SLOVENSKI STANDARD
oSIST prEN 16924:2015

01-december-2015

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

Ta slovenski standard je istoveten z: **prEN 16924**

ICS:

67.200.10	Rastlinske in živalske maščobe in olja	Animal and vegetable fats and oils

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

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

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

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European foreword

This document (prEN 16294:2015) has been prepared by Technical Committee CEN/TC 275 “Food analysis - Horizontal methods”, the secretariat of which is held by DIN.

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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 — Suitable precaution and protection measures need to be taken when carrying out working steps with harmful chemicals. The hazardous substances ordinance (EU) 1907/2006, [3] should be taken into account as well as appropriate National statements e.g. such as in Bibliographical Reference [4].

WARNING — 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 — 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 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 oils (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 oil with dilution solvent, it 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 (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, CAS-No: 17924-92-4, e.g. crystalline, as a film 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). Alternatively, transfer the complete content of the standard vial containing zearalenone as a film with acetonitrile quantitatively into a 100 ml volumetric flask. The stock solution is stable for at least 4 weeks to 12 weeks if deep frozen 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, and determination of concentration.

Pipette 1 ml of the stock solution (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. To check the concentration of the stock solution of zearalenone, 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);

ε is the molar extinction coefficient in acetonitrile: 1262,3 m²/mol;

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

δ is the layer thickness of the cuvette in cm.

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 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 stored in the refrigerator at 4 °C for approximately 1 month.

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) CAS-No: 5975-78-0 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-labelled (fully).

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). Alternatively, transfer the complete content of the standard vial containing zearalanone (ISTD 1) as a film with acetonitrile (4.3) quantitatively into a 100 ml volumetric flask.

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

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).

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 as ISTD 2 (4.11.7) instead of zearalanone ISTD 1, 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 a LC vial, 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 stored in the refrigerator at 4 °C for approximately 1 month.

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

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

5 Instruments 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 system, including:

5.13.2 LC pump, suitable for gradient elution;

5.13.3 Injection system;

5.13.4 Column thermostat;

5.13.5 Fluorescence detector, with variable wavelengths;

5.13.6 Data evaluation system;

5.13.7 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.

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

5.14.1 LC system, including:

5.14.2 LC pump, suitable for gradient elution;

5.14.3 Injection system;

5.14.4 Column thermostat;

5.14.5 Data evaluation system;

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.