SLOVENSKI STANDARD
SIST EN 16924:2017

01-september-2017

Živila - Določevanje zearalenona v jedilnih rastlinskih oljih z LC-FLD ali LC-MS/MS

Foodstuffs - Determination of zearalenone in edible vegetable oils by LC-FLD or LC-MS/MS

Lebensmittel - Bestimmung von Zearalenon in pflanzlichen Speiseölen mit LC-FLD oder LC-MS/MS

Denrées alimentaires - Dosage de la zéaralénone dans les huiles végétales alimentaires par CL-FLD ou CL-SM/SM

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Foodstuffs - Determination of zearalenone in edible vegetable oils by LC-FLD or LC-MS/MS

Produits alimentaires - Dosage de la zéaralénone dans les huiles végétales alimentaires par CL-FLD ou CL-SM/SM

Lebensmittel - Bestimmung von Zearalenon in pflanzlichen Speiseölen mit LC-FLD oder LC-MS/MS

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EUROPEAN COMMITTEE FOR STANDARDIZATION
COMITÉ EUROPÉEN DE NORMALISATION
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European foreword

This document (EN 16924:2017) has been prepared by Technical Committee CEN/TC 275 “Food analysis - Horizontal methods”, the secretariat of which is held by DIN.

This European Standard shall be given the status of a national standard, either by publication of an identical text or by endorsement, at the latest by November 2017, and conflicting national standards shall be withdrawn at the latest by November 2017.

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Introduction

The mycotoxin zearalenone is a resorcylic acid derivative, which is produced by several species of the fungi genus *Fusarium*, in particular by *Fusarium roseum var. graminearum*. Especially cereals like maize and wheat are affected, so that zearalenone can also be detected in the oils produced from them.

WARNING 1 — Suitable precaution and protection measures need to be taken when carrying out working steps with harmful chemicals. The hazardous substances ordinance, Regulation (EC) No 1907/2006 [3], should be taken into account as well as appropriate National statements e.g. such as in [4].

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.

WARNING 3 — Zearalenone is known to have strong oestrogenic effects.

1 Scope

This European Standard describes a procedure for the determination of the zearalenone content in edible vegetable oils specifically maize germ oil by either of the following techniques: High performance liquid chromatography with fluorescence detection (LC-FLD) or high performance liquid chromatography with tandem mass spectrometry (LC-MS/MS) after basic extraction of the diluted oil.

The method has been validated for zearalenone in naturally contaminated maize germ oil at levels of 61,2 µg/kg to 515 µg/kg [5].

Laboratory experiences [6] have shown that this method is also applicable to other vegetable oils such as wheat germ oil (n = 4), sunflower oil (n = 5), pumpkin seed oil (n = 1), soybean oil (n = 5), hemp seed oil (n = 5), rape seed oil (n = 11), and mixed oils including maize germ oil (n = 3). However occasionally, samples can result in interferences in the FLD-chromatograms. In this case, the detection with MS/MS is recommended.

2 Normative references

The following documents, in whole or in part, are normatively referenced in this document and are indispensable for its application. 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 Principle

After diluting the edible vegetable oil, zearalenone is extracted by shaking with an alkaline methanol - ammonium hydrogen carbonate mixture.

For the determination by LC-FLD, an aliquot of the centrifuged methanolic-alkaline extract is evaporated to dryness, then the residue is diluted in acidified LC-eluent and the zearalenone content is determined by LC-FLD.

For the determination by LC-MS/MS, an aliquot of the centrifuged methanolic-alkaline extract is used directly for analysis.

4 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.

4.1 Methanol, p. a. (pro analysis) for extraction.

4.2 Dilution solvent, (defatting solvent), *n*-hexane or, alternatively, *n*-heptane, p. a.

n-Heptane may be used instead of *n*-hexane, however, only *n*-hexane was used in the interlaboratory test.

4.3 Acetonitrile, LC quality.

4.4 Ammonium hydrogen carbonate (NH_4HCO_3).

4.5 Ammonium hydrogen carbonate solution, mass concentration ρ = 10 g/l.

Weigh in 1 g of ammonium hydrogen carbonate (4.4) into a 100 ml volumetric flask and fill up to the mark with water. Prepare a fresh solution each day of analysis.

4.6 Alkaline methanol extraction solution.

Mix 9 volumes of methanol (4.1) with 1 volume of ammonium hydrogen carbonate solution (4.5). Prepare a fresh alkaline methanol extraction solution each day of analysis.

4.7 Zearalenone, e.g. crystalline, purity greater than 98 % mass fraction, or as certified standard solution with 100 µg/ml.

4.8 Stock solution of zearalenone, mass concentration ρ = 100 µg/ml.

Weigh in 10 mg of crystalline zearalenone (4.7) to the nearest 0,1 mg into a 100 ml volumetric flask and fill up to the mark with acetonitrile (4.3). The stock solution is stable for at least 4 weeks to 12 weeks if stored at < −18 °C [7].

This step can be omitted when using the certified standard solution. The certified standard solution then serves as stock solution.

4.9 Standard solution of zearalenone, ρ = 10 µg/ml.

Pipette 1 ml of the stock solution of zearalenone (4.8) into a 10 ml volumetric flask and fill up to the mark with acetonitrile (4.3). Determine the exact concentration of zearalenone in this standard solution spectrometrically.

For this purpose, record the absorption curve of the standard solution from 190 nm to 350 nm against acetonitrile. Calculate the mass concentration of zearalenone in the standard solution ρ_{st} in µg/ml according to Formula (1):

$$\rho_{st} = \frac{E_{max} \cdot M \cdot 100}{\delta \cdot \varepsilon} \quad (1)$$

where

E_{max} is the maximum extinction value determined from the absorption curve (here: 274 nm);

M is the molar mass of zearalenone = 318,4 g/mol;

δ is the layer thickness of the cuvette in cm;

ε is the molar extinction coefficient in acetonitrile: 1262 m²/mol.

4.10 Reagents, mobile phases and calibration solutions for LC-FLD analysis:

4.10.1 Glacial acetic acid for LC-FLD, volume fraction φ (CH_3COOH) approximately 99 %.

4.10.2 Mobile phase A for LC-FLD: Acetonitrile/water/glacial acetic acid (47+52+1, v+v+v).

Mix 47 volumes of acetonitrile (4.3) with 52 volumes of water and 1 volume of glacial acetic acid (4.10.1).

For the stability of the chromatographic separation it is necessary to acidify the mobile phase.

4.10.3 Mobile phase B for LC-FLD: Acetonitrile (100 %) (4.3).

4.10.4 Calibration solutions of zearalenone for LC-FLD.

Prepare a series of calibration solutions from the standard solution (4.9) of zearalenone. According to the pipetting scheme in Table 1, pipette the corresponding volumes of standard solution of zearalenone into volumetric flasks, evaporate the solvent with nitrogen at approximately 40 °C to dryness and dissolve the residue in mobile phase A (4.10.2). Dissolve by means of laboratory shaker (5.4) or ultrasonic bath (5.3) at room temperature. Then fill up to the mark. The calibration range shall be

adapted to the desired working range. The calibration solutions can be used for approximately 1 month if stored in the refrigerator at 4 °C.

Table 1 — Examples of suitable calibration solutions for LC-FLD

Calibration solution	Volume of volumetric flask ml	Volume of standard solution (4.9) µl	Mass concentration of calibration solution ng/ml
1	10	25	25
2	10	50	50
3	10	100	100
4	10	150	150
5	10	200	200
6	10	250	250

4.11 Reagents, mobile phases, and additional standards and standard solutions for LC-MS/MS analysis:

4.11.1 Methanol, LC quality.

4.11.2 Ammonium hydrogen carbonate (NH_4HCO_3), for LC-MS, molecular weight: 79 g/mol.

4.11.3 Mobile phase A for LC-MS/MS: 1 mmol/l NH_4HCO_3 in water/methanol (95+5, v+v).

Dissolve 79 mg of ammonium hydrogen carbonate (4.11.2) in 950 ml of water and mix with 50 ml of methanol (4.11.1).

4.11.4 Mobile phase B for LC-MS/MS: 1 mmol/l NH_4HCO_3 in water/methanol (5+95, v+v).

Dissolve 79 mg of ammonium hydrogen carbonate (4.11.2) in 50 ml of water and mix with 950 ml of methanol (4.11.1).

4.11.5 Working solution of zearalenone for calibration, ρ = 200 ng/ml.

Transfer 0,5 ml of the standard solution (4.9) into a 25 ml volumetric flask and fill up to the calibration mark with methanol (4.11.1).

4.11.6 Zearalanone, internal standard (ISTD 1) for LC-MS/MS, e.g. crystalline or as certified standard solution with 10 µg/ml.

4.11.7 Zearalenone, isotopic labelled internal Standard (ISTD 2) for LC-MS/MS, as optional internal standard, e.g. zearalenone [$^{13}C_{18}$]-labelled.

4.11.8 Stock solution of zearalanone (ISTD 1), ρ = 100 µg/ml.

Weigh in 10 mg of zearalanone (ISTD 1) (4.11.6) to the nearest 0,1 mg into a 100 ml volumetric flask and fill up to the mark with acetonitrile (4.3).

The ISTD 1 stock solution is stable for at least 4 weeks to 12 weeks if stored at < −18 °C [7].

If the certified standard solution is used, the preparation of this stock solution of zearalanone (ISTD 1) is not necessary.

4.11.9 Internal standard solution of zearalanone (ISTD 1), ρ = 10 µg/ml.

Transfer 1 ml of the stock solution of zearalanone (4.11.8) into a 10 ml volumetric flask and fill up to the calibration mark with methanol (4.11.1).

This step can be omitted when using the certified standard solution of zearalanone. The certified standard solution then serves as the internal standard solution of zearalanone (ISTD 1).

4.11.10 Working solution of internal standard zearalanone (ISTD 1) for calibration, ρ = 200 ng/ml.

Transfer 0,5 ml of the internal standard solution of zearalanone (ISTD 1) (4.11.9) into a 25 ml volumetric flask and fill up to the calibration mark with methanol (4.11.1).

However, it is also possible to use isotopic labelled zearalenone (ISTD 2) (4.11.7) as internal standard instead of zearalanone (ISTD 1) (4.11.6), which is sufficient in most cases.

4.11.11 Calibration solutions with zearalenone and zearalanone (ISTD 1) for LC-MS/MS.

Prepare a series of calibration solutions from the working solutions of zearalenone (4.11.5) and zearalanone (ISTD 1) (4.11.10). For this purpose, pipette the corresponding volumes of the working solutions into an LC vial (5.11), evaporate the solvent with nitrogen at approximately 40 °C to dryness and dissolve the residue in the alkaline methanol extraction solution (4.6).

The mass concentration of the ISTDs and the calibration range shall be adapted to the desired working range. Taking into account the dilution steps, the calibration can be carried out as described in Table 2. The calibration solutions can be used for approximately 1 month if stored in the refrigerator at 4 °C.

Table 2 — Examples for suitable calibration solutions for LC-MS/MS

Calibration solution	Volume of working solutions (200 ng/ml)		Final volume ml	Mass concentration of calibration solution ng/ml	
	Zearalenone (4.11.5) µl	ISTD 1 Zearalanone (4.11.10) µl		Zearalenone	ISTD 1 Zearalanone
1	25	100	1	5	20
2	50	100	1	10	20
3	100	100	1	20	20
4	150	100	1	30	20
5	200	100	1	40	20
6	250	100	1	50	20

Control possible reciprocal interferences of zearalenone and zearalanone (ISTD 1) at the chosen measurement conditions, by using one calibration standard with the highest level of zearalenone only and one calibration standard with zearalanone (ISTD 1) with the highest level only.

5 Apparatus and equipment

Usual laboratory apparatus and, in particular, the following:

5.1 Laboratory balance, accuracy: 0,01 g.

5.2 Analytical balance, accuracy: 0,1 mg.

5.3 Ultrasonic bath.

5.4 Laboratory shaker for test tubes.

5.5 Manual dispensers, microlitre syringes or microlitre pipettes for 10 µl to 2 ml.

5.6 Dispenser, suitable for 20 ml.

5.7 Solvent evaporator with heating module.

5.8 Centrifuge, suitable for 3 000 *g*[1)].

5.9 Microcentrifuge, suitable for 14 000 *g*.

5.10 Centrifugation tubes, 50 ml, made of polypropylene with screw cap.

5.11 LC vials.

5.12 UV-spectrometer with quartz cuvettes.

5.13 LC-FLD system with the following components:

5.13.1 LC pump, suitable for gradient elution.

5.13.2 Injection system.

5.13.3 LC column, e.g. LiChroCART® 250-4 filled with LiChrospher®[2)] 100 RP-18 250 mm x 4 mm, particle size 5 µm and corresponding pre-column.

5.13.4 Column thermostat.

5.13.5 Fluorescence detector, with variable wavelengths.

5.13.6 Data evaluation system.

5.14 LC-MS/MS system with the following components:

5.14.1 LC pump, suitable for gradient elution.

5.14.2 Injection system.

5.14.3 LC column, suitable for chromatography under alkaline conditions, e.g. XBridge®[2)] C18 or Shield RP18 100 mm × 3 mm; particle size 3,5 µm, or Gemini®[2)] C18 150 mm × 3 mm; particle size 5 µm and corresponding pre-column.

5.14.4 Column thermostat.

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1) $g = 9{,}81\ \mathrm{m \cdot s^{-2}}$.

2) LiChroCART® 250-4 filled with LiChrospher® are trade names of products supplied by Merck, XBridge® is a trade name of a product supplied by Waters. Gemini® is a trade name of a product supplied by Phenomenex. This information is given for the convenience of users of this European Standard and does not constitute an endorsement by CEN of the products named. Equivalent products may be used if they can be shown to lead to the same results.

5.14.5 Tandem mass spectrometer (MS/MS):

Interface: electrospray ionization (ESI);

acquisition mode: multiple reaction monitoring (MRM);

Ion mode: negative ion mode.

5.14.6 Data evaluation system.

6 Procedure

6.1 Extraction of zearalenone from the sample

6.1.1 General

Weigh in 2 g of the sample into a 50 ml centrifugation tube (5.10) to the nearest 0,01 g, add 2,0 ml of dilution solvent (4.2) and 20,0 ml of alkaline methanol extraction solution (V_e) (4.6).

If the sample is measured by LC-MS/MS, add 40 µl of zearalanone internal standard solution (ISTD 1) (4.11.9) to the sample prior to extraction (corresponds to a mass fraction of 200 µg/kg zearalanone in the sample).

To determine the recovery rate, add 40 µl of the standard solution of zearalenone (4.9) to the sample prior to extraction (corresponds to a mass fraction of 200 µg/kg zearalenone in the sample) and leave for approximately 5 min at room temperature.

Extract the mixture for approximately 20 min using a laboratory shaker (5.4). To separate the phases, centrifuge for 10 min at approximately 3 000 *g* (5.8).

6.1.2 Preparation for LC-FLD

Evaporate 5,0 ml of the clear, alkaline methanol supernatant of the extract to dryness at approximately 40 °C and dissolve in 1,0 ml of mobile phase A (4.10.2).

Dissolve the residue using an ultrasonic bath (5.3). If necessary, centrifuge the sample solution again in a microcentrifuge (5.9) for 2 min at 14 000 *g* and transfer into an LC vial (5.11).

6.1.3 Preparation for LC-MS/MS

Transfer a part of the clear, alkaline methanol supernatant into an LC vial (5.11).

When isotopic labelled zearalenone (ISTD 2) (4.11.7) is used to identify the matrix effect, dilute this supernatant. In this case, the methanol content in this diluted supernatant shall still be 90%.

6.2 LC-FLD analysis

Inject equal suitable volumes of the sample test solution and of each calibration solution (4.10.4) into the LC-FLD system.