SLOVENSKI STANDARD
oSIST prEN 17251:2018

01-september-2018

Živila - Določevanje ohratoksina A v svinjskem mesu in predelanih proizvodih s tekočinsko kromatografijo visoke ločljivosti s fluorescenčno detekcijo (HPLC-FLD)

Foodstuffs - Determination of ochratoxin A in pork meat and derived products by high performance liquid chromatography with fluorescence detection (HPLC-FLD)

Lebensmittel - Bestimmung von Ochratoxin A in Schweinefleisch und Schweinefleischerzeugnissen mit Hochleistungsflüssigchromatographie und Fluoreszenzdetektion (HPLC-FLD)

Produits alimentaires - Dosage de l'ochratoxine A dans la viande de porc et les produits carnés issus du porc par chromatographie liquide à haute performance couplée à la détection par fluorescence (CLHP-DFL)

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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 17251:2018) 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

Ochratoxins are a class of pentaketides molecules made up of dihydroisocumarin linked to β-phenylalanine. Ochratoxin A (OTA) is mainly produced by *Aspergillus ochraceus*, *A. carbonarius* and *A. niger* in tropical regions and by *Penicillium verrucosum* in temperate climates. Especially cereals and their derivatives are major contributors, but it is also found in a variety of food products from coffee to nuts, wine, beer, dried fruits, and spices. Ochratoxin A can also be detected in pork meat and pork based products.

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.

WARNING 3 — Ochratoxin A has been classified as substance of Group 2B by International Agency for Research on Cancer (IARC) meaning the existence of sufficient evidence of its renal carcinogenicity to animals and possibly to humans.

1 Scope

This document describes a procedure for the determination of ochratoxin A (OTA) in pork products specifically ham, pork based products (canned chopped pork) and pork liver using high performance liquid chromatography with fluorescence detection (HPLC-FLD).

The method has been validated for ochratoxin A with naturally contaminated ham, pork based products (canned chopped pork) and pork liver containing 0,5 µg/kg to 11 µg/kg [4, 5, 6].

Laboratory experiences have shown that this method is also applicable to pâté and kidney [4].

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

Ochratoxin A is extracted by mixing a test portion with a mixture of methanol and aqueous sodium hydrogen carbonate solution. The extract is centrifuged, diluted with a mixture of phosphate buffered saline (PBS) and a polysorbate 20 solution, and applied to an immunoaffinity column containing antibodies specific to OTA.

The purified extract is quantified by reverse-phase high performance liquid chromatography (RP-HPLC) coupled with fluorescence detection (FLD).

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. Commercially available solutions with equivalent properties to those listed may be used.

5.1 Methanol, (CH_3OH) technical grade.

5.2 Methanol, (CH_3OH) HPLC grade.

5.3 Acetonitrile, (CH_3CN) HPLC grade.

5.4 Glacial acetic acid, volume fraction $\varphi(CH_3COOH)$ approximately 99 %.

5.5 Toluene, UV grade.

5.6 Solvent mixture of toluene and glacial acetic acid.

Mixture of toluene (5.5) and glacial acetic acid (5.4) (99+1, v+v).

5.7 Sodium hydrogen carbonate ($NaHCO_3$), minimum 99 % purity.

5.8 Sodium chloride (NaCl), minimum 99 % purity.

5.9 Disodium hydrogen ortophosphate ($Na_2HPO_4x12H_2O$), minimum 99 % purity.

5.10 Potassium dihydrogen phosphate (KH_2PO_4), minimum 99 % purity.

5.11 Potassium chloride (KCl), minimum 99 % purity.

5.12 Sodium hydroxide (NaOH), minimum 99 % purity.

5.13 Hydrochloric acid solution (HCl), φ(HCl) = 37 % (acidimetric).

5.14 Hydrochloric acid solution, c(HCl) = 0,1 mol/l.

Dilute 8,28 ml of hydrochloric acid solution (5.13) to 1 l with water.

5.15 Sodium hydroxide solution, substance concentration c(NaOH) = 0,2 mol/l.

Dissolve 8,0 g NaOH (5.12) into 1 l volumetric flask (6.11) and fill up to the mark with water.

5.16 Acetic acid solution, mass concentration $\rho(CH_3COOH)$ = 20 g/l (2 %).

Add 20 g of glacial acetic acid (5.4) to l000 ml of water.

5.17 Phosphate buffered saline (PBS), pH = 7,4.

Weigh with the analytical balance (6.2) 8,0 g of NaCl (5.8), 2,9 g of $Na_2HPO_4x12H_2O$ (5.9), 0,2 g of KH_2PO_4 (5.10) and 0,2 g of KCl (5.11) and transfer into a 1 l volumetric flask (6.11). Dissolve in water and add 900 ml of water.

After dissolution adjust the pH to 7,4 with hydrochloric acid solution (5.14) or sodium hydroxide solution (5.15) as appropriate, then fill up to the mark with water.

Alternatively, a PBS solution with equivalent properties may be prepared from commercially available PBS material.

5.18 Polysorbate 20, e.g. Tween® 20[1] **($C_{18}H_{34}O_6$),** lauric acid ≥ 40 %.

5.19 PBS 0,01 %/Polysorbate solution, ρ(Tween®20) = 0,1 g/l (0,01 %).

Weigh 100 mg of Polysorbate 20 (5.18) with the analytical balance (6.2), transfer quantitatively into a 1 l volumetric flask (6.11) and fill up to the mark with PBS solution (5.17).

[1] Tween 20 is a trade name of a polysorbate 20-type nonionic surfactant supplied by Croda Americas. This information is given for the convenience of users of this European standard and does not constitute an endorsement by CEN of this product. Equivalent products may be used if they can be shown to lead to the same results.

5.20 Sodium hydrogen carbonate solution ($NaHCO_3$), $\rho(NaHCO_3)$ = 10,0 g/l (1 %).

Add 10 g of sodium hydrogen carbonate (5.7) into a 1 l volumetric flask (6.11) and fill up to the mark with water.

5.21 Extraction solvent.

Mix methanol (5.1) and sodium hydrogen carbonate solution (5.20) (3+2, v+v).

5.22 HPLC mobile phase.

Mix methanol (5.2), acetonitrile (5.3) and acetic acid solution (5.16) (25+35+40, v+v+v).

5.23 Injection solvent.

Mix methanol (5.2) and water. (1+1, v+v)

5.24 Immunoaffinity column (IAC).

The immunoaffinity column (IAC) contains antibodies raised against ochratoxin A. The IAC shall have a capacity of not less than 100 ng of ochratoxin A and shall give a recovery of not less than 85 % when applied as a standard solution of ochratoxin A in a mixture of 3 volume of methanol (5.1) and 17 volumes of PBS solution (5.17) containing 3 ng of OTA. Immunoaffinity columns shall be stored in the refrigerator (+4 °C) and shall be allowed to equilibrate at room temperature before use.

5.25 Ochratoxin A (OTA), e.g. crystalline or as a film, purity greater than 98 % mass fraction, or as certified standard solution.

5.26 Stock solution of ochratoxin A, ρ = 20 µg/ml.

Dissolve OTA in crystal form (5.25) or the contents of 1 ampoule (if OTA has been obtained as a film) in solvent mixture (5.6) to give a solution containing approximately 20 µg/ml to 30 µg/ml of OTA.

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

To determine the exact concentration, record the absorption curve between a wavelength of 300 nm and 370 nm in 5 nm steps in 1 cm quartz cells with an UV spectrometer (6.13) and solvent mixture (5.6) as reference.

Identify the wavelength for maximum absorption and calculate the mass concentration of ochratoxin A, ρ, in micrograms per millilitre, according to Formula (1):

$$\rho = \frac{E_{max} \times M \times 100}{\delta \times \varepsilon} \qquad (1)$$

where

E_{max} is the maximum extinction value determined from the absorption curve (here: 333 nm);

M is the molar mass of ochratoxin A, in g/mol (here: 403,8 g/mol);

ε is the molar absorption coefficient of ochratoxin A in solvent mixture (5.6), in m^2/mol (here: 544 m^2/mol);

δ is the path length of the quartz cell, in cm.

This solution can be used for approximately 12 months if stored in the freezer at −18 °C. Allow to reach room temperature before opening. Confirm the concentration of the solution if it is older than six months.

5.27 Ochratoxin A standard solution, mass concentration ρ = 100 ng/ml.

Dilute the stock solution (5.26) or a certified solution of ochratoxin A (5.25) with the injection solvent (5.23) to obtain a standard solution with a mass concentration of OTA of 100 ng/ml. This solution is stable for at least one month if stored in the refrigerator at 4 °C.

5.28 Calibration solutions of ochratoxin A for HPLC.

Prepare six HPLC calibration solutions from the standard solution (5.27).

With appropriate calibrated pipettes or microlitre pipettes (6.4) transfer e.g. the volumes of the ochratoxin A standard solution (5.27) separately each into volumetric flask as listed in Table 1. Fill each volumetric flask up to the mark with injection solvent (5.23), close and mix manually. This will result in six ochratoxin A solutions with approximately the concentrations listed in Table 1. These six solutions cover a range from approximately 0,10 ng/ml to 4,5 ng/ml which cover a range from 0,32 µg/kg to 14,40 µg/kg for ochratoxin A.

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 freezer at −18 °C.

Table 1 — Examples of suitable calibration solutions

Calibration solution	**Standard solution (5.27)** µl	**Final volume** ml	Mass concentration of calibration solution[a] ng/ml	**Contamination level** µg/kg[a]
1	10	10	0,10	0,32
2	50	25	0,20	0,64
3	50	10	0,50	1,60
4	50	5	1,00	3,20
5	125	5	2,50	8,00
6	225	5	4,50	14,40

[a] Nominal concentrations are calculated taking into account the mass concentration of the OTA standard solution (5.27), 100,0 ng/ml; corresponding contamination levels are calculated applying the method as described and injection volume 100 µl.

The exact mass concentrations of ochratoxin A in the ochratoxin A calibration solutions are calculated from the initial concentration of the stock solution (5.26) and the subsequent volumes used.

Transfer calibration solutions (5.28) into LC-vials (6.10) before injection.

6 Apparatus and equipment

Usual laboratory apparatus and, in particular, the following:

6.1 Laboratory balance, accuracy: 0,01 g.

6.2 Analytical balance, accuracy: 0,1 mg.

6.3 Laboratory shaker for test tubes and **horizontal shaker.**

6.4 Manual dispensers or microlitre pipettes for 10 µl to 20 ml with appropriate tips.

6.5 Dispenser, suitable for 20 ml.

6.6 Disposable syringe barrels and syringe, to be used as reservoirs of 50 ml capacity and 10 ml capacity, Luer locks and attachments to fit to immunoaffinity columns.

6.7 Glass microfibre filter paper, 125 mm diameter, 1,6 µm retention size, or equivalent.

6.8 Centrifuge, suitable for relative centrifugal force of at least 3 500 g[2].

6.9 Centrifuge tubes, 50 ml, made of polypropylene with screw cap.

6.10 LC vials.

6.11 Volumetric flasks, of various capacities (e.g. 5 ml, 10 ml, 25 ml, 1 l).

6.12 SPE vacuum manifold/elution system.

6.13 UV-spectrometer with quartz cuvettes.

6.14 HPLC-FLD system, with the following components:

6.14.1 LC pump, capable of maintaining a volume flow of 1 ml/min.

6.14.2 Injection system.

6.14.3 LC column, e.g. C18 RP-column, fully endcapped and with column dimension preferably 150 mm × 4,6 mm I.D. stationary phase with particle size 5 µm, or equivalent.

6.14.4 Guard column, with the same stationary phase material or similar as the analytical column.

6.14.5 Column oven.

6.14.6 Fluorescence detector.

6.14.7 Data evaluation system.

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