SLOVENSKI STANDARD
oSIST prEN 17252:2018

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Živila - Določevanje fomopsina A v semenih volčjega boba in predelanih proizvodih z LC-MS/MS

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

Lebensmittel - Bestimmung von Phomopsin A in Lupinensamen und Lupinenerzeugnissen mit LC-MS/MS

Produits alimentaires - Détermination de la teneur en phomopsine A dans les graines de lupin et les produits dérivés du lupin par CL-SM/SM

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67.060	Žita, stročnice in proizvodi iz njih	Cereals, pulses and derived products

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

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EUROPEAN COMMITTEE FOR STANDARDIZATION
COMITÉ EUROPÉEN DE NORMALISATION
EUROPÄISCHES KOMITEE FÜR NORMUNG

CEN-CENELEC Management Centre: Rue de la Science 23, B-1040 Brussels

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European foreword

This document (prEN 17252:2018) has been prepared by Technical Committee CEN/TC 275 “Food analysis - Horizontal methods”, the secretariat of which is held by DIN.

This document is currently submitted to the CEN Enquiry.

This document has been prepared under a mandate given to CEN by the European Commission and the European Free Trade Association.

Introduction

Phomopsins are mycotoxins produced by the fungus *Diaporthe toxica*. There are several phomopsins of which phomopsin A is the major toxic congener. The main host of the fungus are lupins (*Lupinus L.*). Lupin seeds are being used as food ingredient and therefore phomopsin A might occur in food ingredients and food products containing lupin seeds or lupin flour.

WARNING 1 — Suitable precaution and protection measures need to be taken when carrying out working steps with harmful chemicals. The latest version of the hazardous substances ordinance (EU) 1907/2006, [3] should be taken into account as well as appropriate National statements.

WARNING 2 — The use of this document can involve hazardous materials, operations and equipment. This document does not purport to address all the safety problems associated with its use. It is the responsibility of the user of this document to establish appropriate safety and health practices and determine the applicability of regulatory limitations prior to use.

1 Scope

This document describes a procedure for the determination of phomopsins in lupin seeds and lupin-derived products based on liquid chromatography with tandem mass spectrometry (LC-MS/MS). 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 method has been validated for phomopsin A in naturally contaminated lupin seeds, lupin flour and crisp bread at levels ranging from approximately 5 µg/kg to 60 µg/kg.

2 Normative references

The following documents are referred to in the text in such a way that some or all of their content constitutes requirements 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, *Water for analytical laboratory use - Specification and test methods (ISO 3696)*

3 Terms and definitions

No terms and definitions are listed in this document.

ISO and IEC maintain terminological databases for use in standardization at the following addresses:

- IEC Electropedia: available at http://www.electropedia.org/
- ISO Online browsing platform: available at http://www.iso.org/obp

4 Principle

The phomopsins are extracted from the homogenized sample material by shaking with a mixture of acetonitrile/water/acetic acid (80+19+1, v+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). Phomopsins are quantified by multi-level matrix-matched calibration.

5 Reagents

Use only reagents of recognized analytical grade and water complying with grade 1 of EN ISO 3696, unless otherwise specified. Solvents shall be of quality for LC analysis, unless otherwise specified.

5.1 Water, deionised.

5.2 Water, LC-MS grade.

5.3 Acetonitrile, p.a.

5.4 Methanol, LC-MS grade.

5.5 Acetic acid, purity greater than $w \geq 98$ %.

5.6 Ammonium formate, p.a.

5.7 Extraction solution acetonitrile/water/acetic acid, (80+19+1, v+v+v).

Mix 800 ml of acetonitrile (5.3), 190 ml of water (5.1 or 5.2) and 10 ml of acetic acid (5.4) in a bottle of 1000 ml. This solution is stable for 3 months if stored at room temperature.

5.8 Phomopsin A, isolated from *Phomopsis leptostromiformis.*

5.9 Phomopsin A stock solution (STD 1), mass concentration ρ = 500 mg/l.

Accurately weigh between 5 mg and 6 mg of the phomopsin A standard (5.8) into an amber-coloured glass bottle of 30 ml. Add a volume of methanol (5.4) to produce a solution with a concentration of 500 mg/l. Take into account the weight and the purity of the standard. The solution is stable for 3 months if stored in the refrigerator at 4 °C.

5.10 Standard solution of phomopsin A (STD 2), ρ = 10 mg/l.

Pipette 100 µl of the standard solution (STD 1) (5.9) into a calibrated volumetric flask of 5 ml and make up the volume with methanol (5.4). The solution is stable for 3 months if stored in the refrigerator at 4 °C.

5.11 Standard solution of phomopsin A (STD 3), ρ = 250 µg/l.

Pipette 250 µl of the standard solution (STD 2) (5.10) into a calibrated volumetric flask of 10 ml and make up to the volume with methanol (5.4).

5.12 Intermediate solutions for preparation of the matrix-matched standards.

To seven glass vials (6.9) add different volumes of the standard solution of phomopsin A (5.11) and methanol (5.4) according to Table 1. Close with screw cap and mix. Prepare these solutions freshly for each batch of analysis.

Table 1 — Intermediate standard solutions of phomopsin A in methanol

Intermediate solution	Standard solution STD 3 (5.11)	Methanol	Mass concentration
no	µl	µl	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

5.13 Matrix matched calibration solutions

Prepare matrix-matched calibration solutions in vials (6.9) according to Table 2.

The matrix matched calibration solutions may also be prepared directly in auto sampler vials with insert or filter vials. In that case, proportionally reduce the volumes indicated in Table 2.

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

Table 2 — Matrix matched calibration solutions of phomopsin A in blank matrix extract

Calibration solution	Mass concentration	Blank extract	Phomopsin A intermediate solutions (5.12) see Table 1	Water (5.2)	Equivalent to mass fraction in sample
no	µg/l	µl	µl	µl	µg/kg
0	0	500	0	500	0
1	0,3125	450	50 µl no 1	500	2,5
2	0,625	450	50 µl no 2	500	5,0
3	1,25	450	50 µl no 3	500	10,0
4	2,5	450	50 µl no 4	500	20,0
5	4,375	450	50 µl no 5	500	35,0
6	6,25	450	50 µl no 6	500	50,0
7	8,125	450	50 µl no 7	500	65,0

6 Apparatus and equipment

Usual laboratory glassware and equipment, in particular, the following:

6.1 Conical polypropylene screw cap centrifuge tubes, 50 ml with caps.

6.2 Volumetric flasks, 5 ml and 10 ml.

6.3 Analytical balance, accuracy 0,1 mg.

6.4 Laboratory balance, accuracy 0,01 g.

6.5 Pipettes, e.g. 10 µl to 1000 µl, for organic solvents.

6.6 Adjustable mechanical vertical or horizontal shaker or rotary tumbling machine.

6.7 Laboratory shaker.

6.8 Centrifuge, capable of generating a relative centrifugal force of 3 500 *g*.

6.9 Vials, 1,5 ml to 2 ml, used for intermediate solutions (5.12), made of glass or polypropylene, with screw cap.

6.10 Syringe filter, 0,20 µm to 0,45 µm, nylon or PTFE (for optional filtration of final extracts).

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

6.12 LC-MS/MS system, with the following components:

6.12.1 LC pump, capable of delivering a binary gradient at flow rates appropriate for the analytical column in use with sufficient accuracy.

6.12.2 Injection system, capable of injecting an appropriate volume of injection solution with sufficient accuracy, and cross-contamination below 0,1 %.

6.12.3 LC column, capable of retaining phomopsin A with a retention factor of at least two and that ensures base line separation to distinguish peaks of phomopsin A from all other signals. Examples of suitable columns are listed in Annex B.

6.12.4 Column oven, capable of maintaining a constant temperature.

6.12.5 Tandem mass spectrometer (MS/MS), capable of performing ionization of phomopsins and selected reaction monitoring (SRM), with a sufficiently wide dynamic range.

Any ionization source providing sufficient yield may be used.

6.12.6 Data evaluation system.

7 Procedure

7.1 Preparation of the test sample

Finely grind the laboratory sample, homogenize and store in the dark before analysis.

7.2 Extraction

Weigh a test portion of 5 g of the homogeneous laboratory sample into 50 ml centrifuge tube (6.1) to the nearest 0,01 g.

Add any spikes for quality control purposes (e.g. recovery determinations) at this point, e.g. 100 µl of standard solution STD 3 (5.11) to 5 g of sample to obtain a spike level of 5 µg/kg.

Add 20,0 ml extraction solution (5.7) to the tube, close and shake vigorously by hand or using a laboratory shaker (6.7).

Place the tubes in a mechanical shaker (6.6) and extract for approximately 60 min at a speed that ensures complete suspension and mixing of the sample into the extraction solvent liquid.

Centrifuge the tubes for 10 min at approximately 3 500 *g* (6.8).

If wanted for possible repeats: transfer part of the clear extract into a clean vial for storage of up to seven days in the refrigerator at 4 °C.

7.3 Preparation of sample test solutions

Either transfer 500 µl of the extract (7.2) into a vial (6.9), add 500 µl of water (5.2) and mix (6.7) or transfer 200 µl of extract into a filter vial (6.11) and add 200 µl of water (5.2) without filtering.

Cool the extract in the refrigerator at 4 °C.

If the extract in the vial (6.9) is turbid after cooling, filter through a syringe filter (6.10) or centrifuge it. When using filter vials, press the filter unit down into the vial.

7.4 LC-MS/MS analysis

7.4.1 General

Optimize analytical parameters (selection of the ionization mode, selection of the masses of precursor and product ions, optimization of cone voltages and collision energies) by infusion and injection of standard solutions of phomopsin A.

A combination of analytical column, mobile phase composition, gradient settings and injection volume shall be such that it allows obtaining acceptable separation and reliable results at the required levels, with sufficient selectivity to obtain an acceptably low false suspect rate.

Examples of instruments and conditions that have been shown to achieve this and extracted ion chromatograms are included in Annex B.

7.4.2 Batch composition and analytical 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 number 4 (Table 2) (2,5 µg/l) after every approximately 10 samples. At the end of the batch, re-inject the calibration series.

7.4.3 Identification

Identify phomopsin A in the test solutions by comparing retention time and ion ratio of matrix matched calibration solutions with that of the sample test solution. Phomopsin A is considered identified when:

a) the reagent blank does not show a peak for the quantifier ion at the retention time of phomopsin A that exceeds 30 % of the reporting limit;

b) 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;

c) 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.

   For calculation of the reference ion ratio use only responses with an $S/N > 10$. For the higher concentrations, exclude peak areas exceeding the linear range from calculation of the reference ion ratio.

7.4.4 Calibration

Plot the peak areas of the quantifier (y-axis) of all individual calibration solutions (5.13, calibration solutions 0 to 7) against the corresponding mass concentrations (µg/l) (x-axis). The quantifier is the transition which, overall, gives the best S/N. Construct a calibration curve using (weighted) least-square regression with all individual data points obtained. 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. If higher deviations/nonlinearity is observed, identify the cause and, if necessary, re-run the analyses.

In this method, calibration is performed using matrix-matched calibration solutions to compensate for ion suppression or enhancement that may occur in LC-MS/MS analysis. This is required unless it has been demonstrated that matrix effects are not significant and as long as no isotopically labelled internal standard is available. For the matrices tested during the interlaboratory study, extracts from phomopsin-free milled lupin seeds were found to be suited for calibration. For other matrices, the suitability of the matrix-matched calibration solutions needs to be verified.