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Foodstuffs – Determination of ochratoxin A in wine and beer – HPLC method with immunoaffinity column clean up

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EUROPEAN STANDARD
NORME EUROPÉENNE
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EN 14133

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ICS 67.160.10

English version

Foodstuffs - Determination of ochratoxin A in wine and beer - HPLC method with immunoaffinity column clean-up

Produits alimentaires - Dosage de l'ochratoxine A présente dans le vin et la bière - Méthode par CLHP et par purification en colonne d'immunoafinité

Lebensmittel - Bestimmung von Ochratoxin A in Wein und Bier - HPLC-Verfahren mit Reinigung an einer Immunoaffinitätssäule

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Foreword

This document (EN 14133:2003) 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of an identical text or by endorsement, at the latest by January 2004, and conflicting national standards shall be withdrawn at the latest by January 2004.

Annex A is informative.

WARNING — Ochratoxin A is a potent nephrotoxin and liver toxin and has been reported to have immunosuppressant properties. It is classified by the International Agency for Research on Cancer (IARC) as possibly carcinogenic to humans (Group 2B). Acetonitrile is hazardous. Toluene is highly flammable and harmful. Observe appropriate safety precautions for handling such compounds. Gloves and safety glasses shall be worn at all times and all standard and sample preparation stages shall be carried out in a fume cupboard. Operation outside the fume cupboard, such as measurement of standards by UV spectrometer, shall be performed with the standard in closed containers. Decontamination procedures for laboratory wastes have been reported by the International Agency for Research on Cancer (IARC), see [1].

According to the CEN/CENELEC Internal Regulations, the national standards organizations of the following countries are bound to implement this European Standard: Austria, Belgium, Czech Republic, Denmark, Finland, France, Germany, Greece, Hungary, Iceland, Ireland, Italy, Luxembourg, Malta, Netherlands, Norway, Portugal, Slovakia, Spain, Sweden, Switzerland and the United Kingdom.

1 Scope

This European Standard specifies a method for the determination of ochratoxin A content in wine and beer using immunoaffinity column clean up and high performance liquid chromatography (HPLC), see [2], [3].

This method has been validated in an interlaboratory study according to AOAC International Guidelines [4] for collaborative study procedures to validate characteristics of a method of analysis for the determination of ochratoxin A in wine and beer via the analysis of naturally contaminated and spiked samples of wine and beer at levels ranging from 0,1 ng/ml to 3 ng/ml.

2 Normative references

This European Standard incorporates by dated or undated reference, provisions from other publications. These normative references are cited at the appropriate places in the text and the publications are listed hereafter. For dated references, subsequent amendments to or revisions of any of these publications apply to this European Standard only when incorporated in it by amendment or revision. For undated references the latest edition of the publication referred to applies (including amendments).

EN ISO 3696:1995, *Water for analytical laboratory use — Specification and test methods (ISO 3696:1987).*

3 Principle

Wine and beer samples are diluted with a solution containing polyethylene glycol (PEG) and sodium hydrogen carbonate, filtered and cleaned up by immunoaffinity column. Ochratoxin A is eluted with methanol and quantified by reversed-phase HPLC with fluorescence detection.

NOTE The use of PEG is essential to increase ochratoxin A recoveries and to reduce the number and intensity of other chromatographic peaks.

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4 Reagents

4.1 General

Unless otherwise stated, use only reagents of recognized analytical grade and only distilled water or water of grade 1 as defined in EN ISO 3696:1995.

Commercially available reagents with equivalent properties to the ones listed may be used.

4.2 Sodium chloride

4.3 Sodium hydrogen carbonate

1.4 Polyethylene glycol, (average molecular weight of 8000)

4.5 Methanol, HPLC grade

4.6 Acetonitrile, HPLC grade

4.7 Water, HPLC grade

4.8 Glacial acetic acid, $\varphi(CH_3COOH) \approx 99$ %

4.9 Diluting solution

Dissolve 10 g polyethylene glycol (4.4) and 50 g sodium hydrogen carbonate (4.3) in approximately 950 ml water and bring up to 1000 ml with water.

4.10 Washing solution

Dissolve 25 g sodium chloride (4.2) and 5 g sodium hydrogen carbonate (4.3) in approximately 950 ml water and bring up to 1000 ml with water.

4.11 HPLC mobile phase

Mix 990 ml HPLC water (4.7) with 990 ml acetonitrile (4.6) and 20 ml glacial acetic acid (4.8), filter through 0,45 µm filter and degas (e.g. with helium).

4.12 Toluene

4.13 Solvent mixture of toluene and glacial acetic acid

Mix 99 parts per volume of toluene (4.12) with 1 part per volume of glacial acetic acid (4.8).

4.14 Ochratoxin A stock solution

Dissolve 1 mg of ochratoxin A (in crystal form) or the contents of 1 ampoule (if ochratoxin A has been obtained as a film) in solvent mixture (4.13) to give a solution containing approximately 20 µg/ml to 30 µg/ml of ochratoxin A. 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 (5.10) and solvent mixture (4.13) as reference. Identify the wavelength for maximum absorption and calculate the mass concentration of ochratoxin A, ρ_{OTA}, in micrograms per millilitre, using equation (1):

$$\rho_{\mathrm{ota}} = \frac{\mathrm{A}_{\mathrm{max}} \times M \times 1000}{\varepsilon \times \delta} \qquad (1)$$

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where:

A_{max} is the absorption determined at the maximum of the absorption curve (here: at 333 nm);

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

ε is the relative molar absorption coefficient of ochratoxin A in solvent mixture (4.13) (here: ε = 5440 m^2/mol);

δ is the path length of the quartz cell in centimetres.

This solution is stable at –18 °C for at least 4 years.

4.15 Ochratoxin A standard solution

Dilute the stock solution (4.14) with solvent mixture (4.13) to obtain a standard solution with a mass concentration of ochratoxin A of 2 µg/ml. Store this solution in a refrigerator at approximately 4 °C and check the stability.

4.16 Ochratoxin A calibration solution

Pipette 0,5 ml of the 2 µg/ml ochratoxin A standard solution (4.15) into a 10 ml volumetric flask (5.3) and evaporate the solvent under a stream of nitrogen. Redissolve in 10 ml of mobile phase (4.11). This gives a mass concentration of 100 ng/ml ochratoxin A.

Prepare six HPLC calibrants in separate 5 ml volumetric flasks (5.3) according to Table 1. Dilute each solution to 5 ml with HPLC mobile phase (4.11).

Table 1 — Preparation of calibration solutions

	Std 1	Std 2	Std 3	Std 4	Std 5	Std 6
µl of filtered mobile phase (4.11)	4970	4900	4700	4000	3000	2000
µl of 100 ng/ml OTA solution	30	100	300	1000	2000	3000
OTA mass concentration (ng/ml)	0,6	2,0	6,0	20	40	60
injected ng OTA	0,06	0,20	0,60	2,00	4,00	6,00

Prepare the calibration solutions at the beginning of every day of the analysis.

4.17 Immunoaffinity columns

The immunoaffinity column shall contain antibodies raised against ochratoxin A. The column shall have a total binding capacity of not less than 100 ng of ochratoxin A and shall give a recovery of not less than 85 % when a diluted wine solution containing 100 ng of ochratoxin A is applied.

5 Apparatus

Usual laboratory equipment and, in particular, the following:

5.1 Microbalance, capable to measure 0,01 mg

5.2 Glass vials, approximately 4 ml, with polytetrafluoroethylene (PTFE)-lined screw cap, or appropriate sealable screw cap.

Certain types of vials can lead to losses of ochratoxin A during evaporation. To avoid this, silanization can be applied. Prepare vials by filling them with silanizing reagent and leave this reagent in the vial for one minute. Rinse the vial twice with appropriate solvent (toluene, acetone or hexane) followed by water (twice) and dry the vial.

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5.3 Volumetric flasks, 5 ml and 10 ml volume

5.4 Vacuum manifold, to accommodate immunoaffinity columns

5.5 Reservoirs and attachments, to fit to immunoaffinity columns

5.6 Glass microfibre filters, pore size 1,6 µm, (e.g. Whatman GF/A1) or equivalent)

5.7 Solvent evaporator

5.8 Calibrated microliter syringe(s) or microliter pipette(s)

5.9 HPLC apparatus consisting of

5.9.1 Injection system, a syringe-loading injection valve with 100 µl injection loop or equivalent

5.9.2 HPLC pump, isocratic, capable of maintaining a volume flow rate of 1,0 ml/min

5.9.3 Analytical reverse phase separating column, for example stainless steel (150 mm length, 4,6 mm inner diameter) packed with 5 µm C_{18} reverse-phase material preceded by a suitable corresponding reverse-phase guard column or guard filter (0,5 µm). Columns of different dimensions can be used provided that they ensure a baseline resolution of the ochratoxin A peak from all other peaks.

5.9.4 Fluorescence detector, fitted with a flow cell and set at 333 nm (excitation) and 460 nm (emission). Detection of at least 0,02 ng of ochratoxin A shall be possible.

5.9.5 Data system

5.10 UV spectrometer, with suitable quarz cells

6 Procedure

6.1 Sample preparation

Degas sparkling wine and beer prior to dilution. Pour 10 ml of wine (beer) into a sealable 100 ml conical flask. Add 10 ml of the diluting solution (4.9) and mix vigorously. Filter through glass microfibre filter (5.6) when cloudy solutions or solid residues are formed after dilution.

If there is a problem with degassing, degassing should be performed by sonicating samples for 1 h, previously cooled at +4 °C for 30 min to prevent fast foam formation.

6.2 Immunoaffinity column clean-up

Prepare the immunoaffinity column according to the suppliers instructions.

Connect the immunoaffinity column (4.17) to the vacuum manifold (5.4) and attach the reservoir (5.5) to the immunoaffinity column. Add 10 ml (equivalent to 5 ml wine or beer) of the diluted solution to the reservoir and pass through the immunoaffinity column at a flow rate of about 1 drop per second. The immunoaffinity column shall not be allowed to run dry. Wash the immunoaffinity column with 5 ml of washing solution (4.10) and then with 5 ml of water at 1 to 2 drops per second flow rate. Dry the column by passing air through it. Remove the immunoaffinity column from the vacuum manifold and place it over a vial (5.2).

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[1] Whatman is an example of a suitable product available commercially. This information is given for the convenience of users oh this European Standard and does not constitute an endorsement by CEN of these products. Equivalent products can be used if they can be shown to give equivalent results.