SLOVENSKI STANDARD
SIST EN 17251:2020

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

Foodstuffs - Determination of ochratoxin A in pork meat and derived products by IAC clean-up and 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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67.120.10 Meso in mesni proizvodi Meat and meat products

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Foodstuffs - Determination of ochratoxin A in pork meat and derived products by IAC clean-up and HPLC-FLD

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EUROPEAN COMMITTEE FOR STANDARDIZATION
COMITÉ EUROPÉEN DE NORMALISATION
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European foreword

This document (EN 17251:2020) 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of an identical text or by endorsement, at the latest by July 2020, and conflicting national standards shall be withdrawn at the latest by July 2020.

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Introduction

Ochratoxins are a class of pentaketide molecules made up of dihydroisocoumarin 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. It is found in a variety of food products, especially cereals and their derivatives which are major contributors to exposure, but it is also found in coffee, 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 in 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 https://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 ochratoxin A.

The purified extract is analyzed by reversed-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. Solutions shall be of quality for LC analysis, unless otherwise specified. Commercially available solutions with equivalent properties to those listed may also 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 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 phosphate ($Na_2HPO_4 \cdot 12H_2O$), 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 (HCl), φ(HCl) = 37 % (acidimetric).

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

Dilute 8,28 ml of hydrochloric acid (5.13) to 1000 ml with water in a 1 l volumetric flask.

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

Dissolve 8,0 g sodium hydroxide (5.12) in a 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 %).

Dilute 20 g of glacial acetic acid (5.4) to 1000 ml with water in a 1 l volumetric flask.

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

Weigh 8,0 g of sodium chloride (5.8), 2,9 g of disodium hydrogen phosphate (5.9), 0,2 g of potassium dihydrogen phosphate (5.10) and 0,2 g of potassium chloride (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], lauric acid ≥ 40 %.

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

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

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1 Tween®20 is a trade name of a polysorbate 20-type nonionic surfactant available from various suppliers. 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 solution.

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 solution.

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 volumes of methanol (5.1) and 17 volumes of PBS solution (5.17) containing 3 ng of ochratoxin A. Immunoaffinity columns shall be stored following the instructions of the producers and shall, if necessary, 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 Ochratoxin A stock solution, ρ = 20 µg/ml.

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

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 mixture (5.6) as reference.

Identify the wavelength for maximum absorption and calculate the mass concentration of ochratoxin A, ρ, in µg/ml, according to Formula (1):

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

where

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

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

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

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

This solution can be used for 12 months if stored at −18 °C. Allow to reach room temperature before opening. Confirm the concentration when it is used after 12 months.

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

Dilute the stock solution (5.26) or a certified solution of ochratoxin A (5.25) with the injection solution (5.23) to obtain a standard solution with a mass concentration of ochratoxin A 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.

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

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 solution (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 0,32 µg/kg to 14,4 µg/kg.

The calibration range shall be adapted to the desired working range.

The calibration solutions can be used for approximately 1 month if stored 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	14,4

[a] Nominal concentrations are calculated taking into account the mass concentration of the ochratoxin A 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 glassware and equipment 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 centrifuge tubes.

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*.

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

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, 15 ml, 1 l).

6.12 SPE vacuum manifold/elution system.

6.13 UV-spectrometer with quartz cuvettes.

6.14 HPLC-FLD system, comprising the following:

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 of 150 mm or 250 mm × 4,6 mm I.D. stationary phase with particle size 5 µm, or equivalent.

6.14.4 Pre-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.

7 Procedure

7.1 Preparation of the test sample

Finely grind the laboratory sample, homogenize before analysis. If necessary, store at approximately −18 °C.

7.2 Extraction of ochratoxin A

7.2.1 General

Weigh 5,0 g [m] of the sample to the nearest 0,1 g into a 50 ml centrifuge tube (6.9).

Add 20 ml [V_1] of extraction solution (5.21) with a suitable dispenser (6.5). Shake vigorously for 5 s and then shake for approximately 40 min on a shaker (6.3).

To separate the phases, centrifuge (6.8) the tubes for 10 min at approximately 3 500 *g* at room temperature.