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Results of an interlaboratory validation study

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Contents

Summary

An interlaboratory study was performed for the determination of phomopsin A in lupin seeds and lupin derived products. This was done in the frame of the 2nd Standardization Mandate (M/520) in the field of methods of analysis for mycotoxins in food, which had been issued by the European Commission in the framework of the Regulation EC 882/2004.

The study was organized and carried out in accordance with collaborative study guidelines of AOAC. Test materials (seeds, flour, bakery product) were prepared by addition of naturally contaminated lupin seeds. The method was based on extraction with acetonitrile/water and analysis by LC-MS/MS. The method protocol described sample preparation and the quantification method. For LC-MS/MS analysis, the laboratories used their own, i.e. different, LC-MS/MS equipment and operational conditions suitable to achieve the required sensitivity and selectivity. In total five test materials (three seed materials at various concentrations, lupin flour, and crisp bread), covering non-contaminated seed and contaminated materials in the concentration range of approximately 5 to 60 µg/kg, were analysed as blind duplicates by eleven laboratories. In addition, one sample of blank lupin seed and one sample of blank crisp bread were spiked by each laboratory for recovery determination. Besides precision parameters, other performance parameters were assessed (matrix-effects, recovery, consistency of identification parameters).

The interlaboratory reproducibility of the method, determined as RSD_R, varied from 10-26%. The HorRat values, using the Thompson modification of the predicted Horwitz RSD_R, varied from 0.4-1.2.

Matrix effects observed by the different laboratories for lupin seeds were variable (from non-significant to up to a factor of two suppression and enhancement). The average recovery of phomposin A in lupin seeds (10 µg/kg) and crisp bread (15 µg/kg) were 81% and 106%, respectively. Method selectivity was adequate, no interfering peaks were detected at the retention time of phomopsin A by any of the laboratories in the blank matrices tested. The identification parameters retention time and ion ratio were consistent. Within the sequence, the individual values varied less than ±0.1 min and less than ±30% (rel) from the average, respectively.

1 Introduction

Phomopsins are mycotoxins produced by the fungus *Diaporthe toxica* [EFSA, 2012]. There are several phomposins of which phomopsin A is the major toxic congener and also the only one for which an analytical reference standard was available at the time this interlaboratory study was conducted.

The main host of the fungus are lupins (*Lupinus L.*). Lupin seeds are being used as food ingredient, e.g. as an alternative to soybeans. Phomopsin A has been reported to be hepatotoxic and to be hepatocarcinogenic in rats. To assess the safety of the use of lupin seeds in food with regard to phomopsins, EFSA (2012) has carried out a risk assessment for the European situation. In absence of dose-response information on toxicities associated with phomposins and lack of exposure/occurrence data, no proper risk assessment could be done but it was concluded that exposure should be kept as low as possible. Maximum levels for phomopsins have only be established in Australia, the major producer of lupin seeds for food and feed, where there is a limit for phomopsins in lupin seeds and products thereof of 5 µg/kg.

The development of a standardized method for the determination of phomopsins in lupin and lupin-derived products has been included in the 2nd Standardization Mandate (M/520) in the field of methods of analysis for mycotoxins in food which has been issued by the European Commission in the framework of the Regulation EC 882/2004.

This report describes the set up and results of the interlaboratory study performed to develop an EN standard. The study was organized and carried out in accordance with collaborative study guidelines of AOAC International [AOAC 2002]. The analysis protocol used for the study was based on a method developed and in-house validated by RIKILT. The method involved a single extraction, dilution with water and direct analysis by LC-MS/MS. Details have been published in the scientific literature and can be found in [Nijs et al, 2013].

The published method was able to detect phomopsin A down to 1 µg/kg. The use of a sensitive LC-MS/MS system is a prerequisite to achieve such detection limits with the method used in this study.

2 Method and test materials

2.1 Analysis method

The detailed method protocol can be found in Annex 1. In brief, 5 grams of homogenised test material is extracted with 20 mL of acetonitrile/water/acetic acid (80/19/1) for one hour using mechanical shaking. After centrifugation, a 500 µL aliquot of the extract is diluted with 500 µL water, filtered or centrifuged, and then analysed by LC-MS/MS. Quantification is performed using multi-level matrix-matched calibration.

2.2 Pre-study

Before the interlaboratory study was performed, a pre-study was conducted. This was done because not many laboratories had experience with the determination of phomopsin A, i.e. did not have the analytical reference standard and had no LC-MS/MS conditions optimized. Another reason for the pre-study was to verify whether the LC-MS/MS equipment and conditions used by the laboratories participating in the study would be sufficient to achieve limits of quantification of 5 µg/kg or lower, and to verify selectivity and linearity of response in the relevant concentration range.

Laboratories were invited to participate in the pre-study in April 2015. Eleven laboratories responded positively. These laboratories received an aliquot of stock solution of phomopsin A (10 mg/L methanol), blank extract of lupin seeds, and a vial with a lupin-seed extract containing phomoposin A at a level corresponding to 10 µg/kg. The stock solution could be used for tuning the instrument and was further used for preparation of calibration standards in both solvent and blank extract (matrix-matched calibrants) in the range equivalent to 2.5-100 µg/kg. Calibrants and extracts were analysed, and results reported in an Excel sheet to be sent to the organizer. The completed sheets were received by August 2015. In general, the results from all laboratories were meeting the expectations. A few laboratories were provided with suggestions to improve method LOD. With that, the LC-MS/MS instrumentation and conditions anticipated to be used for the interlaboratory study were considered suitable for all eleven laboratories.

2.3 Test materials

The target matrix for this interlaboratory study was lupin seeds and lupin-derived products. For lupin-derived products a commercial lupin flour and crisp bread containing lupin flour as an ingredient were used. The target concentration range of phomopsin A contamination was 5 to 50 µg/kg, with emphasis on the lower range.

For the study, 5 materials were prepared (Table 1) in February 2016. Lupin seeds and crisp bread were grinded to <0.5 mm particle size, lupin flour was used as such. Contaminated materials were prepared by addition of naturally contaminated lupin seeds (kindly provided by the Department of Agriculture and Food, Perth, Australia) to blank material and mixing thoroughly for 48 hours. A particle size of <0.5 mm and the long extensive mixing were required to obtain sufficiently homogeneous test materials. For the lupin flour and crisp bread material, the percentage of the original matrix was kept as high as possible (76% for flour, 84% for crisp bread). The test materials were divided into sub-portions of ~6 grams in polypropylene tubes with screw cap.

Table 1 *Test materials and target mass fraction Phomopsin A*

Material	Target levels phomopsin A in µg/kg
Lupin seeds	Control (<1)
Lupin seeds	5
Lupin seeds	50
Lupin flour	15
Crisp bread	10

2.4 Homogeneity and stability

The homogeneity of the materials was tested according to ISO 13528 and the international harmonized protocol for proficiency testing of analytical chemistry laboratories [Thompson, 2006], taking into account the insights discussed by Thompson [Thompson et al, 2000] regarding the Horwitz equation. For each test material, 10 tubes were randomly selected and analysed in duplicate. With this procedure the between-sample standard deviation (s_s) and the within-sample standard deviation (s_w) were compared with the standard deviation for proficiency assessment derived from the Horwitz equation (σ_H). The method applied for homogeneity testing was considered suitable if $s_w < 0.5*\sigma_H$ and a material was considered adequately homogeneous if $s_s < 0.3* \sigma_H$.

Results of the homogeneity study are summarized in Annex 4. For the two contaminated lupin seed materials and the lupin flour, the homogeneity of the materials was adequate. For the crisp bread material, the statistics indicated insufficient homogeneity. In an earlier pre-evaluation of homogeneity involving eight instead of ten replicates, the crisp bread material did comply with the homogeneity criteria. It was therefore decided to proceed with this material for the interlaboratory validation, keeping in mind that in case poor precision would be obtained for this material, this issue would need to be taken into account and/or resolved.

The decision whether or not to perform a stability test was postponed until after the results of all participants were received (August 2016). The results showed no signs of instability of the test materials that would unacceptably affect the precision. Therefore, sample stability tests were omitted.

2.5 Sample distribution and instructions

The sample sets were sent by courier at room temperature to eleven laboratories (see Annex 3) on 19th April 2016. The analysis method (see Annex 1), instructions (see Annex 2), and an Excel reporting sheet were sent by email.

The samples for the participants were randomly selected. The five test materials were provided as blind duplicates. In addition to the test materials, each participant also received a milled lupin seed sample labelled as blank material for preparation of matrix-matched calibrants, and blank samples of lupin seed and crisp bread to be spiked in the laboratory for recovery determination. For spiking of the two blank samples, separate vials containing 1.0 mL of a solution of phomopsin A with a concentration unknown to the participants were provided. The content needed to be quantitatively transferred to 5 gram of the blank material. For preparation of calibration standards, a phomopsin A solution of 10.0 mg/L methanol was provided.

The vials containing the stock solution and the spiking solutions were weighed before sending. Each ampule was double packed in a polypropylene tube.

2.6 Set up of the interlaboratory study

The interlaboratory validation was set up in accordance with collaborative study guidelines of AOAC International [AOAC 2002]. Five test materials representing several matrix/concentration combinations had to be measured as blind duplicates. In addition, a blank lupin seed and a blank crisp bread sample were provided for recovery determinations.

The sequence to be measured (study samples and calibrants) was prescribed in the instructions. After LC-MS/MS analysis, peak assignment and integration were checked by the participants and adjusted if necessary. Then the retention time data and peak areas of both transitions were entered into the reporting Excel spreadsheet and sent to the organiser. In the spread sheet, a calibration curve of the standards prepared in blank lupin-seed extract (non-weighed linear regression) was constructed. Concentrations of phomopsin A in the test materials were calculated based on the calibration curve.

For inclusion in the data set used for further evaluation, the following requirements had to be met:

- The signal-to-noise ratio of both transitions at the level equivalent to 5 µg/kg should be > 6
- The back-calculated concentrations of the individual calibration standards using the equation of the calibration curve should not deviate more than 20% of the actual concentration

Although the main purpose of the interlaboratory study was to assess method precision, additional parameters of interest were also determined. These included the recovery of phomopsin A spiked to a blank sample by the participating laboratory, the matrix effect, and the variability of the retention time and ion ratio of the two transitions measured.

3 Statistical evaluation

The statistical evaluation of the quantitative results was carried out according to the Collaborative Study Guidelines of AOAC (2002). The use of blind duplicates facilitated the use of the Cochran test to identify laboratories showing significant greater variability among replicates (within day) when compared to the other participants (1-tail test at a probability value of 2.5%).
The Grubbs test identifies laboratories with extreme averages, the single value test (2-tail, P = 2.5%) followed by a paired value test (P = 2.5%) were performed.

Precision

The repeatability standard deviation (s_r) was calculated as $S_r = \sqrt{\left(\frac{\sum d_i^2}{2L}\right)}$

where d_i is the difference between the individual values for the pair in laboratory I and L is the number of pairs.

The reproducibility standard deviation (s_R) was calculated as $S_R = \sqrt{\left(\frac{S_d^2 + S_r^2}{n_i}\right)}$

where $S_d^2 = \frac{\sum(T_i - T_{avg})^2}{n_i\ (L-1)}$

T_i is the sum of the individual values for the pair in laboratory I, T_{avg} the mean of the T_i across all the laboratories or pairs, L the number of pairs and n_i the "effective" number of replicates per laboratory. In case all laboratories performed the duplicate analysis $n_i = 2$.

In order to facilitate comparison of the variability for different test materials included in the study, the relative standard deviation (RSD) under repeatability (RSD_r) and reproducibility (RSD_R) conditions were calculated as follows

$$RSD_r\,(\%) = \frac{S_r}{\bar{x}} \times 100\% \quad \text{and} \quad RSD_R\,(\%) = \frac{S_R}{\bar{x}} \times 100\%$$

HorRat

The HorRat value is the ratio of the RSD_R(%) to the predicted $PRSD_R$ (%). The ratio was calculated as

$$HorRat = \frac{RSD_R}{PRSD_R} \text{ where } PRSD_R = 2^{(1-0.5\,Log\,C)}\ (\approx 2C^{-0.1505})$$

where C is the estimated mean concentration expressed as a decimal fraction. In this study, the decimal fraction for each material was calculated as: C = (mean µg toxin/kg) multiplied by10^{-9}.

Thompson (2000) has reported that for concentrations below 120 µg/kg the $PRSD_R$ as predicted by the Horwitz equation is less applicable and suggested to use a fixed $PRSD_R$ of 22%. Since all concentrations tested in this study were below 120 µg/kg, HorRat values were also calculated using this Thompson modification.

4 Results

4.1 Sample analysis by the laboratories

The test materials were received in good condition. One laboratory reported leakage of one of the vials containing the spiking solution. A new vial was sent. In two cases leakage of spike solution was suspected because the weight of the vial as received differed substantially from the weight before sending.

Eleven laboratories reported results for the interlaboratory validation study of Phomopsin A in lupin seeds and lupin-derived products. All laboratories adhered to the method as provided in Annex 1 without significant deviations. It should be noted that laboratories used different LC-MS/MS systems and different operational conditions, although the same transitions (precursor ion m/z 789 with product ions m/z 226 and 323) were used in all cases.

All laboratories were able to detect phomopsin A at the required LOQ level of 5 µg/kg. Example extracted ion chromatograms for the calibration standard in lupin extract corresponding to this concentration are shown in Annex 6. In reagent blank and blank lupin seed no peaks were detected at the retention time of phomopsin A, indicating adequate selectivity under the various LC-MS/MS conditions applied. The linearity in the range corresponding to 2.5-65 µg/kg was generally compliant with the requirement set in section 2.6 (only few exceptions for the lowest calibration level). This meant that the data from all eleven laboratories were considered compliant and to be included for further method evaluation.

4.2 Matrix effects

In LC-MS/MS co-extracted matrix constituents can affect the ionisation of the analyte, and with that the response. This can result in a difference in response when analysing a standard in clean solvent compared to the same concentration of analyte in a blank sample extract. This can introduce a bias in the quantification. Ideally, matrix effects are compensated for by using isotopically labelled standards but these are not (yet) available for phomopsin A. In this study matrix-matched calibration was performed using standards prepared in lupin-seed extracts, not only for lupin seed samples, but also for lupin flour and crisp bread. To gain insight in the extend of matrix effect for phomopsin A in lupin seeds, the response in solvent and lupin seed extract (one level, 6.25 ng/mL, in duplicate) were compared in the measurements by all participating laboratories. The matrix effect, expressed as percent response in extract vs solvent is provided in Table 2.

From Table 2 it can be concluded that both ion suppression and enhancement occur. In five cases the matrix effects are minor (<20% difference between response in matrix and solvent), in four cases modest (20-40%), and in two cases strong (factor 2 suppression or enhancement). No clear relation to type of instrument or measurement conditions could be made. Different matrices may result in different matrix effects. It was not investigated whether this was the case for lupin flour and crisp bread, relative to lupin seeds.

Table 2 *Matrix effect for phomopsin A in lupin seed extract as observed by 11 laboratories*

Laboratory	Response matrix/Response solvent (%)
lab01	87%
lab02	91%
lab03	107%
lab04	91%
lab05	140%
lab06	122%
lab07	203%
lab08	50%
lab09	128%
lab10	74%
lab11	89%

4.3 Identification parameters

Phomopsin A in samples is identified based on its retention time and response obtained for the two transitions measured. Retention time and the ratio of the peak areas obtained in the extracted ion chromatograms for samples should match with those of the calibrants. The retention time of phomopsin A in samples and calibrants (both in extract and solvent) were generally very consistent: differences from average were typically within 0.05 min and virtually always within 0.1 min, within the sequence measured by the laboratory. The average ion ratio of the two product ions as observed by the different laboratories varied from 0.44 to 0.92. Within the sequence of a laboratory, the ion ratio was consistent across the investigated concentration range, as can be seen from Table 3. With one exception, all individual ion ratios obtained for phomopsin A in the test materials were within ±30% (relative) of the average value which was taken as reference.

Table 3 *Ion ratios of phomopsin A as observed by 11 laboratories*

Laboratory	Average ratio*	RSD	Minimum	Maximum
lab01	0.58	6%	0.49	0.65
lab02	0.58	11%	0.34	0.67
lab03*	0.44	18%	0.31	0.65
lab04	0.75	7%	0.64	0.89
lab05	0.45	17%	0.32	0.66
lab06	0.80	4%	0.73	0.87
lab07	0.54	7%	0.48	0.64
lab08	0.93	8%	0.66	1.07
lab09	0.52	6%	0.48	0.62
lab10*	0.92	6%	0.86	1.07
lab11	0.56	3%	0.54	0.61

* Average ion ratio calculated using the individual injections of all standards and sample extracts, except for the lowest concentrations of lab03 and lab10 because of low response for (1 of) the product ions.

4.4 Recovery

Since no certified reference material was available for phomopsin A in lupin seeds or other matrices, no trueness could be determined and recovery determinations were done instead. Lupin seeds and crisp bread were spiked by each laboratory by quantitatively transferring the content of vials provided by the organiser to 5 gram of sample. The spike levels obtained this way were 10 and 15 µg/kg, respectively. The recoveries obtained by the laboratories are shown in Table 4. For crisp bread it cannot be excluded that that recovery values are partially affected by a different degree in matrix

effect between the calibrants prepared in blank lupin seeds and the crips bread sample. The acceptable average recovery indicates that the bias because of this seems minor, although it might have contributed to a higher interlaboratory RSD.

Table 4 *Recovery obtained after analysis of spiked samples*

Laboratory	Lupin seed (10 µg/kg)	Crisp bread (15 µg/kg)
lab01	63%	94%
lab02	103%	100%
lab03	52%	87%
lab04	77%	76%
lab05	85%	73%
lab06	52%	92%
lab07	79%	106%
lab08	98%	142%
lab09	78%	149%
lab10	99%	138%
lab11	100%*	113%
Average	**81%**	**106%**
RSD%	**23%**	**25%**

* Corrected for lower amount spiked due to partial loss of vial content.

4.5 Interlaboratory reproducibility and HorRat values

All individual results and statistical evaluation of the blind duplicate samples are included in Annex 5. Statistical evaluation was performed according to AOAC guidelines 'appendix D' [AOAC 2002].

In Table 5 the interlaboratory reproducibility (RSD_R) and the HorRat values, based on a predicted RSD_R according to Thompson (2000) of 22%, are presented. According to [AOAC 2002], HorRat values should typically fall within the range 0.5-1.5 (acceptable up to 2.0). The HorRat values obtained for this interlaboratory validation study varied from 0.4-1.2 and are within this range.

With the statistics used, one outlier (lab05) was identified in the data set for lupin flour. Excluding this laboratory from the data set resulted in lower RSD_R and HorRat values, i.e. similar to those of the crisp bread and lupin seeds (higher concentration).

The interlaboratory reproducibility ranged from 10 to 26%. The lowest level was relatively close to the limit of detection for a number of laboratories, which is the most likely explanation for the higher RSD_R obtained in that case.

Table 5 *Interlaboratory reproducibility (RSD_R) and HorRat values for phomopsin A as obtained in this interlaboratory validation*

	Lupin seeds		Lupin flour	Crisp bread
Overall mean (µg/kg)	6.82	62.4	12.0	16.4
RSD_R (%)	26	10	18 (12**)	10
HorRat value*	1.2	0.4	0.8 (0.6**)	0.5

* Based on predicted RSDR of 22% (Horwitz modified by Thompson).

** Between brackets values when excluding lab05 which was identified as outlier.

5 Conclusion

An interlaboratory validation was carried out for the determination of phomopsin A in lupin seeds and lupin-derived products. HorRat values between 0.4-1.2 were obtained for test materials containing phomopsin A in the range 6.8-62 µg/kg. With interlaboratory reproducibilities in the range 10-26%, the method is considered fit-for-purpose.

Besides precision parameters, other performance parameters were assessed. The selectivity of the method was adequate, i.e. no peaks at the retention time of phomopsin A were observed in blank samples by any of the laboratories. The average recovery in lupin seeds (10 µg/kg) and crisp bread (15 µg/kg) were 81% and 106%, respectively.

The identification parameters retention time and ion ratio were consistent. Within the sequence, the individual values varied less than ±0.1 min and less than ±30% (rel) from the average, respectively.

Matrix effects observed by the different laboratories/instruments varied from minor/insignificant to strong (factor 2, suppression and enhancement). This means that laboratories applying this method need to establish the matrix effects for their instrument/conditions in order to address matrix effects for proper quantification. Although in this study, matrix-matched calibration using lupin seed extracts seems suited also for lupin flour and the crisp bread sample, this might be different for other matrices and other instruments, and exact matrix-matching or alternative quantification approaches (e.g. standard addition) might be required to achieve equivalent performance.

Acknowledgement

Dr. Jeremy Allen from the Department of Agriculture and Food, Perth, Australia is greatly acknowledged for providing naturally contaminated lupin seeds.

The staff of the laboratories participating in this study (see Annex 3) are acknowledged for their time and efforts to set up the method in their laboratory and for carrying out the analyses for this interlaboratory validation.

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Annex 1 Analysis method for determination of Phomopsin A in Lupin and Lupin-derived products

CEN/TC 275/WG5 M520 item 10

Determination of phomopsin A in lupin seeds and lupin-derived products by HPLC-MS/MS

Version 12.04.2016

CEN/TC 275/WG5 M520 item 10

Determination of phomopsin A in lupin seeds and lupin derived products by HPLC-MS/MS

1 Scope

This proposed standard describes an LC-MS/MS-based method for the determination of phomopsins in lupin seeds and lupine containing foodstuffs. Several phomopsins exist, i.e. phomopsin A, B, C and D, but the method only deals with the quantitative measurement of phomopsin-A due to lack of commercially available analytical reference standards for the other phomopsins. The target working range is 5-60 µg/kg.

2 Normative reference

This International Standard incorporates, by dated or undated reference, provisions from other publications. These normative references are cited at the appropriate places in the text and the publications are listed hereafter. The following referenced documents are indispensable for the application of this document. For dated references, only the edition cited applies. For undated references, the latest edition of the referenced document (including any amendments) applies.

EN ISO 3696:1995, Water for analytical laboratory use – Specification and test methods (ISO 3696:1987)

CEN/TR 15298:2006, Foodstuffs - Sample comminution for mycotoxins analysis - Comparison between dry milling and slurry mixing.

3 Principle

The phomopsins are extracted from the homogenised sample material by shaking with a mixture of acetonitrile/water/acetic acid 80/19/1 (v/v). After centrifugation, an aliquot of the extract is diluted with water, optionally filtered, and analysed by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS). Multi-level matrix-matched calibration is used for quantification.

4 Reagents

WARNING The method described in this standard implies the use of reagents that pose a hazard to health. The standard does not claim to address all associated safety problems. It is the responsibility of the user of this standard to take appropriate measures for the health and safety protection of the personnel prior to use of the standard and to ensure that regulatory and legal requirements are complied with.

4.1 Water (deionised)

4.2 Water (LC-MS grade, double-distilled or water of grade 1 as defined in EN ISO 3696:1995)

4.3 Acetonitrile (p.a.)

4.4 Methanol (LC-MS grade)

4.5 Acetic acid (98-100%)

4.6 Ammonium formate (p.a.)

4.7 Extraction solution acetonitrile/water/acetic acid (80/19/1) (v/v/v)

Mix 800 mL acetonitrile (4.3), 190 ml water (4.1 or 4.2) and 10 ml acetic acid (4.4) in a bottle of 1000 ml. This solution is stored at room temperature and can be used for 3 months.

4.8 Phomopsin A, isolated from Phomopsis leptostromiformis **<not needed for the interlab study>**

4.9 Phomopsin A stock solution (500 mg/L) <not needed for the interlab study>

Accurately weigh between 5 and 6 mg of the phomopsin-A standard (4.8) into an amber-coloured glass bottle of 30 mL. Add a volume of methanol (4.4) to produce a solution with a concentration of 500 mg/L. Take into account the weight and the purity of the standard.

4.10 Standard solution of phomopsin-A (10 mg/L) <this solution will be provided>

Pipette 100 µL of the standard solution 4.9 (500 mg/L) into a calibrated volumetric flask of 5 ml and make up the volume with methanol (4.4).

4.11 Standard solution of phomopsin-A (250 µg/L)

Pipette 250 µL of the standard solution 4.10 (10 mg/L) into a calibrated volumetric flask of 10 mL and make up to the volume with methanol (4.4).

4.12 Intermediate solutions to be used for preparation of the matrix-matched standards

To seven glass vials (5.10) add different volumes of the of standard solution phomopsin-A (250 µg/L (4.11) and methanol (4.4) according to Table 1. Close with screw cap and mix. These solutions are freshly prepared for each batch of analysis.

NOTE: Once it has been shown that there is linearity, the number of levels may be adjusted to local needs and requirements.

Table 1. Intermediate standard solutions of phomopsin-A in methanol.

Intermediate solution	Volume (µL) of standard solution 4.11 (250 µg/L)	Volume (µL) of methanol	Concentration obtained (µg/L)
1	25	975	6.25
2	50	950	12.5
3	100	900	25
4	200	800	50
5	350	650	87.5
6	500	500	125
7	650	350	162.5

5 Apparatus

General laboratory glassware and equipment, in particular the items listed below.

5.1 Mill

Single mill or multiple mills capable of comminuting test materials to particle sizes of < 0.5 mm.

5.2 Conical polypropylene screw cap centrifuge tubes, 50 mL with caps

5.3 Volumetric flasks

5 and 10 mL

5.4 Analytical balance with a precision of 0,01 mg

5.5 Laboratory balance with a precision of 0,01 g

5.6 Pipettors

Adjustable, e.g. 10-100 and 100-1000 µL, suited for organic solvents (e.g. positive displacement pipettors), properly calibrated, with appropriate tips

5.7 Adjustable mechanical vertical or horizontal shaker or rotary tumbling machine

5.8 Vortex mixer

5.9 Centrifuge, capable of generating a relative centrifugal force of 3500 g

5.10 Vials (1.5-2 mL)

Used for intermediate solutions 4.12, (deactivated) glass vials or polypropylene vials, with screw cap

5.11 Syringe filter, 0,20-0,45 µm, nylon or PTFE (for optional filtration of final extracts)

5.12 Auto sampler vials

of appropriate size for the auto sampler in use, e.g. glass with insert vials, filter vials (polytetrafluoroethylene (PTFE) 0,45 µm), with crimp cap or equivalent

5.13 LC-MS/MS system consisting of:

5.13.1 Auto Liquid Sampler, capable of injecting an appropriate volume of injection solution with sufficient accuracy, and cross-contamination below 0,1%.

5.13.2 Solvent delivery system, capable of delivering a binary gradient at flow rates appropriate for the analytical column in use with sufficient accuracy.

5.13.3 Column oven, capable of maintaining a constant temperature.

5.13.4 Analytical column, capable of retaining phomopsin-A with a retention factor of at least two.

5.13.5 Mass spectrometer, capable of performing selected reaction monitoring (SRM) with a sufficiently wide dynamic range. Any ionisation source providing sufficient yield may be used.

Note: in agreement with the tender requirement, tandem MS using a triple quadrupole or quadrupole-linear ion trap (Q-trap) is required.

6 Procedure

6.1 Sample preparation

Laboratory samples should be taken and prepared in accordance with European legislation [3] where applicable or, in any other case with EN ISO 6498. The laboratory sample should be finely ground and thoroughly mixed using a mill (5.1) or another process for which complete homogenization has been demonstrated before a test portion is removed for analysis.

The recommended way is to comminute the laboratory sample in several steps. Beginning with the totality of the laboratory sample, each step consists of taking a representative aliquot of the previous step after sufficient homogenization. This aliquot is then comminuted to the next smaller particle size until a subsample of ca. 50 g of the final particle size is obtained. It is of utmost importance that the test portion is taken from a subsample which is sufficiently homogenous with a particle size of ≤ 0.5 mm. Care should be taken to not overheat the sample during this process.

In all instances everything should be at room temperature before any kind of manipulation takes place.

6.2 Extraction

Some of the steps described below are more critical for the accuracy of the results than others. These steps are marked as such and should be carried out with the necessary attention. A scale-up of the test portion size is deemed to be acceptable if such a need is assumed. In that case the amounts of extraction solvent need to be increased at the same rate, f.i. scale-up by factor of 2: 10 g test portion, 40 mL of extraction solvent. In no way shall a scale-up be seen as replacement for proper sample preparation (6.1).

- Weigh a test portion of 4.9 to 5.1 g of the homogeneous sample into a conical polypropylene screw-cap tube (5.2), round and record the weight to the second decimal (the accuracy of this weight is critical for the accuracy of the final result!)

- [any spikes for quality control purposes (e.g. recovery determinations) are added at this point, e.g. 100 µL of 250 µg/L phomposin-A (4.11) to 5 gram sample to obtain a spike level of 5 µg/kg] For this interlab study, please follow the instructions provided.

- Add 20 mL extraction solution (4.7) to the tube (the accuracy of this volume is critical for the accuracy of the final result!), close and shake vigorously by hand or by vortexing.

- Place the tubes in a mechanical shaker (5.7) and extract during 60 minutes at a speed that ensures complete suspension and mixing of the sample into the extraction solvent liquid.

- Centrifuge the tubes during 10 minutes at 3500 g at room temperature (5.9).

- If wanted for possible repeats: transfer part of the clear extract into a clean vial for storage of up to seven days at 2°C to 10°C in the dark.

6.3 Test solutions

- Transfer 500 µL of the clear extract into a clean vial (5.10) and add 500 µL of water (4.2) (the accuracy of these volumes are critical for the accuracy of the final result!). Mix using vortexing.

NOTE: in case of filter vials: transfer 200 µL of extract and add 200 µL of water (4.2)

- Cool the extract to 2°C to 10°C in a refrigerator

- If the extract is turbid after cooling, it may be filtered through a syringe filter (4.11). Alternatively, the extract can be centrifuged.

NOTE: in case of filter vials, filtration is inherent to use of these vials by pressing the filter unit down into the vial

6.4 Calibration standards

In LC-MS/MS, sample-dependent matrix effects (signal suppression/enhancement) may occur. Unless it has been demonstrated that matrix effects are not significant for the matrix analysed and the instrument used, these matrix effects need to be compensated for. Ideally this is done by use of an isotopically labelled standard. However, for phomopsin-A this standard is not yet available. For this reason, quantification is performed using matrix-matched standards. These are prepared by addition of a small volume of the intermediate phomopsin-A standard solutions (4.12) to a blank extract.

For the samples of the interlaboratory trial milled lupin seed, free of phomopsins, is used as blank sample. Prepare the blank extract as described in 6.2. Then prepare matrix-matched calibration standards in vials (5.10) according to Table 2.

Table 2. Calibration solutions of phomopsin-A in blank matrix extract.

#	Calibration solution in matrix, µg/L	Blank extract (6.2)	Phomopsin-A intermediate solutions (4.12)	Water (4.2)	Equivalent to concentration in sample
0	0	500 µL	-	500 µL	0 µg/kg
1	0.3125	450 µL	50 µL 4.11 **(1)** 6.25 µg/L	500 µL	2.5 µg/kg
2	0.625	450 µL	50 µL 4.11 **(2)** 12.5 µg/L	500 µL	5.0 µg/kg
3	1.25	450 µL	50 µL 4.11 **(3)** 25 µg/L	500 µL	10.0 µg/kg
4	2.5	450 µL	50 µL 4.11 **(4)** 50 µg/L	500 µL	20.0 µg/kg
5	4.375	450 µL	50 µL 4.11 **(5)** 87.5 µg/L	500 µL	35.0 µg/kg
6	6.25	450 µL	50 µL 4.11 **(6)** 125 µg/L	500 µL	50.0 µg/kg
7	8.125	450 µL	50 µL 4.11 **(7)** 162.5 µg/L	500 µL	65.0 µg/kg

NOTE: the calibration standards can also be prepared directly in auto sampler vials with insert or filter vials, in that case, the volumes indicated in Table 2 can be proportionally reduced.

7 Measurement

The LC-MS system must meet the requirements laid out in 5.13.

The LC-MS system, injection volume, mobile phase composition and gradient, acquisition conditions, should be such that a gausian peak is obtained and that phomopsin-A in the 0.31 µg/L calibration solution (6.4 #1 in Table 2) is detected with a S/N≥6 for the qualifier ion.

7.1 LC conditions

Choose an analytical column, mobile phase, gradient settings, and injection volume should be such that the requirement laid out in 5.13.4 is met and a Gaussian peak is obtained (for an example see Annex B).

7.2 MS conditions

Choose an ion source with sufficient ionisation yield for phomopsin-A and ion source conditions such that a stable spray is obtained.

Choose an appropriate precursor ion (under most conditions the protonated molecule in positive mode) and select two products ions in the MS/MS spectrum. Set up SRM transitions with these precursor/product ion combinations. For an SRM example, see Annex B.

7.3 Batch composition / analysis sequence

Always start a batch of measurements with a reagent blank run to prove non-contamination of the system. Then inject the calibration solutions, followed by a reagent blank to check for possible carry-over. Subsequently inject the test solutions. For larger batches of samples, inject calibration standard 6.4 (#4) (2.5 µg/L) after every ~10 samples. At the end of the batch, re-inject the calibration series.

<For the interlab study, the intermediate solution 4.12 (1) 6.25 µg/L is included in the sequence. Please use the following sequence for injection (this sequence list is included in the Excel report file>:

1 reagent blank
2 6.4 cal 0 in lupin matrix 0
3 6.4 cal 1 in lupin matrix 0.3125
4 6.4 cal 2 in lupin matrix 0.625
5 6.4 cal 3 in lupin matrix 1.25
6 6.4 cal 4 in lupin matrix 2.5
7 6.4 cal 5 in lupin matrix 4.375
8 6.4 cal 6 in lupin matrix 6.25
9 6.4 cal 7 in lupin matrix 8.125
10 int. solution 4.12 (1) 6.25 µg/L
11 reagent blank
12 material 1
13 material 2

14 material 3
15 material 4
16 material 5
17 material 6
18 6.4 cal 4 in lupin matrix 2.5
19 material 7
20 material 8
21 material 9
22 material 10
23 material spike with vial A (11)
24 material spike with vial B (12)
25 int. solution 4.12 (1) 6.25 µg/L
26 6.4 cal 7 in lupin matrix 8.125
27 6.4 cal 6 in lupin matrix 6.25
28 6.4 cal 5 in lupin matrix 4.375
29 6.4 cal 4 in lupin matrix 2.5
30 6.4 cal 3 in lupin matrix 1.25
31 6.4 cal 2 in lupin matrix 0.625
32 6.4 cal 1 in lupin matrix 0.3125
33 6.4 cal 0 in lupin matrix 0

7.4 Determination of phomopsin-A in calibration and test solutions

Process the data using the appropriate integration software. Peak areas will be used for all subsequent calculations. For peak area determination, integrate the extracted ion current for both transitions. Check peak assignment and integration and adjust if needed. In case noise is integrated, delete the 'peak'.

<for this interlab validation: after checking correct integration, for each injection, copy retention time and peak area of both transitions into the Excel reporting sheet>

7.5 Phomopsin-A identification

Identification of phomopsin-A is in the test solutions is based on matching retention time and ion ratio. Phomopsin-A is considered identified when:

a) the retention time of the peak observed for the test solution differs less than 0.1 min from the average retention time as calculated from the calibration solutions

b) the ratio of the area of the two transitions (lowest area/highest area) of the peaks observed for the test solution deviates less than 30% (relative) from the average ion ratio of the calibration standards.

NOTE: for calculation of the reference ion ratio only responses with an S/N>10 are used. For the higher concentrations: peak areas exceeding the linear range must be excluded from calculation of the reference ion ratio.

<the above identification criteria are default criteria, not to be used for this interlab validation (for the purpose of this study: do not exclude any data when not compliant with the default criteria>

7.6 Calibration

Plot the peak areas of the quantifier (Y-axis) of all individual calibration solutions (6.4, cal 0 to 7) against the corresponding concentrations (µg/L) (X-axis). The quantifier is the transition which, overall, gives the best S/N. Do not use means of multiple injections. Construct a calibration curve using (weighted) least-square regression. The calibration curve is fit-for-purpose when the calculated concentration for the individual calibration solutions using the calibration equation are within 20% of the actual concentration in the range 5-65 µg/kg. If higher deviations/non-linearity is observed, identify the cause and, if necessary, re-run the analyses.

8 Determination of the concentration phomopsin-A in the sample

Calculate the concentration of the analyte in the sample, using linear regression, as follows:

$$C_{sample} = \left(\frac{A_{sample} - Intercept}{Slope} \right) x \frac{V_{extr}}{Sample\ weight} x\ D$$

Where:

C_{sample} = concentration of the analyte in the sample (in µg/kg)

A_{sample} = area of the quantifier ion of phomopsin-A in the sample

Intercept = intercept, estimated with (weighted) least square regression from calibration data

Slope (area/µg/L) = slope, estimated with (weighted) least-square regression from calibration data

Sample weight = sample weight in gram (4.9-5.1 gram)

V_{extr} = volume of extraction solvent (20 mL)

D = dilution of the initial extract (default is 2)

9 Precision

9.1 Interlaboratory study

Details of an interlaboratory study on the precision of the method will be shown in <ILV report>. The values derived from this interlaboratory study may not be applicable to concentration ranges and/or matrices other than those stated.

9.2 Repeatability

<to be added after the study>

9.3 Reproducibility

<to be added after the study>

10 Test report

The test report shall contain at least the following data:

a) information necessary for the identification of the sample

b) a reference to this European Standard

c) the date of sample receipt

d) the test results and the units in which they have been expressed; where necessary the recovery shall be stated along with the test results and whether the test results were corrected with those recoveries.

Annex A (informative)

Precision data

<to be inserted after completion of interlab study>

Annex B (informative)

LC-MS/MS conditions

An LC-MS system consisting of a Shimadzu DGU-20A3 degasser, Shimadzu SIL-20AC XR auto sampler, a Shimadzu LC-20AD pump, a Shimadzu CTO-20A column oven, and a Sciex API 5500 Qtrap tandem mass spectrometer with an ESI interface, and the settings below, have shown to satisfy the performance requirements and provide overall acceptable results

B.1.1 LC conditions

- Vial tray temperature	10°C
- Injection volume	10 µL
- Analytical column	Atlantis T3, 3 µm, 100 x 3 mm ID
- Temperature column thermostat	35°C
- Flow rate	0,4 mL/min.
- Mobile phase A	5 mM Ammonium formate (4.6) in water (4.2) (pH 5)
- Mobile phase B	5 mM Ammonium formate (4.6) in methanol/water 95/5 (v/v) (4.4/4.2)

Table B.1: Gradient program

Time (min)	Mobile phase A (%)	Mobile phase B (%)
0	90	10
1	90	10
9	0	100
11	0	100
11.1	90	10
15	90	10

B.1.2 MS conditions

The conditions given below have been optimised for the Sciex 5500 Qtrap mass spectrometer and should be used as guidelines for this particular instrument under the LC conditions specified under B.1.1.

Table B.2: MS settings for Sciex 5500 Qtrap

Tune parameters product scan measurements	*Q trap*
Scan type	MRM
Ionization mode	ESI+
Ion Source	Turbo spray
Resolution Q1	Unit
Resolution Q2	Unit
Setting Time	5 msec
MR Pause	5 msec
Dwell Time	500 msec
Curtain Gas (CUR)	20 psig
Collision Gas (CAD)	Medium
Source temperature (°C)	500
Ion Source Gas 1 (GS1)	60 psig
Ion Source Gas 2 (GS2)	50 psig
Ionspray Voltage	3000 V
Entrance Potential (EP)	10 V
Declustering Potential (cone voltage)	90 V
Optimalization for TIS probe; Vertical Y axis	2.5 mm
Optimalization for TIS probe; Horizontal X axis	6 mm

Suitable MS/MS transitions for phomopsin-A:

component	$[M+H]^+$	Product ion	CE* (eV)	CXP**(V)
Phomopsin A	789.2 ± 0.5	<u>226.1</u> ± 0.5	47	16
Phomopsin A	789.2 ± 0.5	323.1 ± 0.5	35	22

* CE Collision Energy, ** Cell Exit Potential

The product ion with the highest intensity is underlined.

NOTE: any tradenames, trademarks, product names and/or suppliers mentioned above are only there for the convenience of the users of this International Standard and do not constitute an endorsement by CEN of the products named. Equivalent products may lead to the same results.

Bibliography

[1] EN ISO 3696, Water for analytical laboratory use – Specification and test methods (ISO 3696:1987).

[2] CEN/TR 15298:2006, Foodstuffs - Sample comminution for mycotoxins analysis - Comparison between dry milling and slurry mixing.

[3] European Commission. Commission Regulation (EC) No 401/2006 of 23 February 2006 laying down the methods of sampling and analysis for the official control of the levels of mycotoxins in foodstuffs (Text with EEA relevance). Official Journal of the European Union, 2006. L 70: p. 12–34.

Annex 2 Study instructions

CEN/TC 275/WG5 M520 item 10

Determination of phomopsin A in lupin seeds and lupin-derived products by HPLC-MS/MS

Dear Participant,

Thank you for your collaboration in this inter-laboratory validation.

Please read this document and the method protocol before starting the analysis!

For this ILV you will receive the following materials from RIKILT (Wageningen, Netherlands):

Description	amount	storage conditions
Phomopsin blank material for cal standard	~6 gram	RT, dark
Phomopsin material 1	~6 gram	RT, dark
Phomopsin material 2	~6 gram	RT, dark
Phomopsin material 3	~6 gram	RT, dark
Phomopsin material 4	~6 gram	RT, dark
Phomopsin material 5	~6 gram	RT, dark
Phomopsin material 6	~6 gram	RT, dark
Phomopsin material 7	~6 gram	RT, dark
Phomopsin material 8	~6 gram	RT, dark
Phomopsin material 9	~6 gram	RT, dark
Phomopsin material 10	~6 gram	RT, dark
Phomopsin Spike with vial A (11)	~6 gram	RT, dark
Phomopsin Spike with vial B (12)	~6 gram	RT, dark
Phomopsins calibrant 10.0 µg/mL (mg/L) 4.10	~1 mL	≤ -18°C
Phomopsins spike vial A	~1 mL	≤ -18°C
Phomopsins spike vial B	~1 mL	≤ -18°C

Upon receipt, please check that the content in the package is complete and not damaged, and store the samples and the vials as noted under storage conditions.

Upon shipment, you will receive an email from hans.mol@wur.nl that the package is underway. Please reply to this email to confirm the receipt of the package in good shape, or to report any damage or missing content.

For this ILV, note the following:

- The method protocol must be followed, any deviations should be recorded in the 'general info sheet' of the Excel reporting file.

- Weigh the vials standard solution, spike A and spike B and record the weight on the sheet 'general info' of the Excel reporting file.

- Provide the LC-MS/MS details on the sheet 'general info' of the Excel reporting file.

- The sample tubes contain approx. 6 gram of material. For analysis, weigh 4.9-5.1 gram into your own extraction tube and record the weight in the sheet 'reporting sheet' of the Excel reporting file

- In total 14 extracts need to be prepared:

*Reagent blank: for this use an empty extraction tube from your laboratory and proceed as indicated in 6.2 without weighing in any sample

*Blank extract for preparation of standards in matrix: for this use the material provided in the tube labelled 'Phomopsin blank material for cal standard'. Weigh 4.9-5.1 gram into your extraction tube and proceed as in indicated in 6.2/6.4.

*Test materials: samples in tube 1 to 12

Test material 'Phomopsin Spike with vial A' (11) and 'Phomopsin Spike with vial B' (12) need to be spiked with the spiking solutions provided:

Weigh 4.9-5.1 g of test material (11) into your extraction tube. Quantitatively transfer the content of vial 'Phomopsins spike vial A' to the your extraction tube. This is done by emptying the content of the vial into the tube and rinsing the vial with part of the extraction solvent. For example: measure 20.0 mL of extraction solvent into a calibrated cylinder. Transfer ca. 2 mL into the, now empty, vial, close with cap and shake, empty into the extraction tube. Repeat two more times. Add the remaining extraction solvent from the cylinder to the extraction tube.

For test material 12 the same is done, but in this case the content of vial 'Phomopsin spike vial B' is added to your extraction tube in which to weighed 4.9-5.1 g of material (12).

- Before analysing the samples, check instrument sensitivity for phomopsin-A by injection of the calibration solutions 6.4 #1. The S/N of the quantifier should be ≥6.

- Inject the samples according to the sequence as provided in the reporting sheet of the Excel reporting file.

- After analysis, check peak assignment/integration as indicated in 7.4. For all injections, copy/paste the retention time and peak area of both transitions into the 'reporting sheet' of the Excel reporting file.

- Construct the calibration curve using your instrument software or Excel and check linearity of the response vs concentration as indicated in 7.6.

- Determine the concentration of phomopsin-A in the samples and report the values in µg/kg (3 significant figures) in the 'reporting sheet' of the Excel reporting file.

- Send the Excel reporting file to: hester.vandentop@wur.nl with cc to hans.mol@wur.nl

The deadline for submission of results is Friday 3rd June 2016.

We are looking forward for your results.

Comments to the method, questions and remarks can be send to:

Hans Mol, email: hans.mol@wur.nl

Annex 3 Participating laboratories

In total 11 laboratories participated in this interlaboratory study, 10 from Europe and one from Singapore.

Laboratory	Country
Eurofins WEJ	Germany
CODA-Cerva	Belgium
Fera Science Ltd	United Kingdom
BOKU Tulln	Austria
HSE	Ireland
JRC	Belgium
Czech Agriculture and Food Inspection Authority	Czech Republic
Central Institute for Supervising and Testing in Agriculture (UKZUZ)	Czech Republic
Health Sciences Authority Singapore	Singapore
NVWA	The Netherlands
RIKILT	The Netherlands

Annex 4 Homogeneity study results

Homogeneity Lupin seeds 5 µg/kg target level

	replicate 1	replicate 2
1	**6.8**	**6.3**
2	**6.5**	**5.2**
3	**5.3**	**4.7**
4	**5.3**	**4.9**
5	**6.5**	**6.0**
6	**5.7**	**5.5**
7	**5.7**	**5.3**
8	**5.4**	**5.6**
9	**5.8**	**5.5**
10	**5.9**	**5.4**
Cochrans test		
C		0.513678
ccrit		0.602
c<ccrit		NO OUTLIERS
target		Horwitz / Thompson 1.25
sx		0.47
sw		0.41
ss		0.37
critical		0.37
ss,critical		ACCEPTED
sw<0.5sigmah		ACCEPTED

Homogeneity Lupin seeds 50 µg/kg target level

	replicate 1	replicate 2
1	**53.8**	**43.9**
2	**45.8**	**44.9**
3	**46.4**	**54.7**
4	**51.3**	**53.5**
5	**50.1**	**51.5**
6	**46.0**	**43.6**
7	**49.4**	**46.6**
8	**50.1**	**62.3**
9	**47.3**	**55.0**
10	**47.7**	**46.4**
Cochrans test		
C		0.374036
ccrit		0.602
c<ccrit		NO OUTLIERS
target		Horwitz / Thompson 10.89
sx		3.44
sw		4.46
ss		1.39
critical		3.27
ss,critical		ACCEPTED
sw<0.5sigmah		ACCEPTED

Homogeneity Lupin flour 15 µg/kg target level

	replicate 1	replicate 2
1	**11.5**	**9.2**
2	**9.2**	**10.2**
3	**9.6**	**12.1**
4	**8.6**	**9.8**
5	**8.8**	**10.2**
6	**8.6**	**9.2**
7	**8.5**	**9.3**
8	**10.5**	**9.0**
9	**10.6**	**9.4**
10	**9.7**	**10.8**
Cochrans test		
C		0.286172
ccrit		0.602
c<ccrit		NO OUTLIERS
target		Horwitz / Thompson 2.14
sx		0.64
sw		1.04
ss		0.00
critical		0.64
ss,critical		ACCEPTED
sw<0.5sigmah		ACCEPTED

Homogeneity Crisp bread 10 µg/kg target level

	replicate 1	replicate 2
1	**11.3**	**12.1**
2	**9.6**	**10.5**
3	**10.5**	**12.9**
4	**10.2**	**10.6**
5	**13.3**	**11.2**
6	**14.2**	**13.9**
7	**12.2**	**13.2**
8	**11.0**	**11.1**
9	**12.9**	**11.7**
10	**10.9**	**10.1**
Cochrans test		
C		0.385026738
ccrit		0.602
c<ccrit		NO OUTLIERS
target		Horwitz / Thompson 2.51
sx		1.22
sw		0.86
ss		1.06
critical		0.77
ss,critical		NOT ACCEPTED
sw<0.5sigmah		ACCEPTED

Annex 5 Statistical evaluation and results

Lupin seeds, target concentration 5 µg/kg

Laboratory	Replicate 1	Replicate 2
1	5.12	4.28
2	7.32	12.58
3	6.83	8.34
4	5.82	6.44
5	5.17	6.72
6	7.41	7.19
7	6.22	6.37
8	7.23	4.39
9	6.60	6.23
10	7.48	9.87
11	6.45	5.97

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AOAC International Interlaboratory Study Workbook			***Version: 2.0***
Blind (Unpaired) Replicates			
Study Reported Values			
Seq.	Item	Symbol	Value
	Study name:		Phomopsins in Lupin seeds and products
	Study date:		22-sep-2016
	Sample ID:		seeds 5 µg/kg
1	Total number of laboratories	p	11
2	Total number of replicates	Sum(n(L))	22
3	Overall mean of all data (grand mean)	XBARBAR	6.8195
4	Repeatability standard deviation	s(r)	1.4718
5	Reproducibility standard deviation	s(R)	1.8015
6	Repeatability relative standard deviation	RSD(r)	21.58
7	Reproducibility relative standard deviation	RSD(R)	26.42
8	**HORRAT value**		**0.78**
9	**HorRat based on Thompson**		**1.20**

Lupin seeds, target concentration 50 µg/kg

Laboratory	Replicate 1	Replicate 2
1	55.42	58.46
2	66.23	73.32
3	59.16	59.5
4	61.42	56.86
5	72.52	71.8
6	61.05	56.11
7	58.72	58.77
8	54.79	59.87
9	66.81	63.71
10	65.24	73.16
11	57.79	62.87

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AOAC International Interlaboratory Study Workbook ***Version:*** ***2.0***

Blind (Unpaired) Replicates

Study Reported Values

Seq.	Item	Symbol	Value
	Study name:		Phomopsins in Lupin seeds and products
	Study date:		22-sep-2016
	Sample ID:		seed 50 µg/kg
1	Total number of laboratories	p	11
2	Total number of replicates	Sum(n(L))	22
3	Overall mean of all data (grand mean)	XBARBAR	62.4355
4	Repeatability standard deviation	s(r)	3.2284
5	Reproducibility standard deviation	s(R)	6.0330
6	Repeatability relative standard deviation	RSD(r)	5.17
7	Reproducibility relative standard deviation	RSD(R)	9.66
8	**HORRAT value**		**0.40**
9	**HorRat based on Thompson**		**0.44**

Lupin flour, target concentration 15 µg/kg

Laboratory	Replicate 1	Replicate 2
1	13.12	12.38
2	9.63	12.31
3	12.82	11.34
4	9.26	10.99
5	15.99	17.75
6	11.4	11.12
7	9.30	9.43
8	11.85	11.64
9	13.90	11.46
10	13.41	12.71
11	10.18	11.39

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AOAC International Interlaboratory Study Workbook ***Version:*** ***2.0***

Blind (Unpaired) Replicates

Study Reported Values

Seq.	Item	Symbol	Value
	Study name:		Phomopsins in Lupin seeds and products
	Study date:		22-sep-2016
	Sample ID:		Lupin flour 15 µg/kg
1	Total number of laboratories	p	11 (10)
2	Total number of replicates	Sum(n(L))	22 (20)
3	Overall mean of all data (grand mean)	XBARBAR	11.9718 (11.4820)
4	Repeatability standard deviation	s(r)	1.0457 (1.0237)
5	Reproducibility standard deviation	s(R)	2.1199 (1.4062)
6	Repeatability relative standard deviation	RSD(r)	8.73 (8.92)
7	Reproducibility relative standard deviation	RSD(R)	17.71 (12.25)
8	**HORRAT value**		**0.57 (0.39)**
9	**HorRat based on Thompson**		**0.81(0.56)**

Lab 5 was detected as outlier in the single Grubbs test. The data when excluding lab 5 are given between brackets.

Crisp bread, target concentration 10 µg/kg

Laboratory	Replicate 1	Replicate 2
1	14.79	14.51
2	17.78	15.36
3	14.79	15.72
4	17.21	16.32
5	14.87	16.29
6	13.84	14.94
7	14.86	19.28
8	20.47	16.22
9	15.97	17.96
10	19.09	16.59
11	16.85	17.02

AOAC International Interlaboratory Study Workbook			***Version: 2.0***
Blind (Unpaired) Replicates			
Study Reported Values			
Seq.	Item	Symbol	Value
	Study name:		Phomopsins in Lupin seeds and products
	Study date:		22-sep-2016
	Sample ID:		Crispbread 10 µg/kg
1	Total number of laboratories	p	11
2	Total number of replicates	Sum(n(L))	22
3	Overall mean of all data (grand mean)	XBARBAR	16.3968
4	Repeatability standard deviation	s(r)	1.6328
5	Reproducibility standard deviation	s(R)	1.7173
6	Repeatability relative standard deviation	RSD(r)	9.96
7	Reproducibility relative standard deviation	RSD(R)	10.47
8	**HORRAT value**		**0.35**
9	**HorRat based on Thompson**		**0.48**

Annex 6 Example chromatograms

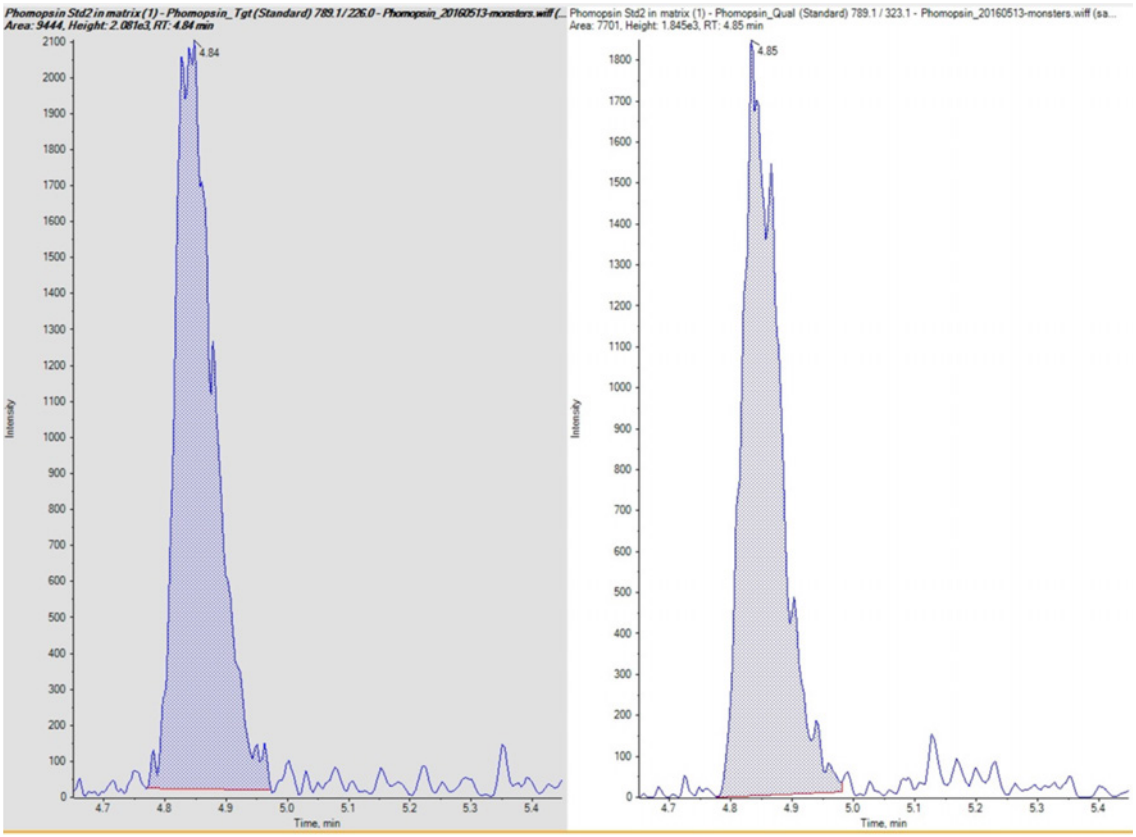

Figure 1 *Example extracted ion chromatograms of a calibration standard of phomopsin A in lupin seed extract at a concentration corresponding to 5 µg/kg.*

Column: 100 x 2.1 mm i.d., 1.8 µm HSS T3; 40°C
Injection volume: 5 µL
Gradient: water/methanol, 5 mM ammonium formate/0.5% (v/v) formic; 0.40 mL/min
MS/MS: Sciex 6500 Qtrap, ESI^+

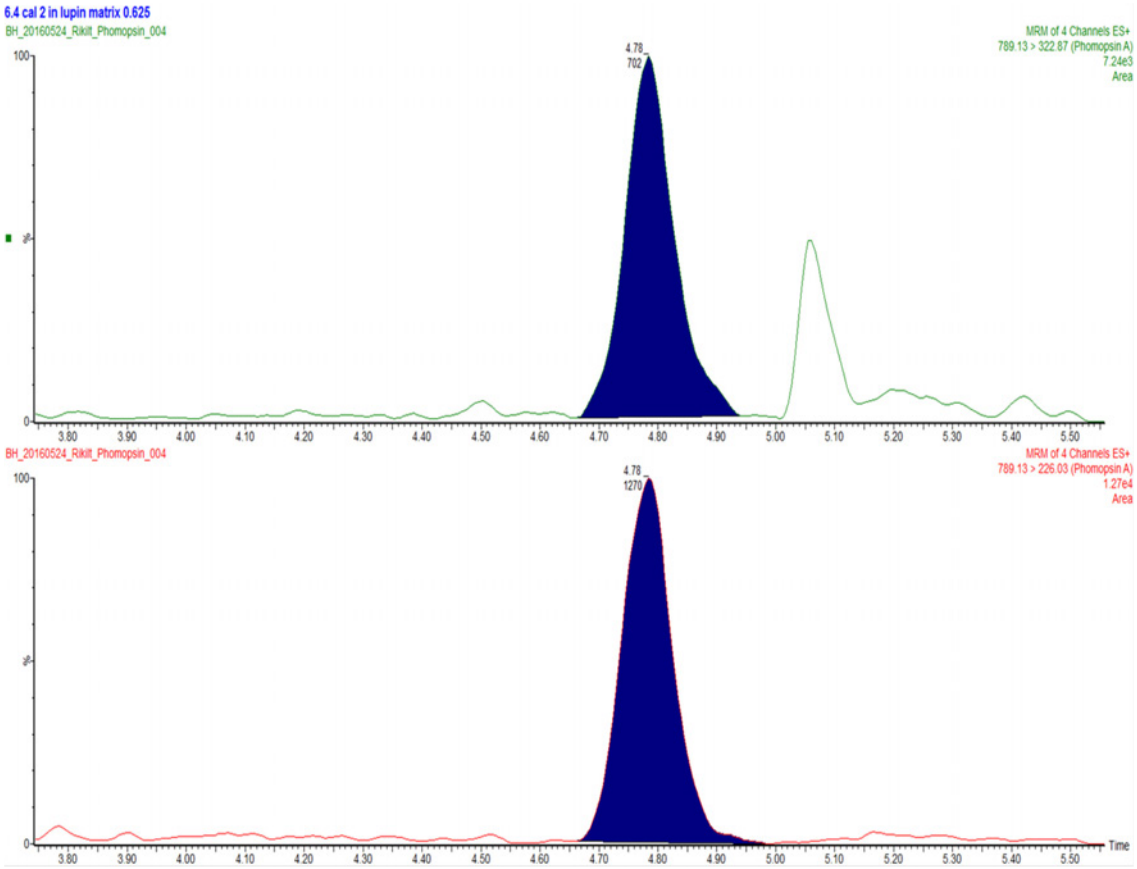

Figure 2 *Example extracted ion chromatograms of a calibration standard of phomopsin A in lupin seed extract at a concentration corresponding to 5 µg/kg.*

Column: 100 x 4.6 mm i.d., Phenomenex Kinetex 2.6 µm XB-C18; 40°C
Injection volume: 10 µL
Gradient: water/methanol, 5 mM ammonium acetate/0.05% (v/v) acetic acid formate; 1.0 mL/min
MS/MS: Waters Xevo TQ-S, ESI^+

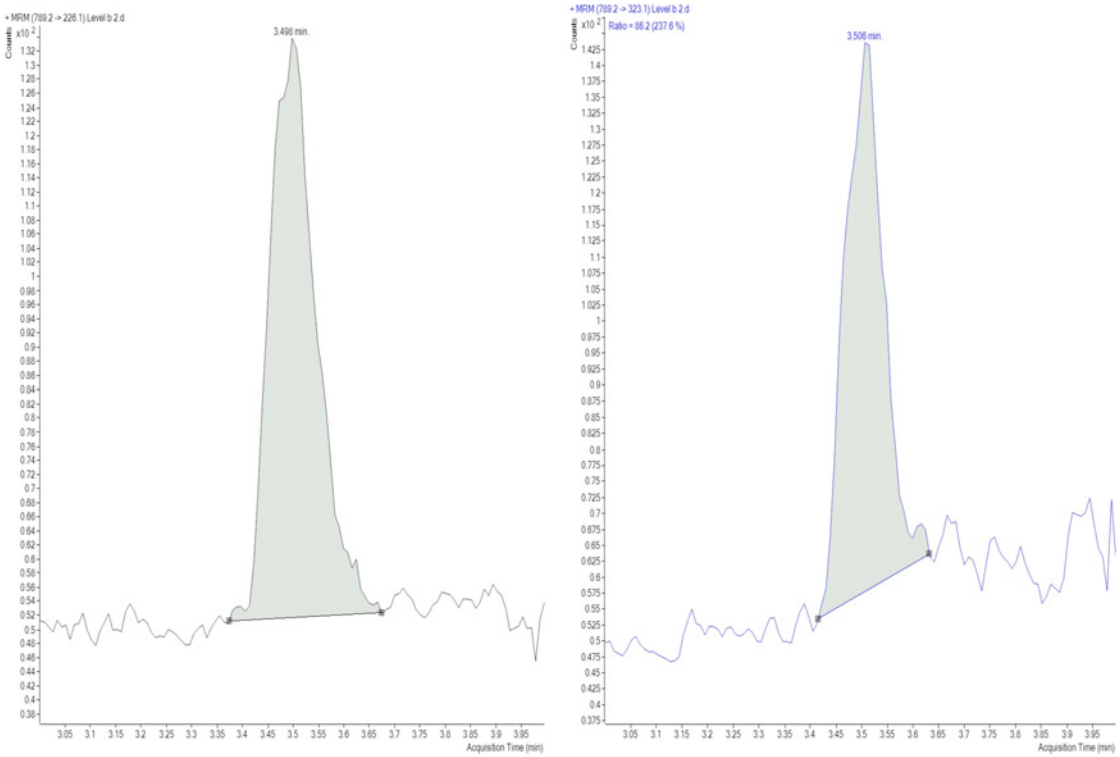

Figure 3 *Example extracted ion chromatograms of a calibration standard of phomopsin A in lupin seed extract at a concentration corresponding to 5 µg/kg.*

Column: 100 x 2 mm i.d., 2.7 µm MN Nucleoshell RP 18 plus; 30°C
Injection volume: 10 µL
Gradient: water/methanol, 5 mM ammonium formate/0.1% (v/v) formic acid; 0.35 mL/min
MS/MS: Agilent 6490, ESI^+

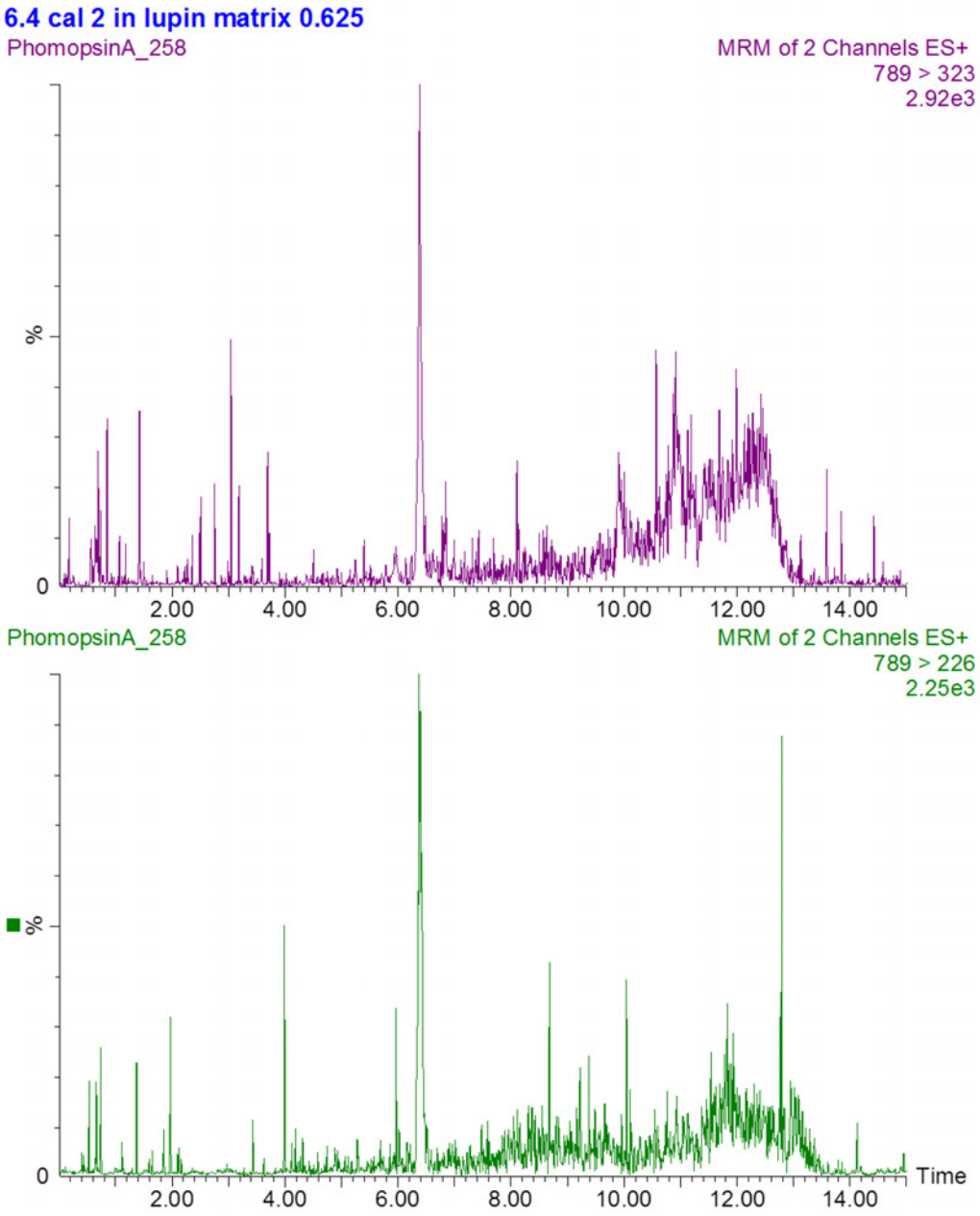

Figure 4 *Example extracted ion chromatograms of a calibration standard of phomopsin A (6.4 min) in lupin seed extract at a concentration corresponding to 5 µg/kg.*

Column: 100 mm x 2.1 mm i.d., 2.7 µm Supelco Ascentis Express C18; 45°C
Injection volume: 10 µL
Gradient: water/methanol, 5 mM ammonium formate; 0.40 mL/min
MS/MS: Micromass Quattro Ultima Pt, ESI^+

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