) Addendum to the study report Zehr, P.S. (2013) – PROBLAD PLUS: Preliminary analysis (PSL Study Number #32342) - containing the validation data of the HPLC method for the quantification of BLAD lead component and GC method for the quantification of Lupanine in PROBLAD PLUS Fungicide</p> <p>2) PROBLAD PLUS - Preliminary Analysis. Converde SA</p> <p>3) PROBLAD PLUS : Storage Stability and Corrosion Characteristics Study –24-Month Interim Report</p>
Author(s) & Year:	<p>1) A. Carreira (2018a)</p> <p>2) P. S. Zehr, 2013</p>

	3) W. D. Gravelle, 2016
Document No, Authority registration No	1) CEV-QCLR-18.07-02 2) 32342 3) 35987
Guideline(s):	Yes The applicant used SANCO 3030/99 rev. 4, the current version is SANCO 3030/99 rev. 5
Deviations:	No
GLP or GEP:	1) No 2) Yes 3) Yes
Acceptability:	Yes
Study relied upon:	Yes

Determination of lupanine by gas chromatography with flame ionisation detection

Principle of the method

Sample preparation

The test sample was prepared in triplicate by accurately weighing approximately 1 g of the test substance into a 50 mL volumetric flask, adding 0.5 mL of internal standard (caffeine in chloroform 500 µg/mL), diluting to volume with chloroform and sonicating for 10 minutes, mixing well. The solution was filtered through 0.45 µm syringe filters into autosampler vials for analysis under the following GC-FID conditions.

It is noted that chloroform which is listed as a hazardous reagent which should not be used in methods as outlined in SANCO/3030/99 rev. 5. The applicant has provided an alternative method reported below.

Principle of the method**Determination of lupanine by GC-FID:**

Gas Chromatograph	Hewlett Packard 6890
Detector temperature	FID @ 250 °C
Column	J&W DB – 1.15 m x 0.25 mm ID x 0.25 µm
Oven program	Initial 120°C for 0 min Increase to 185°C with ramp of 3°C/min, hold 1 min Increase to 300°C with ramp of 30°C/min, hold for 8.5 min
Run time	35 min
Injector temperature	250°C
Flow rate	2.0 mL/min
Injection volume	2.0 µL
Retention time	Lupanine ~20.9 min Internal standard (Caffeine) ~10.7 min

The above conditions were modified slightly for the determination of lupanine content in the phys-chem properties study (flow rate of 1.5 or 0.9 mL/min). This is not expected to impact the validity of the method and the consideration of validation data from all three study reports where this GC-FID method has been considered have been reported here.

Quantification of lupanine was against external standards.

Specificity

Representative chromatogram of lupanine standard and lupanine in PROBLAD PLUS show a retention time match. There is no significant interference with the blank sample.

Confirmation of identity

GC-FID is not considered a highly specific method. An alternative method which is highly specific (GC-MS/MS) has been reported below.

Linearity

Linearity was demonstrated by analysing 5 standard solutions of increasing concentration in the range of 0.32-1.62 µg/mL (equivalent to 0.016 to 0.081 g/kg). The coefficient of determination (R^2) was 0.9976 and the linear calibration equation was $y = 0.3406x - 0.0085$. (Zehr 2013)

Linearity was demonstrated by analysing 5 samples with increasing lupanine content in the range of 0.60-1.80 µg/mL. The samples were prepared in duplicate, the coefficient of determination (R^2) was 0.9938 and calibration equation was $y = 0.3227x - 0.0591$. (Gravelle 2016)

Precision (Repeatability)

The applicant provided data for five replicate injections of 1.08 µg/mL standard solution, with a %RSD of 3.32 (Zehr 2013) and five replicate injections of 1.20 µg/mL, with a %RSD of 1.69 (Gravelle 2016). However, this is not an acceptable approach for the determination of repeatability. The method has been superseded by the GC-MS/MS method described below.

The content of lupanine in three samples of PROBLAD PLUS was determined in duplicate as part of the storage stability study (Gravelle 2016). These determinations were made on the same samples at 3, 6, 12 and 24 month time points. The results of these analysis may be considered repeatability determinations, as they are replicate determinations of lupanine content in samples. The content of lupanine was not known prior to analysis (the samples were not spiked at a known level), but as an indication of method precision, these data are supportive. The data are summarised in Table B.5.1.1-4. The majority of %RSD values are below the acceptable Horwitz value of 6.1 at a concentration of approximately 0.004% w/w. This gives a Horrat ratio of < 1.

Table B.5.1.1-4. Summary of lupanine content determination data

Time period of analysis	Sample name	Lupanine content (% w/w)	%RSD
Initial	A1	0.0042, 0.0045	3.68
	A2	0.0045, 0.0046	
	A3	0.0043, 0.0044	
3 months	A1	0.0040, 0.0041	6.07
	A2	0.0046, 0.0045	
	A3	0.0045, 0.0046	
6 months	A1	0.0041, 0.0040	6.38
	A2	0.0044, 0.0042	
	A3	0.0045, 0.0048	
12 months	A1	0.0042, 0.0043	1.94
	A2	0.0044, 0.0043	
	A3	0.0044, 0.0042	
24 months	A1	0.0042, 0.0041	3.22

	A2	0.0045, 0.0043	
	A3	0.0043, 0.0044	

Recovery (Accuracy)

Recovery samples were prepared by spiking a solution of PROBLAD PLUS with standard solution of lupanine. Two independent samples were prepared at one fortification level, in each study report. Both were injected in replicate. The average recoveries were 102.6% and 101.5%. Two independent weighings and determinations were made at appropriate level considering the proposed specification for lupanine (max. 0.035 g/kg, equivalent to 0.7 µg/mL). This is sufficient in line with SANCO/3030/99 rev. 5.

Table B.5.1.1-5. Summary of recovery data

Study reference	Sample ID	Lupanine calculated (µg/mL)	Expected amount (µg/mL)	Recovery (%)	Mean recovery (%)
Zehr 2013	Spike 1	0.84	0.77	109.2	102.6
		0.78	0.77	101.0	
	Spike 2	0.73	0.76	95.5	
		0.80	0.76	104.6	
Gravelle 2016	Spike 1	0.95	1.00	95.3	101.5
		1.05	1.00	105.1	
	Spike 2	1.06	1.01	104.1	
		1.03	1.01	101.6	

LOQ

According to assimilated Regulation No. 283/2013, the experimental determination of the limit of quantification (LOQ) is not required for impurities in the technical active substance. However, the method has to be validated at least 20% less than the level included in the specification for relevant impurities. This has not been achieved as there is no precision data demonstrating repeatability of the method.

Conclusion

The method is not fully validated in accordance with SANCO/3030/99 rev. 5 due to the method not being highly specific and able to confirm the identity of the analytes, and the lack of data to address precision and therefore the LOQ of the method.

This method used a hazardous reagent which is not permitted in line with SANCO/3030/99 rev. 5. This method should not be used and has been superseded by the GC-MS/MS method described below for the determination of the QAs in PROBLAD PLUS.

This method was used to support determination of the QA content in the pilot scale batches and in the phys-chem properties studies determining the content of the QAs before and after storage. Confirmation of identity should 'ideally' be demonstrated for methods used for this purpose. The method cannot be considered fully validated due to the lack of precision data. There is some confidence that the method can determine the content of lupanine in PROBLAD PLUS from the accuracy data and precision data determined using the analytical standards. The method is considered sufficient for regulatory purposes considering the supporting pilot scale data and supporting determinations of levels in the formulation before and after storage (see Volume 4 section C.1.3.1 for more information).

Reference:	1.2.3
Report Title:	1) Validation Report for the Analytical Method for the Determination of Quinolizidine Alkaloids by GC-MS/MS 2) Quantification of quinolizidine alkaloids (QAs) in PROBLAD PLUS and <i>Lupinus albus</i> seeds
Author(s) & Year:	1) R. Li, 2021 2) A. Carreira, 2019
Document No, Authority registration No	1) MVR 3.2.40_01 2) CEV-QCLR-19.11-01

Guideline(s):	SANCO/3030/99 rev.5
Deviations:	No
GLP or GEP:	1) Yes 2) No
Acceptability:	Yes
Study relied upon:	Yes

Determination of lupanine by gas chromatography with tandem mass spectrometry

Principle of the method

Sample preparation: The sample is weighed (0.5 g) and mixed in water (4 mL) with ammonium hydroxide (0.5 mL, 1:1 v/v solution with deionised water) and dichloromethane (10 mL). Vortex for 2-3 min. Add sodium sulfate (3 g), vortex for 30 s. Centrifuge at 2800 rpm for 10 min, filter the sample using a 0.45 µm PTFE syringe filter if needed. After centrifuging the mixture, 1 mL of the organic layer is analysed by GC-MS/MS under the following conditions:

Instrument	Shimadzu GCMS-TQ8040 (GC-MS/MS)
Column	Restek RXI-5 ms, 30 m, 0.25 mmID, 0.25 µm
Column flow	1.0 mL/min
Split ratio	10.0
Oven temperature	Start at 64°C Ramp at 15 °/min to 100°C, hold for 20s Ramp at 18 °/min to 320°C, hold for 3 min
Retention time	Lupanine 7.7 min Sparteine 10.3 min Lupanine 12.8 min

	13-Hydroxy-Lupanine 14.0 min
MS parameters	Ion Source temperature: 200°C Interface temperature: 300°C
Masses to identify compounds	Lupanine: 152, 138, 168 m/z Sparteine: 137, 98, 193 m/z Lupanine: 136, 149, 248 m/z 13α -OH-Lupanine: 152, 246, 134 m/z 13α-OH-Lupanine 2: 264, 165, 112 m/z (additional ion transitions) p-Tetraphenyl-d ₁₄ (Internal Standard): 244, 243, 122 m/z

Dichloromethane, which is classified as a category 2 carcinogen, is used in the sample preparation. This is not recommended in line with SANCO/3030/99 rev. 5. The applicant has explained that Quinolizidine Alkaloids are notoriously difficult to analyse. As a consequence, chlorinated solvents are the only appropriate solvents to extract the analytes. However, full safety precautions are employed with extractions being performed in fume cabinets to prevent operator exposure. This is acceptable in this specific case.

Due to the unavailability of reference standards for seven other quinolizidine alkaloids of interest (13α-angeloyloxylupanine, Albine, Angustofoline, 13α-tigloyloxylupanine, α-isolupanine, Tetrahydrorhombifoline, Multiflorine), the identification of these seven analytes would be initially screened with selected ion monitoring for the characteristic fragment ions of each analyte:

Component	m/z	Retention time (mins)
Lupanine	152	7.8
Sparteine	137	10.3
Albine	191	-

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Tetrahydrorhombifoline*	207	-
Angustofoline*	193	-
α -isolupanine*	136	-
Lupanine (perchlorate)	136	12.8
Multiflorine*	134	-
13-OH-lupanine	152	14.0
13 α -angeloyloxylupanine*	246	-
13 α -tigloyloxylupanine*	246	-

*no reference standard available.

The quinolizidine alkaloids are expected to elute in the above listed order based on research literature and should be identified by their fragment m/z. For the compounds 13 α -angeloyloxylupanine and 13 α -tigloyloxylupanine, which are isomers and, therefore, have the same m/z value (along with isolupanine which is related to lupanine perchlorate without the perchlorate group), if the sample is screened by mass spectroscopy and those compounds are found present in a sample, it would become important to source a standard for these compounds. However, no positive m/z detections were found for those four compounds in the analyzed samples.

Table B.5.1.1-6. Summary of validation data for the determination of quinolizidine alkaloids in PROBLAD PLUS and aqueous extract from the germinated seeds of sweet *Lupinus albus*

Matrix	Analyte	LOQ (mg/kg)	Level present prior to fortification (mg/kg)	Recovery fortification level (mg/kg)	% Recovery	Repeatability % RSD (n)	Linearity	Specificity
PROBLAD PLUS	Lupinine	4	Not detected < 4	4 10 20 30 40	97.38 105.16 119.52 120.77 113.85	11.25 @ ~4 mg/kg (n = 6) Modified Horwitz = 8.74 @ 3.9 mg/kg therefore $H_r = 1.29$ 1 result (0.003 g/kg) identified as an outlier using Grubbs test. Removing this from the dataset gives %RSD of 3.63, therefore $H_r = 0.42$	2.41 to 192.96 mg/mL (equivalent to 2 to 160 mg/kg) (n = 6) $Y = -169288125.24x^3 + 42945809.93x^2 + 345,266.79x - 798.01$ $R^2 = 0.99998$ Note : this was based on a 3rd order polynomial in the study report but a linear relationship can be demonstrated by plotting the reported values, giving the equation : $y = 3015020x - 27594$ $R^2 = 0.9931$ The results determined using the two equations are not significantly different but it is clear from the results at lower concentrations that a polynomial fit is better.	Retention time match to reference standard. No significant interfering peaks observed in the fortified and test sample chromatograms

Matrix	Analyte	LOQ (mg/kg)	Level present prior to fortification (mg/kg)	Recovery fortification level (mg/kg)	% Recovery	Repeatability % RSD (n)	Linearity	Specificity
	Sparteine	2	Not detected < 2	2 5 10 15 20	90.95 79.86 77.44 76.29 78.76	4.51 @ ~1.8 mg/kg (n = 6) Modified Horwitz = 9.80 @ 1.8 mg/kg therefore $H_r = 0.42$	1.21 to 96.48 mg/ml (equivalent to 1 to 80 mg/kg) $y = 20000000x + 34848$ $R^2 = 0.9947$ (n = 6)	Retention time match to reference standard. No significant interfering peaks observed in the fortified and test sample chromatograms
	Lupanine (free, standard is perchlorate salt, but GC-MS-MS measures free lupanine, conversion factor 0.712) ¹	1.6	1.6	1.4 3.6 7.1 10.7 14.2 32.0	100.34 98.96 97.20 95.93 97.85 93.91	1.49 @ ~1.6 mg/kg (n = 6) Modified Horwitz = 7.07 @ 1.6 mg/kg therefore $H_r = 0.21$	0.86 to 68.69 mg/ml (equivalent to 1 to 80 mg/kg) $y = 20000000x + 20508$ $R^2 = 0.9977$ (n = 6)	Retention time match to reference standard. No significant interfering peaks observed in the fortified and test sample chromatograms

Matrix	Analyte	LOQ (mg/kg)	Level present prior to fortification (mg/kg)	Recovery fortification level (mg/kg)	% Recovery	Repeatability % RSD (n)	Linearity	Specificity
	13-hydroxy-lupanine	6.6	6.6	2 5 10 15 20	91.81 94.74 84.49 80.64 76.37	2.64 @ ~6.6 mg/kg (n = 6) Modified Horwitz = 8.08 @ 6.6 mg/kg therefore H _r = 0.33	1.21 to 96.48 mg/ml (equivalent to 1 to 80 mg/kg) $y = 20000000x + 12265$ $R^2 = 0.9986$ (n = 6)	Retention time match to reference standard. No significant interfering peaks observed in the fortified and test sample chromatograms !

¹ Full details of the calculation of a conversion from the salt to the free lupanine is given in the study report.

Specificity:

Specificity was demonstrated by retention time match with a reference standard for lupinine, sparteine, lupanine, and 13-hydroxy-lupanine. No significant interferences were detected in solvent blanks in the region of the impurity peaks.

Confirmation of identity:

The identity of each component was confirmed by the fragment mass and retention time matching with certified reference standards. SANCO/3030/99 rev. 5 states that for GC-MS/MS methods, at least two mass fragments should be used for identification. However, in this case, the range of fragments of the separate QA components and confidence in the single fragments used for each component provides sufficient reassurance regarding the identity and further confirmatory analysis is not considered necessary.

Linearity:

Linearity was demonstrated by the analysis of six standards of increasing concentration. The response was linear for all analytes with a coefficient of determination (R^2) above 0.99 for all analytes. The range of linear calibration for all analytes extends over a suitable range considering the levels determined in the samples (<LOQ) and the levels in the applicant's proposed specification (lupanine 35 mg/kg and total QAs 50 mg/kg).

Accuracy:

Recovery samples were prepared by spiking samples of PROBLAD PLUS formulation with reference standards of all analytes. Five different spike levels were prepared for each analyte and total recovery was calculated. Six spike levels were prepared for lupanine (free). This is acceptable in line with the SANCO/3030/99 rev. 5 guidance.

The concentration of each analyte in samples is below 0.01% w/w, thus the acceptable mean recovery range is 70-130% ; all recoveries were within this range.

The fortified sample levels are supportive of the levels of these impurities found in the sample analysis.

Precision:

Lupinine and sparteine were not detected in the sample matrix, therefore precision was calculated utilizing six replicates in the lowest concentration fortification samples from the accuracy evaluation (4 mg/kg for lupinine and 2 mg/kg for sparteine). For other analytes, six replicate samples of 'PROBLAD PLUS' were prepared and

analysed using the method described above, and the %RSD was calculated. The relative standard deviation obtained was within the guideline requirements of a HORRAT (H_r) of ≤ 1 . An outlier was removed from the dataset for lupinine, supported by the Grubbs test.

LOQ:

The LOQ is defined as the lowest fortification level with acceptable recovery and precision data. These are reported in the summary of validation data table above. For lupanine and 13-hydroxy lupanine, the LOQ has been set based on the level tested in the precision data as there is no precision data to support the lowest fortification level tested.

Conclusion

The method is acceptably validated in accordance with SANCO/3030/99 rev.5 and is suitable for the determination of the QAs in PROBLAD PLUS.

This method was used in the determination of QA content in the commercial scale batches analysed (which are identical to the PPP also).

B.5.1.2. Methods for risk assessment

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

No methods provided under this data point.

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

No methods provided under this data point.

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

No methods provided under this data point.

The method 'IM02' referred to in the certificate of analysis in the toxicology study 'PROBLAD PLUS: 13 week oral (gavage) administration toxicity study in the rat', is the same method as the modified Lowry method described in study no. 35987 summarised in section B.5.1.1. This method was used to determine the content of BLAD in PROBLAD PLUS therefore the validation data presented under section B.5.1.1 is applicable here also.

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

No methods provided under this data point.

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

Reference:	
Report Title:	APPENDIX B: ELISA Analytical Methods for Determination of BLAD Protein in Grape and Tomato Residue In Magnitude and Decline of BLAD Residues Following Application of ProBLAD Plus to Grapes, Strawberries, and Tomatoes
Author(s) & Year:	D. Vespestad (2014)
Document No, Authority registration No	Eurofins Agrosience Services, Inc. Study Number S13-04129
Guideline(s):	Yes EPA Residue Chemistry Test Guidelines OPPTS 860.1000: Background OPPTS 860.1500: Crop Field Trials SANTE/2020/12830 rev. 1
Deviations:	No
GLP or GEP:	Yes
Acceptability:	Yes
Study relied upon:	Yes

Principle of the method

Grape and strawberry samples were analyzed for BLAD residues using grape ELISA (Enzyme Linked Immunosorbent Assay) method (EASI Method No.: RA029). Tomato samples were analyzed for BLAD residues using grape ELISA method (EASI Method No.: RA031). The ELISA methods involve a capture antibody; a sample is added, followed by conjugate (anti-lupin-peroxidase, enzyme). The enzymatic reaction occurs and the reaction will change colour which can be detected with UV-Vis.

Apparatus: VersaMax microplate reader (Molecular Devices, California), the absorbance measured at 450 nm (reference wavelength 620 nm)

Lupine ELISA: ImmunoLab; LUP-E01 (ImmunoLab (GmbH), Germany)

Sealing film: PlateMax Ultraclean Sealing Film

Sample preparation:

Homogenized grape and strawberry samples were extracted with poly(vinylpyrrolidone) in a buffer before being analyzed on a VersaMax plate reader at optical density (OD) 450 nm using an ELISA method.

Homogenized tomato samples were extracted with buffer alone before being analysed on a VersaMax plate reader at OD 450 nm using an ELISA method.

Specific sample preparation:

Accurately weigh 0.5 g of matrix into a 15 mL centrifuge tube. Add 0.5 g PVPP to the matrix (for grape and strawberry, not used for tomato), followed by 10 mL of the 1x extraction buffer, mix well to ensure homogeneity of the PVPP in solution. Incubate the suspension into a preheated water bath at 60°C for 15 min. Gently shake the sample 20 times in 2 minute intervals to ensure complete homogeneity. Centrifuge the sample at approximately 1814.2g (2540 rpm) for 10 minutes. Filtration following centrifugation is optional.

Transfer the supernatant of the centrifuged sample into a newly labelled 15 mL centrifuge tube. Leave at least 1 mL in the centrifuge tube over the precipitate PVPP and matrix to get a clear soup. Place the labelled samples at 4°C for 10 min until ready for the ELISA quantification step.

Aliquot 100 µL of the aforementioned prepared samples in duplicates into the appropriate wells of the microtiter plate. Seal the plate carefully before vortexing. Incubate the plate for 20 minutes at room temperature with moderate shaking at speed 3.

Prepare a 1x wash buffer by transferring 1 mL of the 10x wash buffer (provided by ImmunoLab) into a 15 mL centrifuge tube and adding 9 mL of HPLC water. The buffer should be warmed for 30 min in warm water, shake every 5-10 min to ensure homogeneous mixture before preparing the 1x wash buffer. Gently invert the well containing the samples onto a paper towel to ensure the liquid is removed from the wells (do not aspirate). Add 300 µL of the 1x wash buffer to each well, waiting 20s before inverting the well onto a paper towel again to remove the liquid. Repeat this step three more times. Take care to never fully dry the wells during this step (this step is further referred as “washing procedure”).

Pipet 100 µL of conjugate (anti-lupin-peroxidase) into each well, seal the plate carefully and incubate for 20 min at room temperature with moderate shaking at speed 3. Wash the plate according to the washing procedure. Pipet 100 µL of substrate into each well followed by aluminium foil wrapping and incubation at room temperature with moderate shaking at speed 3.

Stop the enzyme reaction by adding 100 µL of stop solution into each well, sealing the plate carefully and mixing for 5-10 minutes at room temperature with moderate shaking at speed 3 (the blue colour will turn yellow upon addition). After thorough mixing, measure the absorbance at 450 nm (reference wavelength 620 nm) using a VersaMax plate reader. If the reading comes beyond the linear region, samples will be diluted up to 1:10.

Selectivity and Specificity

Selectivity and specificity were not addressed in the method validation report. However, the ELISA kit used by the applicant is a commercial kit which is advertised as “The Immunolab Lupine ELISA is a quick and sensitive method for the quantitative determination of lupine in foods.” Ref. [Immunolab Lupine ELISA kit.pdf \(oxfordbiosystems.com\)](https://www.oxfordbiosystems.com/oxfordbiosystems.com)

There is some confidence that the equipment used will be specific to BLAD from data and literature considered in the residues section (i.e. BLAD specific antibodies described study ref: CEV110820 and ‘residues test’ study).

It should be noted that for a complex protein such as BLAD, using the confirmatory techniques proposed in SANTE/2020/12830 are not viable options to address confirmation of identity of the target analyte.

Matrix effects

The applicant confirmed that standard solutions were mixed with plant matrix therefore no further consideration of matrix effects is required.

Calibration

Calibration based on solvent based standards was presented. Six standards in the concentration range 0.005-0.15 ppm were presented showing a linear relationship, with a correlation coefficient of 0.998. A quadratic equation is presented. When plotted, a linear relationship is observed but the correlation coefficient is 0.9664. The applicant provided the following justification for the quadratic relationship: the absence of residual analysis, the acceptable correlation coefficient indicates suitability of the chosen function. Also, polynomial curves are typical for ELISA methods. This is considered acceptable.

Recovery and repeatability

Recovery samples were prepared by spiking grape and tomato matrices with standard solutions of BLAD protein.

Grape (PVPP and matrix ratio 1:1)			
Spike concentration (mg/kg µg/mL)	Results (mg/kg µg/mL)		% Recovery
	Sample 1	Sample 2	
0.02 (~0.4 mg/kg)	0.012	0.007	47.5
0.05 (~1 mg/kg)	0.023	0.020	43.0
0.08 (~1.6 mg/kg)	0.034	0.030	40.0

Tomato		
Spike concentration (mg/kg µg/mL)	Results (mg/kg µg/mL)	% Recovery
0.02 (~0.4 mg/kg)	0.021	105.0
0.025 (~0.5 mg/kg)	0.024	96.0
0.03 (~0.6 mg/kg)	0.032	106.7
0.035 (~0.7 mg/kg)	0.033	94.3
0.04 (~0.8 mg/kg)	0.043	107.5
0.07 (~1.4 mg/kg)	0.08	114.3

0.2 (~4 mg/kg)	0.1	50.0
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Procedural recoveries

Matrix	Fortification level (mg/kg, µg/mL)	% Recovery
Tomato	0.02 (~0.4 mg/kg)	60.5
	0.04 (~0.8 mg/kg)	49.0
	0.05 (~1 mg/kg)	53.4
	0.08 (~1.6 mg/kg)	48.1
	0.1 (~2 mg/kg)	50.1
Grape	0.02 (~0.4 mg/kg)	48.4
	0.05 (~1 mg/kg)	42.0
	0.08 (~1.6 mg/kg)	44.1

The recoveries in tomato are within the acceptable range (70-110%) for the fortification levels up to 0.07 mg/kg 0.04 µg/mL. The recoveries at the 0.07 and 0.2 mg/kg µg/mL fortification levels are outside of this acceptable range therefore the method is not considered validated at these higher levels based on the information provided.

The recoveries in grape are below the acceptable range (70-110%).

The procedural recoveries presented (42.0-60.5%) are all outside of the acceptable range (70-110%). This suggests that the method was not performing as expected at the time of analysis of the test samples.

Repeatability samples and Limit of quantification

Validation data to address the repeatability and precision of the method, and therefore to determine the LOQ of the method have not been presented and are not available.

Stability of standards and extracts

Extracts were all analysed within 24 hours therefore no further consideration of storage stability is required. All standard solutions were prepared fresh on the day of the experiment, therefore no further consideration of storage stability is required.

Extraction efficiency

As the samples are not extracted, no further information is required.

Conclusions

Considering significant deficiencies of the study in terms of recoveries, repeatability and precision, the method is not validated according to SANTE/2020/12830 rev.1.

No validation data has been presented for the strawberry matrix other than what is reported in the field trials data.

There is some confidence that the method can determine the presence of BLAD in treated crops, but a reliable LOQ cannot be established.

B.5.1.2.6. Methods in soil, water, sediment, feed and any additional matrices used in support of ecotoxicology studies

Method ref: S18-08251

The following analytical method was used in the following ecotox studies: Gerke and Schneider (2019): 48 h acute *Daphnia magna*; Gerke and Schneider (2019): Life-cycle toxicity test with *Daphnia magna* and Arnie et al. (2019): Freshwater algae study. ELISA method ref. S18-08251.

The ecotox study 'Huerta (2020): Non-target plants' also utilises ELISA method ref. S18-08251 for the determination of PROBLAD PLUS in water solutions. The validation data presented below can be considered supportive of the validity of the method for use in the 'non-target plants' study due to the similarity of the simple matrices under consideration (distilled water/fresh water matrix).

Reference:	
Report Title:	Validation of an Enzyme Linked Immunoassay (ELISA) Method for the Determination of PROBLAD PLUS in Test Medium used for Acute Toxicity to Green Alga, Acute Toxicity to <i>Daphnia</i> and Chronic Toxicity to <i>Daphnia</i>

Author(s) & Year:	A. Perry (2019)
Document No, Authority registration No	Eurofins Agrosience Services, Inc. Study Number S18-08251
Guideline(s):	Yes SANCO/3029/99 rev. 4
Deviations:	No
GLP or GEP:	Yes
Acceptability:	Yes
Study relied upon:	Yes

Principle of the method

Sample preparation for Algae and Daphnia media: The wash buffer was removed from the ELISA kit and placed in a water bath or on a heat block to warm for at least 15 minutes to ensure that all the buffer constituents were completely dissolved prior to use. Calibration and fortification standards were prepared fresh daily by diluting a known weight of PROBLAD PLUS (recommended amount 0.5 g) in HPLC water followed by serial dilution in HPLC water. All concentrations were recorded as PROBLAD PLUS (mg/L). Working calibration standards were prepared in 1x sample dilution buffer. All analytical specimens were diluted at a minimum 1:2 in 1x sample dilution buffer to eliminate matrix effects. A volume of 0.1 mL of the calibrators, fortifications and diluted specimens were pipetted into a minimum of two duplicate wells of the ELISA plate which was then immediately sealed. The plate was incubated for 60 minutes at room temperature with moderate shaking (approximately 40 rpm). The 1x wash buffer was prepared by adding 50 mL of the x10 concentrate to 450 mL of HPLC water and gently mixing. Volumes were adjusted accordingly if smaller volumes were required. Following the 1 hour incubation, the plate was washed four times with 1x wash buffer using an automated plate washer. A volume of 0.1 mL of conjugate (anti-lupin-peroxidase) was pipetted into each well, the plate was sealed and incubated for 60 minutes at room temperature with moderate shaking at approximately 40 rpm. The plate was washed as per previous steps. A volume of 0.1 mL of substrate was pipetted into each well, the plate was sealed and wrapped in aluminium foil to protect from light. The plate was incubated for

approximately 7-12 minutes at room temperature with moderate shaking at approximately 40 rpm. The reaction was stopped by adding 0.1 mL of Stop Solution to each well. The absorbance at 450 nm was measured using an absorbance plate reader.

A summary of the validation data is presented in Table B.5.1.2-1.

Table B.5.1.2-1 Summary of the method validation data for the determination of BLAD in PROBLAD PLUS in matrix W2 and W4

Matrix	Analyte	LOQ (mg/L)	Recovery fortification level (mg/L)	Recoveries % range (mean)	Repeatability %RSD (n)	Linearity
W2	BLAD (in PROBLAD PLUS)	0.03	0.03	80-120 (99)	15 (5)	0.003-6.67 mg/L $r > 0.99$ (n = 8) $y = (0.0973 - 3.31) / (1 + (x/1.05)^{0.842}) + 3.31$
			4	99-127 (117)	9 (5)	
			130	102-121 (111)	8 (5)	
			Overall	80-127 (109)	12 (15)	
W4	BLAD (in PROBLAD PLUS)	0.03	0.03	73-100 (89)	11 (5)	
			4	90-101 (98)	5 (5)	
			130	84-111 (95)	11 (5)	
			Overall	73-111 (94)	10 (15)	

Specificity:

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

The methodology determines BLAD in PROBLAD PLUS, in the matrices. BLAD is determined using an immuno-assay method (ELISA or Enzyme Linked Immunosorbent Assay). The method is based on coupling anti-bodies to the enzyme (BLAD) which then produces a colourant, which can be detected using UV-VIS equipment. Quantification of BLAD is calculated by comparing the absorbance in sample solutions with the absorbance in reference solutions. The content of PROBLAD PLUS is 21.2% w/w BLAD so the content of BLAD or PROBLAD PLUS can be determined.

The ELISA method is not highly specific, but other validation parameters are appropriately assessed. Also, considering the target analyte is a 210 KDa oligomer protein, the usual confirmatory techniques outlined in SANTE/2020//12830 rev. 1. are not suitable.

Matrix Effects:

Matrix effects on the detection of PROBLAD PLUS in extracts of matrix W2 and W4 were found to be insignificant at the minimum dilution factor, therefore standard solutions were diluted in sample diluent for quantification. No significant matrix effects are observed.

Linearity:

Linearity was demonstrated by the analysis of eight standards of increasing concentration. The range of standard concentrations used was 0.003-6.67 mg/L. Samples were diluted to be within the linear range if required, as per the sample preparation details. The response was best plotted as a polynomial curve with a correlation coefficient (r) of > 0.99. The applicant provided the following justification for the use of a polynomial curve: in the absence of residual analysis, the acceptable correlation coefficient does indicate suitability of the chosen function. Also, polynomial curves are typical for ELISA methods. This is considered acceptable.

Accuracy:

Recovery samples were prepared by spiking control samples of the matrices with PROBLAD PLUS and analysing them by the method described. The spike concentrations were in the range 0.03 to 130 mg/L. Five samples were prepared at each fortification level.

For matrix W4 and the 0.03 mg/L fortification level in matrix W2, the mean recoveries are within the acceptable range (70-110%). For the highest fortification levels (4 and 130 mg/L) in matrix W2, the mean recoveries (111-117%) are outside of the acceptable range (70-110%). However, due to the nature of this method (ELISA) and biological nature of the test substance, this exceedance may be justified.

Precision:

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

Extraction efficiency:

As the matrices being considered are relatively simple water based solutions, no further consideration of extraction efficiency is required.

Conclusion

The analytical method for the determination of PROBLAD PLUS in W4 can be considered fully validated in accordance with SANTE/2020/12830 rev. 1.

For the matrix W2, the method is fully validated at the low fortification level (0.03 mg/L) but not fully validated at the higher fortification levels. This is due to recoveries above the acceptable range.

For matrix W2, further consideration was made for the specific ecotoxicology study where further supporting validation data was presented and was critical to their assessment (PROBLAD PLUS: A 72-HOUR TOXICITY TEST WITH THE FRESHWATER ALGA, study ref: 896P-101).

The fresh procedural recoveries were reported as presented in Table B.5.1.2-2.

Table B.5.1.2-2 Summary of procedural recoveries

Matrix	Analyte	Fortification level (mg/L)	Recovery (%) (mean)
Algae test water referred to as W2 in raw data	BLAD (in PROBLAD PLUS)	0.03	63, 87 (75)
		10	93, 108, 116 (106)
		120	80, 106 (92)

Considering the recovery determinations at 10 mg/L and 120 mg/L, although there is a limited number of samples tested (2 at each level), these provide extra reassurance that the method was working at the time of use. The validation data alone suggested that the method may not be fully validated and may be slightly over-estimating the amount present at these higher levels, however, these procedural recoveries provide sufficient reassurance that the method was working at the time of analysis. The method is considered sufficient for regulatory purposes.

Method ref: S19-21256

The following analytical method was used in the following ecotox studies: Aguilar-Alberola (2019): Acute bumblebee, Aguilar-Alberola (2019): Chronic adult honeybee and Aguilar-Alberola (2019): Honeybee larval study. ELISA method ref. S19-21256.

Reference:	
Report Title:	Validation of an Enzyme Linked Immunoassay (ELISA) Method for the determination of PROBLAD PLUS in test medium used for honey bee larval toxicity and adult honey bee chronic feeding tests.
Author(s) & Year:	A. Perry (2019)
Document No, Authority registration No	Eurofins Agrosience Services, Inc. Study Number S19-21256
Guideline(s):	Yes SANCO/3029/99 rev. 4
Deviations:	No
GLP or GEP:	Yes
Acceptability:	Yes
Study relied upon:	Yes

Principle of the method

Sample preparation: The wash buffer was removed from the ELISA kit and placed in a water bath or on a heat block to warm for at least 15 minutes to ensure that all the buffer constituents were completely dissolved prior to use. Calibration and fortification standards were prepared fresh daily by diluting a known weight of PROBLAD PLUS (recommended amount 0.5 g) in HPLC water followed by serial dilution in HPLC water. All concentrations were recorded as PROBLAD PLUS (mg/L). Working calibration standards were prepared in 1x sample dilution buffer. All analytical specimens were diluted at a minimum dilution factor for the matrix or higher, in 1x sample dilution buffer to eliminate matrix effects. A volume of 0.1 mL of the calibrators, fortifications and diluted specimens were pipetted into a minimum of two duplicate wells of the ELISA plate which was then immediately sealed. The plate was incubated for 60 minutes at room temperature with moderate shaking (approximately 40 rpm). The 1x wash buffer was prepared by adding 50 mL of the x10 concentrate to 450 mL of HPLC water and gently mixing. Volumes were adjusted accordingly if smaller volumes were required. Following the 1 hour incubation, the plate was washed four times with 1x wash buffer using an automated plate washer. A volume of 0.1 mL of conjugate (anti-lupin-peroxidase) was pipetted into each well, the plate was sealed and incubated for 60 minutes at room temperature with moderate shaking at approximately 40 rpm. The plate was washed as per previous instructions. A volume of 0.1 mL of substrate was pipetted into each well; the plate was sealed and wrapped in aluminium foil to protect from light. The plate was incubated for approximately 7 to 15 minutes (adjust accordingly to allow sufficient colour development) at room temperature with moderate shaking at approximately 40 rpm. The reaction was stopped by adding 0.1 mL of Stop Solution to each well. The absorbance at 450 nm was measured using an absorbance plate reader.

A summary of the validation data is presented in Table B.5.1.2-3.

Table B.5.1.2-3 Summary of the method validation data for the determination of PROBLAD PLUS in deionised water and sucrose solution 50% w/v

Matrix	Analyte	LOQ (mg/L)	Recovery fortification level (mg/L)	Recoveries % range (mean)	Repeatability %RSD (n)	Linearity
Deionised water	PROBLAD PLUS	0.2	0.2 12000 Overall	105-115 (110) 77-86 (80) 77-115 (95)	3 (5) 5 (5) 17 (10)	0.01-10 mg/L r > 0.99 (n = 7) $y = (0.139 - 3.43) / (1 + (x/0.867)^{0.931}) + 3.43$
Sucrose solution 50% w/v	PROBLAD PLUS	1.6	1.6 7200 Overall	93-117 (108) 93-104 (96) 93-117 (102)	9 (5) 5 (5) 9 (10)	

Specificity:

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

The methodology determines BLAD in PROBLAD PLUS, in the matrices. BLAD is determined used an immuno-assay method (ELISA or Enzyme Linked Immunosorbent Assay). The method is based on coupling anti-bodies to the enzyme (BLAD) which then produces a colourant, which can be detected using UV-VIS equipment. Quantification of BLAD is calculated by comparing the absorbance in sample solutions with the absorbance in reference solutions. The content of

PROBLAD PLUS is 21.2% w/w BLAD so the content of BLAD or PROBLAD PLUS can be determined.

Matrix Effects:

Matrix effects on the detection of PROBLAD PLUS in extracts of both matrices were found to be insignificant at the minimum dilution factor, therefore standard solutions were diluted in sample diluent for quantification. No significant matrix effects are observed.

Linearity:

Linearity was demonstrated by the analysis of seven standards of increasing concentration. The range of standard concentrations used was 0.01-10 mg/L. Samples were diluted to be within the linear range if required, as per the sample preparation details. The response was best plotted as a polynomial curve with a correlation coefficient (r) of > 0.99. The applicant provided the following justification for the use of a polynomial curve: in the absence of residual analysis, the acceptable correlation coefficient does indicate suitability of the chosen function. Also, polynomial curves are typical for ELISA methods. This is considered acceptable.

Accuracy:

Recovery samples were prepared by spiking control samples of the matrices with PROBLAD PLUS and analysing them by the method described. The spike concentrations were in the range 0.2 to 12000 mg/L. Five samples were prepared at each fortification level. The mean recoveries are all within the acceptable range (70-110%).

Precision:

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

Extraction efficiency:

As the matrix being considered is a relatively simple water based solution, no further consideration of extraction efficiency is required.

Conclusion

The analytical method for the determination of PROBLAD PLUS in sucrose solution and deionised water can be considered fully validated in accordance with SANTE/2020/12830 rev. 1.

B.5.1.2.7. Methods in water, buffer solutions, organic solvents and any additional matrices resulting from the physical and chemical properties tests

See section B.5.1.1 for details of the method used in the physical and chemical properties tests.

B.5.2. Methods for post-approval control and monitoring purposes

B.5.2.1. Methods for residues in or on food and feed of plant origin

Residue definition not required therefore methods for post-approval control and monitoring purposes not required and methods not submitted.

B.5.2.2. Methods for residues in or on food and feed of animal origin

Residue definition not required therefore methods for post-approval control and monitoring purposes not required and methods not submitted.

B.5.2.3. Methods for residues in soil and sediment

Residue definition not required therefore methods for post-approval control and monitoring purposes not required and methods not submitted.

B.5.2.4. Methods for residues in water

Residue definition not required therefore methods for post-approval control and monitoring purposes not required and methods not submitted.

B.5.2.5. Methods for residues in air

Residue definition not required therefore methods for post-approval control and monitoring purposes not required and methods not submitted.

B.5.2.6. Methods for residues in body fluids and tissues

Residue definition not required therefore methods for post-approval control and monitoring purposes not required and methods not submitted.

B.5.3. References relied on

Data Point	Author(s)	Year	Title Company Report No. Source (where different from company) GLP or GEP status Published or not	Vertebrate Study Y/N	Data Protection Claimed Y/N	Justification if Data Protection is claimed	Owner	Previous evaluation
CA 4.1.1/01	Zehr, P.S.	2013	PROBLAD PLUS - Preliminary Analysis Company Report No. 32342 (amended) Eurofins PSL, USA GLP, Unpublished	N	Y	Data protection is claimed in accordance with Article 59 of assimilated Regulation No. 1107/2009	CEV	None
CA 4.1.1/02	Carreira, A.	2018a	Addendum to the study report Zehr, P.S. (2013) – PROBLAD PLUS: Preliminary analysis (PSL Study Number #32342) - containing the validation data of	N	Y	Data protection is claimed in accordance with Article 59 of assimilated Regulation No. 1107/2009	CEV	None

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
			the HPLC method for the quantification of BLAD lead component in PROBLAD PLUS Fungicide Company Report No. CEV-QCLR-18.07-02 CEV, S.A, Portugal GLP, Unpublished					
CA 4.1.2/01	Gravelle, W.D.	2015	PROBLAD PLUS: Storage stability and corrosion characteristics study — 24 month interim report Company Report No. 35987	N	Y	Data protection is claimed in accordance with Article 59 of assimilated Regulation No. 1107/2009	CEV	None

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
			Product Safety Labs, USA GLP, Unpublished					
CA 4.1.2/02	Carreira, A.	2018b	Addendum to the study report Gravelle, W.D. (2016) – PROBLAD PLUS: Storage Stability and Corrosion Characteristics Study (PSL Study Number #35987) - containing the validation data of the modified Lowry method for the quantification of BLAD lead component in	N	Y	Data protection is claimed in accordance with Article 59 of assimilated Regulation No. 1107/2009	CEV	None

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
			PROBLAD PLUS Fungicide Company Report No. CEV-QCLR-18.06-01 CEV, S.A, Portugal GLP, Unpublished					
CA 4.1.2/03	Wo, C	2018	Physical and chemical characteristics: UV/Vis absorption spectra Company Report No. 48182 CEV, S.A, Portugal GLP, Unpublished	N	Y	Data protection is claimed in accordance with Article 59 of assimilated Regulation No. 1107/2009	CEV	None

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
	Vespestad, D.	2014	<p>APPENDIX B: ELISA Analytical Methods for Determination of BLAD Protein in Grape and Tomato Residue</p> <p>In Magnitude and Decline of BLAD Residues Following Application of ProBLAD Plus to Grapes, Strawberries, and Tomatoes</p> <p>Report No. S13-04129</p> <p>CEV, S.A, Portugal</p>	N	Y	Data protection is claimed in accordance with Article 59 of assimilated Regulation No. 1107/2009	CEV	None

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
			GLP, Unpublished					
KCA 4.1.2/04	Perry, A.	2019	Validation of an Enzyme Linked Immunoassay (ELISA) Method for the Determination of PROBLAD PLUS in Test Medium used for Acute Toxicity to Green Alga, Acute Toxicity to Daphnia and Chronic Toxicity to Daphnia Report No. S18-08251 CEV, S.A, Portugal	N	Y	Data protection is claimed in accordance with Article 59 of assimilated Regulation No. 1107/2009	CEV	None

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
			GLP, Unpublished					
KCA 4.1.2/05	Perry, A.	2019	Validation of an Enzyme Linked Immunoassay (ELISA) Method for the determination of PROBLAD PLUS in test medium used for honey bee larval toxicity and adult honey bee chronic feeding tests. Study number: S19-21256 CEV, S.A, Portugal	N	Y	Data protection is claimed in accordance with Article 59 of assimilated Regulation No. 1107/2009	CEV	None

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
			GLP, Unpublished					