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Phomopsin A, lupin-containing food, method development, UHPLC-MS/MS

Abstract

Phomopsin A, a secondary metabolite of the mold Diaporthe toxica, showed severe toxic effects in animal studies with potential relevance for human health. This lead to the establishment of a legal maximum amount for phomopsin A of 5 µg/kg in lupin seeds and their products by the Australian and New Zealand Food Authority (2001) as well as by the Food and Agriculture Organisation (2004).

We therefore developed a sensitive method for the determination of phomopsin A in lightly processed matrices like lupin flour with a limit of quantification (LOQ) of 1.4 µg/kg. The method involves a simple acetonitrile/methanol/water-extraction (3/1/1, v/v/v) without any clean-up step (“dilute & shoot”), followed by UHPLC-MS/MS analysis. Five matrices (lupin flour, lupin steak, lupin coffee, soy flour and wheat flour) were validated in accordance with the criteria set forth in EU-guideline SANCO/12495/2011 for the presented method. Sufficient recoveries and

variation coefficients (cv) for lupin flour at the spiking levels of 1.4 µg/kg (LOQ) with 73 % (cv 4.9 %) and 7.1 µg/kg (5-fold LOQ) with 75 % (cv 4.3 %) were obtained. Wheat flour also showed sufficient results regarding the recovery and the LOQ, while unsatisfactory LOQ's were obtained for lupin steak, lupin coffee and soy flour.

A market survey of twenty-five relevant foods showed that no current contamination of European food with phomopsin A seems to be detectable.

Introduction

Phomopsins are a family of toxic metabolites produced by the mold *Diaporthe toxica* (formerly *Phomopsis leptostromiformis*) [1, 2]. The main host of this mold is the genus of lupins in the legume family, which are used for feed (mainly the stubbles) and food (seeds) [1, 2, 3]. For human nutrition, the lupin seeds are processed to lupin flour, meat surrogates, plant milk, yogurt and cheeses [3, 4]. Therefore, they are used for vegan or allergen free nutrition. Other plant-based food, such as grapes, aubergines, soybeans, chestnuts and mangos could be contaminated with further *Diaporthe*-subspecies as well [5]. It has not yet been examined if these *Diaporthe*-subspecies produce phomopsins or if the particular food is contaminated with phomopsins [5].

Wood and Petterson (1986) asserted that up to 20 % of the harvested lupin seeds in Australia are contaminated with *D. toxica* [6]. Precise conditions for the growth of the mold and the production of its toxin on crops are not known [7]. A higher rate of contamination from *D. toxica* and an increase in the production of phomopsins has so far only been observed with precipitation [7].

Phomopsin A is the most frequently detected mycotoxin of the phomopsin family [1, 2]. Phomopsin A is a macrocyclic hexapeptide, containing rare amino acids, such as N-methyl-3-(3'-chloro-4', 5'-dihydroxyphenyl)serine (**Figure 1**) [8]. It was shown that phomopsin A is sensitive to acidic pH-values, resulting in hydrolysis [9]. In addition, it remains stable during food processing such as heating, e. g. during the baking process [10].

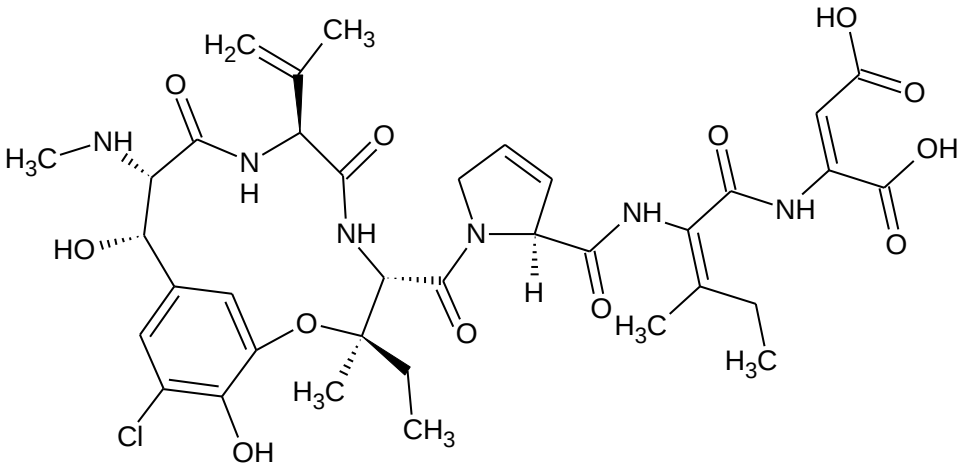

Figure 1: Chemical structure of phomopsin A.

So far, no verified experimental data concerning the toxicokinetics of phomopsins are available [11]. Knowledge about its prevalence in organisms has been derived only from observed effects: oral transmission of the phomopsins causes cytotoxic effects in the liver (associated with the sheep disease “Lupinosis”) and in the kidneys [12, 15, 16, 17]. Carcinogenicity and teratogenicity in rats were associated with the phomopsins as well [16, 19]. However, phomopsin A showed the greatest toxicity within the phomopsin family in animal studies [11]. A “No Observed Advanced Effect Level” (NOAEL) has not yet been determined [11].

The severity of the toxic effects of the phomopsins, especially phomopsin A, on animals suggests relevance for human health. The exposure should thus be kept as low as possible. In response, the Australian and New Zealand Food Authority (ANZFA, 2001) as well as the Food and Agriculture Organization (FAO, 2004) legislated a maximum amount of 5 µg/kg in lupin seeds and their products. Highly sensitive methods for the determination of phomopsin A and its related compounds are required to monitor compliance with this regulation.

Different techniques have been used to determine phomopsin A thus far, including a Nursling Rat Bioassay (NRB), an Enzyme-linked Immunosorbent Assay (ELISA) and, especially, High Performance Liquid Chromatography (HPLC) with different detection methods.

The NRB determined the phomopsins with a limit of quantification (LOQ) of 5 µg/kg. This method is not used anymore because of the time-consuming determination and the non-justifiable use of a large number of experimental animals.

The established phomopsin-ELISA allows the quantification of phomopsin A and B with a LOQ of 1 µg/kg [20, 21]. However, production of the required antibodies has ceased [11].

For determination using HPLC, several clean-up techniques (solid-phase extraction, SPE and liquid-liquid extraction, LLE) as well as detection with UV/Vis (matrix-dependent limit of detection, LOD: 200 – 500 µg/kg), electro-chemical detection (LOD: 50 µg/kg) and two methods using tandem-mass spectrometry detection (LOD: 1 µg/kg and LOQ: 5 µg/kg, respectively) have been described [22, 23, 24].

Some of the methods mentioned in literature often consist of time-consuming extraction steps or are, in part, not sensitive enough to assure quantification of the phomopsins at the maximum permitted amount. In addition, some of the methods are no longer in use in routine analysis. Therefore, we tried to develop a non-elaborate and sensitive UHPLC-MS/MS method for the determination of phomopsin A.

Experimental

Chemicals

The solvents methanol and acetonitrile of Lichrosolv® grade (purity ≥ 99.9 %) were purchased from Merck KGaA (Darmstadt, Germany). Ammonium formate of puriss grade (purity ≥ 98.5 %), used as a modifier for the UHPLC-MS/MS-determination, was purchased from Fluka/Sigma Aldrich (Steinheim, Germany). The phomopsin A standard substance (purity > 98 %, isolated from *D. toxica*) was purchased from BIOMOL (Hamburg, Germany).

Apparatus

For the determination of the standard stock solution concentration, a spectrophotometer DU 800 from Beckman Coulter (Krefeld, Germany) was used.

The shaking machine HS 501 digital (Janke & Kunkel, Staufen, Germany) was used for automated extraction. The centrifuge Rotanta 460 by Hettich (Tuttlingen, Germany) was appropriate for the centrifuge tubes employed in the procedure and was capable of achieving 4000 rpm. Electronic pipettes (Research Pro single channel) applicable for volumes of 5 – 100 µL, 100 – 1000 µL and 100 – 5000 µL including appropriate tips as well as a dispenser (with Combitip 50 mL) applicable for volumes of 0.5 – 10 mL were from Eppendorf (Wesseling-Berzdorf, Germany). An analytical balance (PM2000) capable of weighing substances from 0.5 g to 2100 g with a scope of 0.01 g was from Mettler-Toledo (Greifensee, Switzerland). Milli-Q water was received by a Milli-Q Direct 8 System from Millipore (Billerica, MA, USA).

50 mL borosilicate glass tubes with screw caps (with PTFE seal) for the sample extraction were from Pyrex (England). 1.5 mL HPLC autosampler vials (first hydrolytic class, appropriate for DIN ISO 719) were from Klaus Ziemer GmbH (Langenwehe, Germany).

An Acquity UPLC® system (Waters, Milford, MA, USA) combined with a mass spectrometer QTrap 5500® (AB Sciex, Darmstadt, Germany) using the Analyst-Software (Version 1.6.2, AB Sciex, Darmstadt, Germany) were used for the analysis of the extracts.

Samples and commodities

The described method was developed with lupin flour, lupin coffee and wheat flour and additionally validated with homogenized lupin steak (stored at -24 °C until use) and soy flour. After arriving at the lab the samples were homogenized if necessary and stored at room temperature.
The commodities used for an additional market survey of the German Market are presented in **Table 1**.

Table 1: Commodities used for the market survey.

Lupin containing commodities	Grain commodities	Commodities potentially contaminated with further *Diaporthe*-subspecies
Lupin flour (3x)	Rye flour	Soy flour
Lupin bread	Wholemeal wheat flour	Mango, dried (3x)
Lupin seeds	Rice	Raisins
	Quick cooking noodles	Grapes
	Maize farina	Grape juice
	Maize bread	Sunflower seed (3x)
	Maize snack	Chestnut flour
		Aubergine

Stock solution and standards

A stock solution of 95.0 µg/mL phomopsin A in methanol was prepared with the phomopsin A standard substance. The concentration of the stock solution was determined with a photometer (λ_{Max}: 288 nm; $\varepsilon_{Methanol}$: 16154 L/mol·cm). The stock solution was diluted with methanol to 9.50 µg/mL (working solution). All solutions were stored in the dark at -24 °C. Suitable diluted working solutions were prepared for each measuring day.

Measuring method

An aliquot of 8 µL of the samples were injected on an Acquity UPLC® BEH C_{18} column (50 x 2.1 mm, 1.7 µm) with a VanGuard BEH C_{18} pre-column, both purchased from Waters (Milford, MA, USA). The column temperature was 40 °C and the flow rate 0.4 mL/min. Eluent A consisted of 2 mmol/L ammonium formate in methanol/water (5/95, v/v) and eluent B consisted of 2 mmol/L ammonium formate in methanol/water (95/5, v/v). The used gradient program for the separation is shown in **Table 2**.

Table 2: UHPLC gradient program for the determination of phomopsin A.

Time	Eluent A [%]
Initial	100
0.5	100
5	45
6	45
7	0
8	0
9	100
10	100

The injection probe was washed with 600 µL methanol/water (10/90, v/v, weak wash solvent) and 200 µL acetonitrile/water (1/1, v/v, strong wash solvent) after every injection.

For the MS/MS detection, electrospray ionization (ESI) in positive mode was applied. The source temperature was set at 600 °C. A curtain gas flow of 30 psi, a nebulizer gas flow of 60 psi and an auxiliary gas flow of 60 psi were used. The ion transfer voltage was set to 5300 V. The entrance and declustering potential were 10 V and 51 V, respectively. Nitrogen was used as the CAD-Gas, at medium intensity. Other acquisition details are provided in **Table 3**.

Table 3: MS/MS parameters for phomopsin A.

Q1 mass	Q3 mass	CE [V]	CXP [V]	Dwell time [ms]
789.2	226.0	47	20	500
	323.0	37	20	150
	452.0	29	16	150

Sample extraction

For the extraction 4.0 g (lupin coffee and soy flour) or 8.0 g (lupin flour, lupin steak and wheat flour) were weighed into 50 mL glass tubes. 12 mL of acetonitrile was added, followed by soaking for 20 minutes. Then, 4 mL

methanol and 4 mL water were added. In case of lupin steak, only 0.4 mL water was added because of the higher native water content. The tube was closed and shaken by a mechanical shaker for 30 minutes. Afterwards the tube was centrifuged for 15 min at 3500 rpm. The supernatant was diluted 1:2 with methanol and water (5:45, v/v) to obtain the same solvent composition as in the calibration standard solutions. Finally, the diluted extract was transferred into a glass vial and measured via UHPLC-MS/MS analysis.

Method validation

The criteria stipulated in the EU-guideline for "method validation and quality control – procedures for pesticide residues – analysis in food and feed" (SANCO/12495/2011) were used.

For the determination of the detection linearity of phomopsin A, solvent standards in the concentration range of 0.1 ng/mL – 4,80 µg/mL were prepared. The linearity range of the resulting calibration curve was analyzed by residual analysis, using the relative residual standard deviation.

The method was validated for the matrices lupin flour, lupin steak (homogenized), lupin coffee, soy flour and wheat flour. All blank samples were proven not to contain phomopsin A right before validation.

The LOD and LOQ were measured for each matrix before the proper validation step by spiking blank extracts at appropriate levels (n = 5). The LOD's and LOQ's were determined by the signal-to-noise (S/N) ratio of 3 to 1 and 10 to 1, respectively.

The method was then validated for the mentioned matrices by recovery experiments (n = 5) at the LOQ levels and the 5-fold LOQ levels. In case of lupin coffee, the validation was conducted at the LOQ level and the 2-fold LOQ-level. Blank samples were spiked to the required level with the working solution or a diluted working solution and set aside for one hour until the solvent had evaporated. Because of its native water content, the lupin steak samples were spiked right before the extraction. At least 5-point matrix-matched calibration (MMC) for each spiking level was applied. For this purpose, suitable volumes of the diluted working solutions (con-

sisting of methanol) and water were added to blank extracts of each matrix, resulting in a 1:2 dilution of the matrix extract (end-composition: acetonitrile/methanol/water, 30/15/55, v/v/v). The concentration of the recovery samples were calculated from the known concentration of appropriate calibration standards. Consistent solvent calibration standards were prepared for the determination of the matrix effect. The slopes of the MMC's and the solvent calibration were compared.

Results and Discussion

Method development

The first step of our work was finding the best extraction solvent for phomopsin A. Therefore, we initially determined the recovery rates of an acetonitrile-extraction, followed by salt-induced phase-separation ("QuEChERS", modified; without PSA-Clean-Up) at different pH-values (0 %, 0.1 %, 0.5 % and 2 % formic acid in the water fraction). The QuEChERS-procedure was not favorable for the phomopsin A extraction because the analyte was not transfered completely into the acetonitrile phase when matrix was present. Comparing the low QuEChERS recoveries in lupin flour at any formic acid concentration with the high recoveries without matrix (by using the addition of formic acid), a pH-buffering by the matrix proteins resulting in low recoveries of phomopsin A could be suggested.

Further, we tried more polar extraction solvents without any other respective clean-up step, by just diluting the raw extract 1:2 with water. For this purpose methanol/water (8/2, v/v) and the extraction solvents acetonitrile/methanol/water (4/1/5, v/v/v) and acetonitrile/ methanol/water (3/1/1, v/v/v) developed for multi-mycotoxin analysis were tested [25]. The best recoveries and the lowest limit of quantification (LOQ) in lupin flour (results not shown) were accomplished by extraction with acetonitrile/methanol/water (3/1/1, v/v/v).

On the basis of this extraction solvent, we tried freezing out, salting-out with NaCl and solid-phase extraction (reversed-phase, cation- and anion-exchange materials and Bond Elut mycotoxin) as a clean-up of the extracts. None of these procedures resulted in a lower LOQ or higher recov-

ery rates on spiked samples (data not shown). Another possibility for the removal of matrix influences was tested by using detection with MS^3. Although the more selective MS^3 measurement achieved a lower matrix background, the signals showed little reproducibility in the low concentration region of less than 5 ng/mL phomopsin A (**Figure 2**). Therefore, we could not gain a lower LOQ using MS^3- instead of MS/MS-detection.

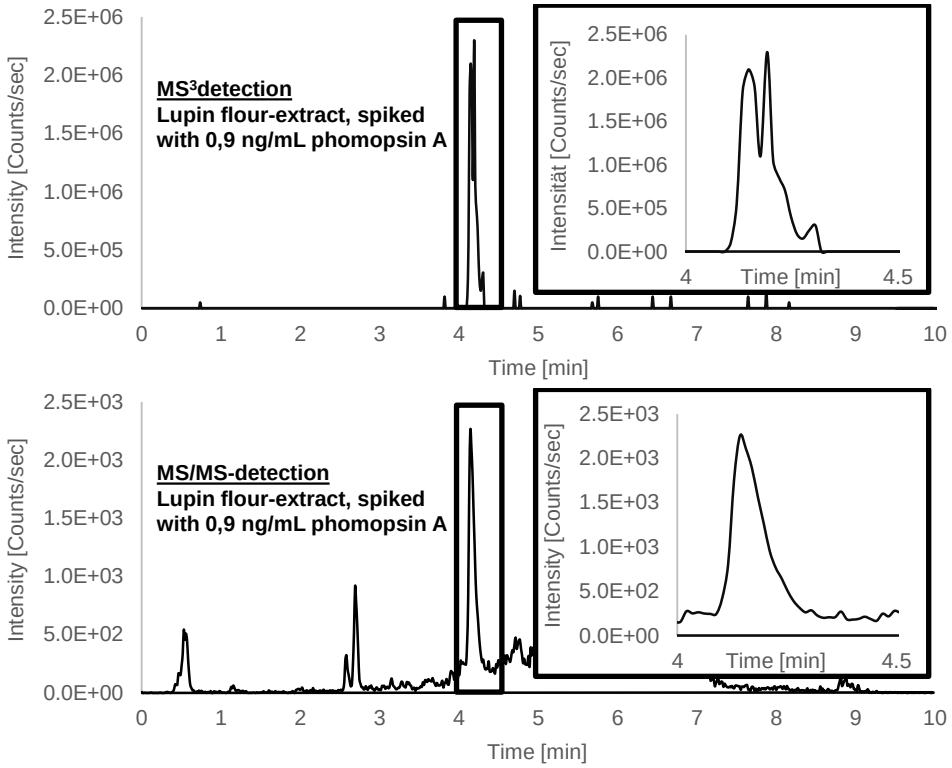

Figure 2: Chromatograms of a spiked lupin extract (0.9 µg/mL), detected with MS^3 and MS/MS. Decreased matrix background around the target peak time with MS^3.

By extraction with acetonitrile/methanol/water (3/1/1, v/v/v) and quantification via matrix matched calibration (MMC), we still obtained recoveries of only 60–80 % (matrix-dependent). Other working groups, e. g. de Nijs *et al*. (2013), had the same experiences [22, 24]. Both methods are “dilute & shoot” methods, containing hardly any steps during extraction where analyte loss could occur. One explanation for the analyte loss could be the adsorption of phomopsin A to the materials used. The influence of different extraction vessel materials on the extraction of different peptides was determined by Goebel-Stengel *et al.* (2011) [26]. They described in their

work how the choice of material could have an influence of up to 70 % on the recovery. Polypropylene tubes (Sarstedt, Nümbrecht, Germany) were used by de Nijs *et al.* (2013) and during our method development [24]. By using borosilicate tubes, we increased the recovery rates for the matrices lupin coffee and wheat flour by 6 % (**Figure 3**). No enhanced recovery rates were obtained for lupin flour. This indicates that there could be a further reason for analyte loss in this matrix, besides adsorption on the extraction vessel surface. Compared to the three tested matrices, lupin flour contains a high amount of proteins. As described by Cornwell *et al.* (1958) regarding amino acids and peptides, loss of phomopsin A could be due to irreversible adsorption on matrix proteins during the extraction process.

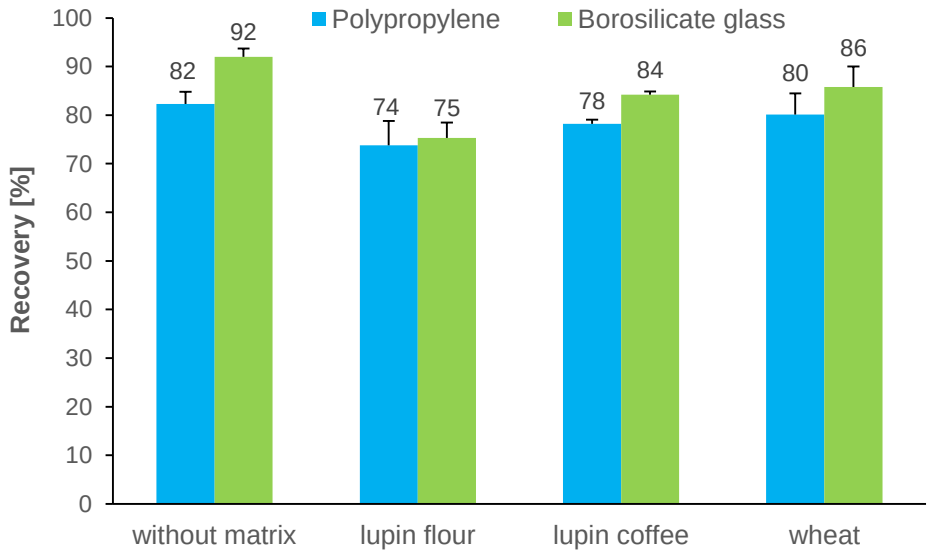

Figure 3: Comparison of the recoveries using polyproplyene and borosilicate glass tubes as extraction vessels.

Method validation

Method linearity and matrix effects

For the determination of linearity, phomopsin A solvent calibration solutions in the range of 0.1 ng/mL – 4.80 µg/mL were prepared.

Phomopsin A has a linear range of between 0.1 ng/mL and 950 ng/mL, resulting from the residual analysis of the calibration curve (data not shown).

The method was validated for the lupin containing commodities lupin flour, steak and coffee, for the grain commodity wheat flour, as well as for soy flour, a commodity that is taxonomically related to lupins.

The matrix effect was determined for all these matrices by comparing the respective MMC with the solvent calibration. Apart from wheat flour, the slopes of the matrix calibration curves tend to be lower compared to the solvent calibration curves, implying signal suppression (**Table 4**). For wheat flour, the slope of the MMC curve is in a range similar to that of the solvent calibration. The strongest matrix effect of 62 % was observed for lupin coffee, a highly complex matrix.

Table 4: Relations of the MMC slopes to the solvent calibration slopes and the resulting matrix effects of the validated commodities.

Matrix	Ratio of the MMC slope to the solvent calibration slope	Resulting matrix effect [%]
Lupin flour	0.76	24
Lupin steak	0.82	18
Lupin coffee	0.38	62
Soy flour	0.79	21
Wheat flour	1.0	-

Methods LOD and LOQ

For the LOD, we requested a S/N-ratio of at least 3 to 1 and a S/N-ratio of at least 10 to 1 for the LOQ by using spiked matrix extracts. The LOD's and LOQ's for the processed matrices lupin steak and coffee were higher compared to the non-processed matrices lupin flour and wheat flour (**Table 5**). For lupin coffee, the LOD and LOQ was even 100-fold higher, due to the strong matrix effect and a multitude of co-extracted reaction products which are related to the reaction products of complex matrices such as coffee. The lupin steak consisted of 40 % lupin seeds and a multitude of other ingredients such as plant oils, spices and vegetables. This indicates a correlation of the 5-fold higher LOD and LOQ of phomopsin A

in this commodity due to co-extraction of these ingredients compared to pure lupin flour. Finally, only the unprocessed lupin flour and wheat flour (both LOQ 1.4 µg/kg) matched the required criteria of the methods LOQ ≤ the maximum level of 5 µg/kg stipulated by ANZFA and FAO. Nevertheless, the developed method currently shows the highest sensitivity of all existing phomopsin A-methods for lupin flour.

Table 5: LOD's and LOQ's of phomopsin A for the validated matrices.

Matrix	LOD [µg/kg]	LOQ [µg/kg]
Lupin flour	0.4	1.4
Lupin steak	2.0	6.8
Lupin coffee	34.2	114
Soy flour	3.4	11.4
Wheat flour	0.4	1.4

Recovery rates and precision

Blank samples were spiked with appropriate dilutions of the phomopsin A working solution to the LOQ and 5-fold LOQ and, accordingly, to the 2-fold LOQ for lupin coffee (n = 5). The recovery rates were determined with an appropriate 5-point MMC for each matrix.

Except from the matrix lupin steak, all recoveries were in the required range between 70 % and 120 % (**Figure 4**). The lower recoveries related to the protein-containing matrices lupin flour, lupin steak and soy flour indicate again, that there is a possible loss of phomopsin A due to irreversible adsorption on matrix proteins during the extraction process. To compensate for the described analyte loss and to obtain sufficient recoveries, the addition of an isotopically labeled internal standard followed by a stable isotope dilution analysis (SIDA) is required. However, no isotopically labeled internal standard for phomopsin A is currently available.

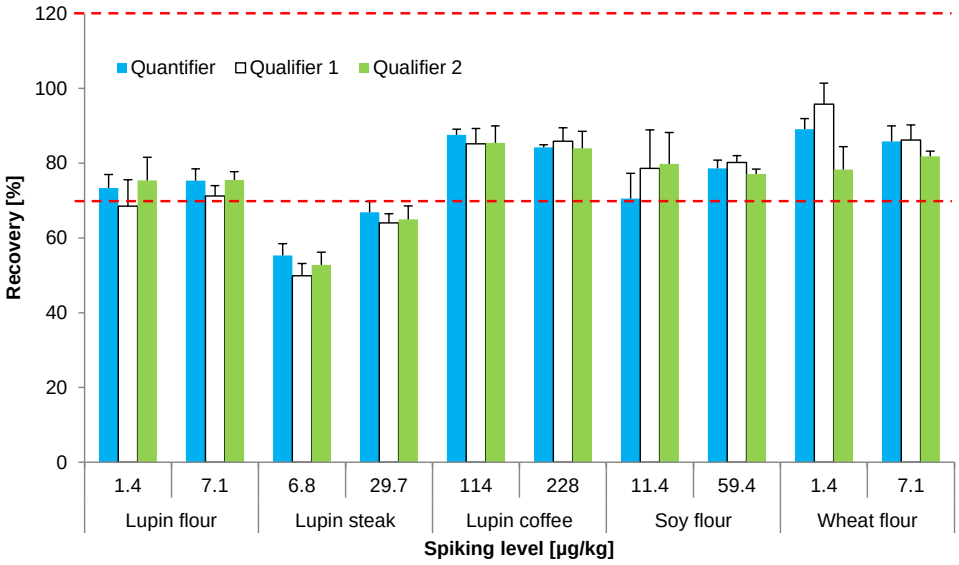

Figure 4: Recoveries of the validated matrices (n = 5).

The required precision (± 20 %) of the method was easily achieved with variation coefficients fewer than 10 %. Only three variation coefficients of the validation were above 10 %. The greatest variation coefficient with 13 % was obtained for the first qualifier mass trace of soy flour.

Market survey

Subsequent to the validation, twenty-five different commodities from the German market were investigated for phomopsin A using the developed method. The commodities could be divided into three groups: lupin-containing commodities, grain commodities and commodities which could be contaminated with further *Diaporthe*-subspecies (other than *D. toxica*).

Phomopsin A was not detected in any commodity. Similar reasults has already been reported by Reinhard *et al.* (2006) and de Nijs *et al.* (2013), who conducted surveys of the Swiss and Dutch markets [23, 24]. It should be taken into consideration that some of the investigated matrices were highly processed (e. g. lupin bread and maize snack), probably resulting in high LOQs and matrix effects. Consequently, a possible phomopsin A contamination would not be detected. However, contamination of European food with phomopsin A doesn't seem to be currently detectable using existing methods.

Conclusion

The presented method demonstrates that phomopsin A can be simply and sensitively analyzed by UHPLC-MS/MS after acetonitrile/methanol/water-extraction (3/1/1, v/v/v) without any clean-up step ("dilute & shoot") for lightly processed matrices like lupin flour and wheat flour. The validation of these matrices showed satisfying recoveries and variation coefficients, as well as the lowest LOQ's, compared to all other methods published so far. Sufficient recoveries were obtained for lupin coffee and soy flour as well, but the LOQ's were unsatisfactory. Neither the LOQ nor the recovery of lupin steak was in the permitted range. For lower LOQ's on highly processed matrices, a clean-up or a more selective detection method (e. g. ion-mobility MS) should be developed. The poor recoveries from protein-containing commodities due to analyte loss can be increased by the use of an isotopically labeled internal standard (not available so far).

A survey of twenty-five relevant commodities from the German market were investigated for phomopsin A using the developed method. Phomopsin A was not detected in any commodity. Thus, contamination of European food with phomopsin A does not seem to be currently detectable with the existing methods. Due to ever-increasing global trade and thus far unclarified climatic parameters of mold growth and the resulting production of its toxins, the continued comprehensive monitoring of phomopsin A is strongly recommended.

Appendix

Table 6: LODs and LOQs of phomopsin A for the validated matrices.

Matrix	LOD [µg/kg]	LOQ [µg/kg]
Lupin flour	0.4	1.4
Lupin steak	2.0	6.8
Lupin coffee	34.2	114
Soy flour	3.4	11.4
Wheat flour	0.4	1.4

Table 7: Validation recoveries, quantifier mass trace.

Quantifier mass trace m/z 789.0-226.0														
Matrix	Spiking at LOQ							Spiking 5-fold or 2-fold LOQ						
	Recovery (%)					Average (%)	RSD (%)	Recovery (%)					Average (%)	RSD (%)
Lupin flour	73.9	76.9	71.5	68.7	77.1	73.4	4.9	70.6	76.8	77.8	74.5	-	75.3	4.3
Lupin steak	57.2	55.0	54.1	59.4	51.0	55.3	5.8	65.2	63.9	68.2	70.3	71.1	66.9	4.4
Lupin coffee	88.9	87.5	89.1	86.8	85.5	87.6	1.7	83.2	85.0	84.1	84.3	86.0	84.2	0.9
Soy flour	80.9	72.9	68.2	66.8	63.5	70.5	9.6	79.8	80.9	77.6	761	-	78.6	2.8
Wheat flour	88.1	889	93.5	89.1	85.8	89.1	3.2	87.6	85.9	80.0	89.8	84.8	85.8	4.9

Table 8: Validation recoveries, first qualifier used.

Qualifier mass trace m/z 789.0-323.0														
Matrix	Spiking at LOQ							Spiking 5-fold or 2-fold LOQ						
	Recovery (%)					Average (%)	RSD (%)	Recovery (%)					Average (%)	RSD (%)
Lupin flour	76.1	59.1	64.6	68.8	74.9	68.5	10.3	68.9	68.2	73.3	73.3	-	71.2	3.9
Lupin steak	49.0	49.5	50.7	45.5	54.7	49.9	6.6	62.2	61.6	66.2	66.2	66.3	64.0	3.9
Lupin coffee	90.1	86.9	86.0	84.3	78.9	85.2	4.8	85.1	83.7	85.6	83.3	92.1	85.9	4.2
Soy flour	94.3	79.6	79.6	67.2	72.2	78.6	2.8	80.8	82.2	77.9	79.6	-	80.2	13.0
Wheat flour	90.4	88.9	100.6	99.8	99.1	95.8	5.9	87.8	83.8	81.8	85.7	92.1	86.2	4.6

Table 9: Validation recoveries, second qualifier used.

Qualifier mass trace m/z 789.0-452.0														
Matrix	Spiking at LOQ							Spiking 5-fold or 2-fold LOQ						
	Recovery (%)					Average (%)	RSD (%)	Recovery (%)					Average (%)	RSD (%)
Lupin flour	74.4	71.3	77.5	68.8	84.9	75.4	8.3	76.6	77.8	72.9	74.8	-	75.5	2.8
Lupin steak	52.7	56.1	47.5	55.3	52.7	52.8	6.4	61.8	64.3	63.8	70.2	66.3	65.0	5.6
Lupin coffee	86.7	86.7	86.7	77.4	89.4	85.4	5.4	77.2	82.6	87.2	84.6	88.6	84.0	5.3
Soy flour	92.0	78.7	70.1	74.9	83.4	79.8	10.5	77.5	78.3	77.5	75.2	-	77.1	1.7
Wheat flour	70.0	84.7	81.7	74.1	81.2	78.3	7.7	82.0	81.0	80.1	82.5	83.7	81.8	1.7

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