BRITISH STANDARD

BS EN 14133:2009

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

ICS 67.160.10

BSI British Standards

National foreword

This British Standard is the UK implementation of EN 14133:2009. It supersedes BS EN 14133:2003 which is withdrawn.

The UK participation in its preparation was entrusted to Technical Committee AW/-/3, Food analysis - Horizontal methods.

A list of organizations represented on this committee can be obtained on request to its secretary.

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This British Standard was published under the authority of the Standards Policy and Strategy Committee on 30 June 2009.

ISBN 978 0 580 64237 1

Amendments/corrigenda issued since publication

Date	Comments

EUROPEAN STANDARD

NORME EUROPÉENNE

EUROPÄISCHE NORM

EN 14133

May 2009

ICS 67.160.10

Supersedes EN 14133:2003

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 dans le vin et la bière - Méthode par purification sur colonne d'immuno-affinité suivie d'une analyse par chromatographie liquide haute performance (CLHP)

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

This European Standard was approved by CEN on 24 May 2009.

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COMITÉ EUROPÉEN DE NORMALISATION
EUROPÄISCHES KOMITEE FÜR NORMUNG

Management Centre: Avenue Marnix 17, B-1000 Brussels

Ref. No. EN 14133:2009: E

Contents

Foreword

This document (EN 14133:2009) 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of an identical text or by endorsement, at the latest by November 2009, and conflicting national standards shall be withdrawn at the latest by November 2009.

Attention is drawn to the possibility that some of the elements of this document may be the subject of patent rights. CEN [and/or CENELEC] shall not be held responsible for identifying any or all such patent rights.

This document will supersede EN 14133:2003.

The 2003 version has been updated with the inclusion of the corrigendum and some minor editorial improvements.

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].

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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] and [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

The following referenced documents are indispensable for the application 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 3696:1987)*

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.

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.

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

4.2 Sodium chloride

4.3 Sodium hydrogen carbonate

4.4 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 of 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 of 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):

$$\rho_{ota} = \frac{A_{max} \times M \times 100}{\varepsilon \times b} \qquad (1)$$

where

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

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

ε is the molar absorption coefficient of ochratoxin A in the solvent mixture (4.13), (here: 544 m^2/mol);

b is the optical 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 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 1 min. Rinse the vial twice with appropriate solvent (toluene, acetone or hexane) followed by water (twice) and dry the vial.

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/A[1] or equivalent)

5.7 Solvent evaporator

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

5.9 HPLC apparatus consisting of

[1] Whatman is an example of a suitable product available commercially. This information is given for the convenience of users of 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.

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).

6.3 Preparation of the sample test solution

Elute ochratoxin A into the vial by passing 2 ml of methanol (4.5) at 1 drop per second flow rate. Evaporate the eluate to dryness under a stream of nitrogen e.g. at approximately 50 °C. Redissolve in 250 µl HPLC mobile phase (4.11) and store at 4 °C until HPLC analysis. This is the sample test solution.

7 HPLC analysis

7.1 Sample analysis

Inject 100 µl of reconstituted extract (equivalent to 2 ml wine or beer) into the chromatographic apparatus using the mobile phase (4.11) at flow rate of 1,0 ml/min.

7.2 Calibration curve

Prepare a calibration curve at the beginning of every day of the analysis and whenever chromatographic conditions change.

Inject 100 µl of each calibrant solution (4.16) into the HPLC apparatus and plot peak area or peak height values of ochratoxin A calibrant solutions against the ochratoxin A masses in nanograms.

If the content of ochratoxin A in the samples falls outside the calibration range, appropriate dilution shall be performed, or lower volumes can be injected; in these cases calculation shall be reconsidered accordingly.

Due to the wide range of concentrations, the calibration curve should be forced through the “zero” for a more accurate quantification at low ochratoxin A concentrations (< 0,1 ng/ml).

7.3 Identification

Identify the analyte by comparing the retention time of the relevant peak in the sample with the peak of the standard substance in the chromatogram.

Sometimes it can be necessary to identify the peak by simultaneous injection of sample test solution and standard solution.

7.4 Determination

To carry out the determination by the external standard method, integrate the peak area or determine the peak height, and compare the results with the corresponding values for the standard substance with the nearest peak area or height, or use a calibration curve. In the case of a calibration curve, additional solutions with concentrations within the linear range may be prepared for the calibration curve.

Inject equal volumes of sample test solution and standard solution used for the calibration curve.

If the ochratoxin A response of the sample is outside the calibration range, adjust the amount of sample injected by diluting the sample test solution.

8 Calculation

Read off from the calibration curve, the amount in nanogram of ochratoxin A in the aliquot of test solution injected into the HPLC column.

Calculate the mass concentration ochratoxin A, ρ_{OTA}, in nanogram per milliliter using Equation (2):

$$\rho_{OTA} = \frac{2 \times m_{OTA} \times V_3}{V_1 \times V_2} \quad (2)$$

where

m_{OTA} is the mass of ochratoxin A in the aliquot of sample test solution injected on column determined from the calibration curve, in nanogram;

2 is the dilution factor;

V_1 is the volume of sample to be analysed, in millilitre (here: 10 ml);

V_2 is the volume of test solution injected on column, in microlitre (here: 100 µl);

V_3 is the volume of mobile phase used to dissolve the dried eluate, in microlitre (here: 250 µl).

9 Precision

9.1 Interlaboratory test

Details of an interlaboratory test on the precision of the method are given in Annex A. The values derived from this interlaboratory test may not be applicable to concentration ranges and matrices other than those given in Annex A.

9.2 Repeatability

The absolute difference between two single test results found on identical test material by one operator using the same apparatus within the shortest feasible interval will exceed the repeatability limit *r* in not more than 5 % of the cases.

The values for white wine are:

$\bar{x}$ = 0,105 ng/ml	r = 0,028 ng/ml	(fortified)
$\bar{x}$ = 0,998 ng/ml	*r* = 0,196 ng/ml	*(fortified)*
$\bar{x}$ = 1,764 ng/ml	*r* = 0,420 ng/ml	*(fortified)*
$\bar{x}$ = 0,283 ng/ml	*r* = 0,084 ng/ml	*(naturally contaminated)*

The values for red wine are:

$\bar{x}$ = 0,186 ng/ml	*r* = 0,028 ng/ml	*(fortified)*
$\bar{x}$ = 0,813 ng/ml	*r* = 0,224 ng/ml	*(fortified)*
$\bar{x}$ = 2,530 ng/ml	*r* = 0,616 ng/ml	*(fortified)*
$\bar{x}$ = 1,690 ng/ml	*r* = 0,504 ng/ml	*(naturally contaminated)*

The values for beer are:

$\bar{x}$ = 0,190 ng/ml	*r* = 0,056 ng/ml	*(fortified)*
$\bar{x}$ = 0,696 ng/ml	*r* = 0,140 ng/ml	*(fortified)*
$\bar{x}$ = 1,403 ng/ml	*r* = 0,196 ng/ml	*(fortified)*
$\bar{x}$ = 0,070 ng/ml	*r* = 0,028 ng/ml	*(naturally contaminated)*

9.3 Reproducibility

The absolute difference between two single test results found on identical test material reported by two laboratories will exceed the reproducibility limit *R* in not more than 5 % of the cases.

The values for white wine are:

$\bar{x}$ = 0,105 ng/ml	*R* = 0,056 ng/ml	*(fortified)*
$\bar{x}$ = 0,998 ng/ml	*R* = 0,364 ng/ml	*(fortified)*
$\bar{x}$ = 1,764 ng/ml	*R* = 0,644 ng/ml	*(fortified)*
$\bar{x}$ = 0,283 ng/ml	*R* = 0,112 ng/ml	*(naturally contaminated)*

The values for red wine are:

$\bar{x}$ = 0,186 ng/ml	*R* = 0,056 ng/ml	*(fortified)*
$\bar{x}$ = 0,813 ng/ml	*R* = 0,280 ng/ml	*(fortified)*
$\bar{x}$ = 2,530 ng/ml	*R* = 0,980 ng/ml	*(fortified)*
$\bar{x}$ = 1,690 ng/ml	*R* = 0,644 ng/ml	*(naturally contaminated)*

The values for beer are:

$\bar{x}$ = 0,190 ng/ml	*R* = 0,112 ng/ml	*(fortified)*
$\bar{x}$ = 0,696 ng/ml	*R* = 0,364 ng/ml	*(fortified)*
$\bar{x}$ = 1,403 ng/ml	*R* = 0,588 ng/ml	*(fortified)*
$\bar{x}$ = 0,070 ng/ml	*R* = 0,056 ng/ml	*(naturally contaminated)*

10 Test report

The test report shall contain the following data:

a) all information necessary for the identification of the sample (kind of sample, origin of sample, designation);

b) reference to this European Standard;

c) date and type of sampling procedure (if known);

d) date of receipt;

e) date of test;

f) test results and units in which they have been expressed;

g) any particular points observed in the course of the test;

h) any operations not specified in the method or regarded as optional, which might have affected the results.

Annex A
(informative)

Precision data

The data in Tables A.1 to A.3 were obtained in an interlaboratory test [5] according to AOAC Guidelines for collaborative study procedures to validate characteristics of a method of analysis [4].

Table A.1 — Precision data for white wine

WHITE WINE	**blank**	**spiked with OTA** (ng/ml)			**n.c.**[b]
		0,100	1,100	2,000	
Year of inter-laboratory test	1999	1999	1999	1999	1999
Number of laboratories	16	16	16	16	16
Number of laboratories retained after eliminating outliers	14[a]	13[a]	14	14	15
Number of outliers (laboratories)	-	1	2	2	1
Number of accepted results	28	26	28	28	30
Mean value, ng/ml	< 0,01	0,105	0,998	1,764	0,283
Repeatability standard deviation s_r, ng/ml	-	0,01	0,07	0,15	0,03
Repeatability relative standard deviation RSD_r, %	-	7,9	6,6	8,4	10,8
Repeatability limit r ($r = 2{,}8 \times s_r$), ng/ml	-	0,028	0,196	0,420	0,084
Reproducibility standard deviation s_R, ng/ml	-	0,02	0,13	0,23	0,04
Reproducibility relative standard deviation RSD_R, %	-	15,9	13,3	13,1	14,6
Reproducibility limit R ($R = 2{,}8 \times s_R$), ng/ml	-	0,056	0,364	0,644	0,112
Recovery, %	-	105,4	90,7	88,2	-

[a] Two laboratories have been excluded from the statistical evaluation because of the high detection limit (= 0,2 ng/ml).

[b] n.c. = naturally contaminated sample

Table A.2 — Precision data for red wine

RED WINE	**blank**	**spiked with OTA** (ng/ml)			**n.c.**[b]
		0,200	0,900	3,000	
Year of inter-laboratory test	1999	1999	1999	1999	1999
Number of laboratories	15	15	15	15	15
Number of laboratories retained after eliminating outliers	14[a]	12[a]	14	15	14
Number of outliers (laboratories)	-	2	1	-	1
Number of accepted results	28	24	28	30	28
Mean value, ng/ml	<0,01	0,186	0,813	2,530	1,690
Repeatability standard deviation s_r, ng/ml	-	0,01	0,08	0,22	0,18
Repeatability relative standard deviation RSD_r, %	-	6,5	9,9	8,9	10,8
Repeatability limit r ($r = 2{,}8 \times s_r$), ng/ml	-	0,028	0,224	0,616	0,504
Reproducibility standard deviation s_R, ng/ml	-	0,02	0,10	0,35	0,23
Reproducibility relative standard deviation RSD_R, %	-	11,9	12,5	13,6	13,6
Reproducibility limit R ($R = 2{,}8 \times s_R$), ng/ml	-	0,056	0,280	0,980	0,644
Recovery, %	-	93,1	90,3	84,3	-

[a] One laboratory has been excluded from the statistical evaluation because of the high detection limit (= 0,2 ng/ml).

[b] n.c. = naturally contaminated sample.

Table A.3 — Precision data for beer

BEER	**blank**	**spiked with OTA** (ng/ml)			**n.c.**[b]
		0,200	0,800	1,500	
Year of inter-laboratory test	1999	1999	1999	1999	1999
Number of laboratories	15	15	15	15	15
Number of laboratories retained after eliminating outliers	14[a]	13[a]	15	13	14[a]
Number of outliers (laboratories)	-	1	-	2	-
Number of accepted results	28	26	30	26	28
Mean value, ng/ml	<0,01	0,190	0,696	1,403	0,070
Repeatability standard deviation s_r, ng/ml	-	0,02	0,05	0,07	0,01
Repeatability relative standard deviation RSD_r, %	-	10,6	7,2	4,7	16,5
Repeatability limit r ($r = 2{,}8 \times s_r$), ng/ml	-	0,056	0,140	0,196	0,028
Reproducibility standard deviation s_R, ng/ml	-	0,04	0,13	0,21	0,02
Reproducibility relative standard deviation RSD_R, %	-	20,9	18,3	15,2	26,1
Reproducibility limit R ($R = 2{,}8 \times s_R$), ng/ml	-	0,112	0,364	0,588	0,056
Recovery, %	-	95,0	87,0	93,6	-

[a] One laboratory has been excluded from the statistical evaluation because of the high detection limit (= 0,2 ng/ml).

[b] n.c. = naturally contaminated sample.

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