# SLOVENSKI STANDARD
# SIST EN 17250:2020

**01-marec-2020**

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**Živila - Določevanje ohratoksina A v začimbah, sladkem korenu, kakavu in kakavovih proizvodih z IAC-čiščenjem in HPLC-FLD**

Foodstuffs - Determination of ochratoxin A in spices, liquorice, cocoa and cocoa products by IAC clean-up and HPLC-FLD

Lebensmittel - Bestimmung von Ochratoxin A in Gewürzen, Süßholz, Kakao und Kakaoerzeugnissen nach IAC-Reinigung mit HPLC-FLD



Produits alimentaires - Dosage de l'ochratoxine A dans les épices, la réglisse, les produits à base de réglisse, le cacao et les produits à base de cacao par purification sur colonne d'immuno-affinité et CLHP-DFL



**Ta slovenski standard je istoveten z:** **EN 17250:2020**

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| 67.140.30 | Kakav | Cocoa |
| 67.220.10 | Začimbe | Spices and condiments |

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EUROPEAN STANDARD

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English Version

# Foodstuffs - Determination of ochratoxin A in spices, liquorice, cocoa and cocoa products by IAC clean-up and HPLC-FLD

Produits alimentaires - Dosage de l'ochratoxine A dans les épices, la réglisse, les produits à base de réglisse, le cacao et les produits à base de cacao par purification sur colonne d'immuno-affinité et CLHP-DFL

Lebensmittel - Bestimmung von Ochratoxin A in Gewürzen, Süßholz, Kakao und Kakaoerzeugnissen nach IAC-Reinigung mit HPLC-FLD

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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 (EN 17250:2020) 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of an identical text or by endorsement, at the latest by July 2020, and conflicting national standards shall be withdrawn at the latest by July 2020.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

The mycotoxin ochratoxin A has a chemical structure comprising a dihydrocoumarin moiety linked to a molecule of L-β-phenylalanine via an amide bond. Ochratoxin A is produced by several fungal species in the Penicillium and Aspergillus genera, primarily *Penicillium verrucosum*, *Aspergillus ochraceus* and *Aspergilli* of the section *Nigri*, especially *A. carbonarius*. Cereals such as wheat are especially affected, as well as a diverse range of other foodstuffs such as dried fruit, spices, cocoa, coffee, wine, beer, liquorice and products thereof.

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 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 — Ochratoxin A is a potent nephrotoxic agent, a carcinogen and has genotoxic properties. Ochratoxin A has been classified by IARC as Group 2B.

# 1 Scope

This document specifies a procedure for the determination of ochratoxin A (OTA) in chilli, paprika, black and white pepper, nutmeg, spice mix, liquorice (root and extracts), cocoa and cocoa products by high performance liquid chromatography (HPLC) with immunoaffinity column clean-up and fluorescence detection (FLD).

This method has been validated in interlaboratory studies via the analysis of both naturally contaminated and spiked samples ranging from 1,0 µg/kg to 84,9 µg/kg for spices (paprika and chili [5], black and white pepper, nutmeg and spice mix [6]), ranging from 7,7 µg/kg to 96,8 µg/kg for liquorice and liquorice products [7] and ranging from 2,1 µg/kg to 26,3 µg/kg for cocoa and cocoa products [6].

For further information on the validation, see Clause 10 and Annex B.

# 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/ui



# 4 Principle

Spices or liquorice and liquorice products are extracted with a mixture of methanol and aqueous sodium hydrogen carbonate solution, whereas cocoa and cocoa products are extracted with aqueous methanol. The extract is filtered, diluted with phosphate buffered saline (PBS), polysorbate 20 (except for liquorice and liquorice products), and applied to an immunoaffinity column containing antibodies specific to ochratoxin A. The ochratoxin A is isolated, purified and concentrated on the column then released using methanol. The purified extract is quantified 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. Commercially available solutions with equivalent properties to those listed may be used.

**5.1** **Nitrogen**, minimum 99,95 % purity.

**5.2** **Methanol**, technical grade.

**5.3** **Methanol**, HPLC grade.

**5.4** **Acetonitrile**, HPLC grade.

**5.5 Glacial acetic acid**, 99 % purity.

**5.6 Toluene**, UV grade.

**5.7 Sodium hydrogen carbonate**, minimum 99,5 % purity.

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

**5.9 Disodium hydrogen phosphate dodecahydrate ($Na_2HPO_4 \cdot 12\ H_2O$)**, 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**, volume fraction $\varphi$(HCl) = 37 % (acidimetric).

**5.14 Hydrochloric acid solution**, substance concentration $c$(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**, $c$(NaOH) = 0,2 mol/l.



Dissolve 8 g of sodium hydroxide (5.12) in 1 l of water.

**5.16 Acetic acid solution**, mass concentration $\rho(CH_3COOH)$ = 10 g/l.



Dilute 9,5 ml of glacial acetic acid (5.5) to 1 l with water.

**5.17 Polysorbate 20**

**5.18 Polysorbate 20 solution**, $\rho$(Tween® 20[1]) = 20 g/l.

Dissolve 20 g of Polysorbate 20 (5.17) in 1 000 ml of water.

**5.19 Phosphate buffered saline solution (PBS)**, pH = 7,4.

Dissolve 8 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) in 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 dilute to 1 l with water.

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

**5.20 Sodium hydrogen carbonate solution**, $\rho(NaHCO_3)$ = 30 g/l.

Dilute 30 g of sodium hydrogen carbonate (5.7) in 1 000 ml of water.

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

**5.21 Extraction solution A** (for spices, liquorice and liquorice products).

Mix methanol (5.2) with sodium hydrogen carbonate solution (5.20) (50+50, v+v). Mix well.

**5.22 Extraction solution B** (for cocoa and cocoa products).

Mix methanol (5.2) with water (80+20, v+v). Mix well.

**5.23 Mobile phase A** (for paprika and chilli).

Mix methanol (5.3) with acetonitrile (5.4), water and glacial acetic acid (5.5) (35+35+29+1, v+v+v+v).

**5.24 Mobile phase B** (for liquorice and liquorice products).

Mix methanol (5.3) with water and glacial acetic acid (5.5) (70+30+1, v+v+v).

**5.25 Mobile phase C** (for black and white pepper, nutmeg, spice mix, cocoa and cocoa products).

Mix methanol (5.3), acetonitrile (5.4), water and glacial acetic acid (5.5) (28+28+39+1, v+v+v+v).

**5.26 Mobile phase D** (HPLC column washing solution for liquorice and liquorice products).

100 % methanol (5.3).

**5.27 Immunoaffinity column**

The immunoaffinity column contains antibodies raised against ochratoxin A. The column 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 15 parts per volume of methanol (5.2) and 85 parts per volume of PBS solution (5.19) containing 3 ng of ochratoxin A.



**5.28 Ochratoxin A**, in crystal form or as a film in ampoules or as a certified standard solution.



**5.29 Ochratoxin A stock solution**, $\rho$(ochratoxin A) = 10 µg/ml.

Prepare a stock solution of ochratoxin A (5.28) in a mixture of toluene (5.6) and glacial acetic acid (5.5) in ratio 99 + 1 (v+v) with a nominal concentration of 10 µg/ml.

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 in a spectrometer with the solvent mixture (toluene + glacial acetic acid, 99 + 1, v+v) as reference. Identify the wavelength for maximum absorption and calculate the mass concentration of ochratoxin A, $\rho$, in µg/ml, using Formula (1):

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

where

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

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

$\varepsilon$ is the molar absorption coefficient of ochratoxin A in the solvent mixture (toluene + glacial acetic acid: 99+1, v+v), in m²/mol (here: 544 m²/mol);

$b$ is the path length of the quartz cell, in cm.

This solution can be used for 6 months if stored at approximately −18 °C. Allow to reach room temperature before opening. Confirm the mass concentration of the solution if it is older than 6 months.

This step may be omitted when using a certified standard solution, provided that the product comes with a certificate, giving sufficient evidence on the correctness of the stated mass fraction. The certified standard solution then serves as stock solution.

The exact mass concentrations of ochratoxin A in the standard, spiking and calibration solutions are calculated from the initial concentration of this stock solution and the subsequent volumes used.

**5.30 Ochratoxin A standard solution**, $\rho$(ochratoxin A) = 1 µg/ml.

Pipette (6.4) 100 µl of ochratoxin A stock solution (5.29) into a 1 ml volumetric flask (6.10), dry by a gentle flow of nitrogen (5.1), then dilute to 1 ml (up to the mark) with the appropriate mobile phase (5.23, 5.24 or 5.25) and shake it vigorously. This gives a standard solution containing 1 µg/ml of ochratoxin A. This solution can be used for 6 months if stored at approximately −18 °C. Allow to reach room temperature before opening. Confirm the mass concentration of the solution if it is older than 6 months.

**5.31 Ochratoxin A spiking solution**, $\rho$(ochratoxin A) = 400 ng/ml.

Pipette 1 ml of ochratoxin A stock solution (5.29) into a 25 ml volumetric flask (6.10) and dilute to the mark with a mixture of acetonitrile (5.4) and glacial acetic acid (5.5) in a ratio of 99 + 1, v+v, and shake. This gives a spiking solution containing 400 ng/ml of ochratoxin A.

This solution can be used for 6 months if stored at approximately −18 °C. Allow to reach room temperature before opening. Confirm the mass concentration of the solution if it is older than 6 months.

**5.32 Calibration solutions**



Prepare six calibration solutions from the standard solution (5.30) as follows:

With appropriate pipettes (6.4) transfer e.g. the volumes of the ochratoxin A standard solution (5.30) separately each into volumetric flasks as specified in Table 1. Fill each volumetric flask up to the mark with the appropriate mobile phase (5.23, 5.24 or 5.25), close and shake manually. This results in six ochratoxin A calibration solutions with approximately the concentrations as listed in Table 1.



These six calibration solutions cover a range from approximately 1,2 µg/kg to approximately 100 µg/kg for ochratoxin A for all spices, cocoa and cocoa products and from approximately 2,4 µg/kg to approximately 200 µg/kg for liquorice and liquorice products.

Protect calibration solutions from light. These solutions can be used for 1 month if stored at approximately −18 °C.

**Table 1 — Preparation of calibration solutions**

| Calibration solution | Standard solution (5.30) µl | Final volume ml | Mass concentration of calibration solution ng/ml |
|---|---|---|---|
| 1 | 15 | 50 | 0,3 |
| 2 | 15 | 25 | 0,6 |
| 3 | 25 | 25 | 1 |
| 4 | 50 | 10 | 5 |
| 5 | 150 | 10 | 15 |
| 6 | 250 | 10 | 25 |

Transfer these calibration solutions into LC vials (6.9) before injection.

## 6 Apparatus and equipment

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

**6.1 Laboratory balance**, accuracy: 0,01 g.

**6.2 Analytical balance**, accuracy: 0,1 mg.

**6.3 Laboratory shaker**, and **shaker for centrifuge tubes**

**6.4 Pipettes**, e.g. 100 µl to 2 000 µl and 4 ml volumetric pipettes, suitable for organic solvents.

**6.5 Disposable syringe reservoir**, of 100 ml capacity, and attachments to fit to immunoaffinity columns.

**6.6 Glass microfibre filter paper**, 1,6 µm retention size, 150 mm diameter, or equivalent. As an alternative, filter paper (6.7) may be used when they have been proven to give equivalent results.

**6.7 Cellulose filter paper**, 11 µm pore size, 150 mm diameter.

**6.8 SPE vacuum manifold/elution station.**

**6.9 LC vials**, approximately 2 ml capacity, or 2 ml LC vial with insert, with crimp caps or equivalent.

**6.10 Volumetric flasks**, of various capacities (e.g. 1 ml, 2 ml, 5 ml, 10 ml, 25 ml, 50 ml).



**6.11 Conical flasks**, 100 ml or 500 ml with screw cap, or similar recipient.

**6.12 HPLC system**, comprising the following:



**6.12.1 HPLC pump**, gradient, capable of maintaining a volume flow rate of 0,8 ml/min pulse free and 1,0 ml/min pulse free.

**6.12.2 Injection system.**

**6.12.3 Pre-column**, of suitable dimensions, with stationary phase material the same or similar to the analytical column.

**6.12.4 Reversed-phase HPLC column.**

A suitable column and appropriate HPLC conditions (isocratic or gradient programme) with a retention factor of at least two that ensures base line separation to distinguish peaks of ochratoxin A from all other signals.

Some examples of columns which have been found to be suitable are given in Annex A.

**6.12.5 Degasser**, optional, for degassing mobile phases (5.23; 5.24; 5.25; 5.26).

**6.12.6 Column oven,** capable of maintaining a constant temperature.

**6.12.7 Fluorescence detector.**

**6.12.8 Data evaluation system.**