RESOLUTION OENO 16/2001

DETERMINATION OF OCHRATOXIN A BY COLUMN OF IMMUNO-AFFINITY

THE GENERAL ASSEMBLY,

HAVING CONSIDERED Article 5 of the October 13, 1954 international convention for the unification of means of analysis and appreciation of wine,

With the proposal of the means of analysis and appreciation of wine sub-committee

DECIDES, to introduce in Appendix A of the Compendium the international methods of analysis for wine and musts, the following method:

MEASURING OCHRATOXINE A IN WINE AFTER GOING THROUGH AN IMMUNOAFFINITY COLUMN AND HLPC WITH FLUORESCENCE DETECTION

1. FIELD OF APPLICATION

This document describes the method used for determining ochratoxine A (OTA) in red, rosé, and white wines, including special wines, in concentrations ranging up 10 µg/l using an immunoaffinity column and high performance liquid chromatography (HPLC) [1].

This method was validated following an international joint study in which OTAs were measured in white and red wines during the analysis of naturally contaminated wines and wines with toxins ranging from 0.01 µg/l to 3.00 µg/l.

This method can apply to semi-sparkling wines and sparkling wines as long as the samples have been degassed beforehand, through sonication, for example.

2. PRINCIPLE

Wine samples are diluted with a solution containing polyethylene glycol and sodium hydrogen carbonate. This solution is filtered and purified on the immunoaffinity column.

OTA is eluted with methanol and quantified by HPLC in inverse state with fluorimetric detection.

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3. REAGENTS

Unless otherwise indicated, use only those reagents known for the quality of analysis, distilled water, or water with the EN ISO 3696. Solvents must be HPLC quality.

3.1. Sodium chloride (NaCl)

3.2. Sodium hydrogen carbonate ($NaHCO_3$)

3.3. Polyethylene glycol (PEG 8000)

3.4. Methanol (CH_3OH)

3.5. Acetonitrile (CH_3CN)

3.6. Purified water for laboratories, for example EN ISO 3696 quality (water for analytical laboratory use - Specification and test method [ISO 3696:1987]).

3.7. Acetic acid 85% (CH_3COOH)

3.8. Dilution solution (1% PEG + 5% $NaHCO_3$)

Dissolve 10 g of PEG (3.3) and 50 g of $NaHCO_3$ (3.2) in 950 ml of water and fill up with water to the 1 litre mark.

3.9. Washing solution (2,5% de NaCl + 0,5 % $NaHCO_3$)

Dissolve 25 g of NaCl (3.1) and 5 g of $NaHCO_3$ (3.2) in 950 ml of water and fill up with water to the 1 litre mark.

3.10. Mobile HPLC phase (water: acetonitrile: glacial acetic acid, 99:99:2, v/v/v)

Mix 990 ml of water with 990 ml of acetonitrile (3.5) and 20 ml of glacial acetic acid (3.7). Filter through a 0,45 µm filter and degas, with helium for example.

3.11. Toluene

3.12. Mixture of solvents (Toluene: a glacial acetic acid, 99:1, v/v).

Mix 99 parts in volume of toluene (3.11) with one part volume of glacial acetic acid (3.7).

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3.13. OTA stock solution

Dissolve 1 mg of OTA or the same content in a bulb, if the OTA was obtained in the form of film after evaporation, in the solvent mixture (3.12) to obtain a solution containing approximately 20 to 30 µg/ml of OTA.

To determine the exact concentration, record the absorption spectrum between 300 and 370 nm in a quartz space with 1 cm of optical path while using the solvent mixture (3.12) as a blank. Identify maximum absorption and calculate the concentration of OTA (c) in µg/ml by using the following equation:

$$c = A_{max} \times M \times 100/\varepsilon \times \delta$$

In which:

A_{max}= Absorption determined by the longest maximum wave (about 333 nm)

M = OTA molecular mass = 403,8 g/mole

ε = coefficient d'extinction molaire de l'OTA dans le mélange de solvant (3.12) (ε = 544/mole)

δ = optical pathway (cm)

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

3.14. Standard OTA solution (2 µg/ml in toluene: acetic acid, 99:1, v/v)

Dilute the stock solution (3.13) with the solvent mixture (3.12) to obtain a standard solution of OTA with a concentration of 2 µg/ml.

This solution can be stored at + 4 °C in a refrigerator. The stability should be tested regularly.

4. EQUIPMENT

Usual laboratory equipment and in particular, the following equipment:

4.1. Glass tubes (4 ml)

4.2. Vacuum pump to prepare the immunoaffinity columns.

4.3. Reservoir and flow tube adapted to immunoaffinity columns.

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4.4. Fibre glass filters (for example Whatman GF/A).

4.5. Immunoaffinity columns specifically for OTA.

The column should have the total link capacity of at least 100 ng OTA. This will allow for a purification yield of at least 85% when a diluted solution of wine containing 100 ng OTA is passed through.

4.6. Rotating evaporator

4.7. Liquid chromatography, a pump capable of attaining a constant flow of 1 ml/mn isocratic, as with the mobile phase.

4.8. Injection system must be equipped with 100 µl loop.

4.9. Column of analytical HPLC in steel 150 x 4.6 mm (i.d.) filled with a stationary phase C18 (5 µm) preceded with a pre-column or a pre-filter (0,5 µm) containing an appropriate phase. Different size columns can be used provided that they guarantee a good base line and background noise enabling the detection of of OTA peaks, among others.

4.10. Fluorescence detector is connected to the column and the excitation wavelength is set at 333 nm and the emitting wavelength at 460 nm.

4.11. Information retrieval system

4.12. U.V. spectrometer

5. PROCEDURE

5.1. Preparation of samples

Pour 10 ml of wine in a 100 ml conical flask. Add 10 ml of the dilution solution (3.8). Mix vigorously. Filter through fibreglass filter (4.4). Filtration is necessary for cloudy solutions or when there is precipitation after dissolving.

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5.2. Purification by immunoaffinity column

Set up the by immunoaffinity column (4.5) to the vacuum pump (4.2), and attach the reservoir (4.3).

Add 10 ml (equivalent to 5 ml of wine) of the diluted solution in the reservoir. Put this solution through the immunoaffinity column at a flow of 1 drop per second. The immunoaffinity column should not become dry. Wash the immunoaffinity column with 5 ml of cleaning solution (3.9) and then with 5 ml of water at a flow of 1 to 2 drops per second.

Blow air through to dry column. Elute OTA in a glass flask (4.1) with 2 ml of methanol (3.4) at the rate of 1 drop per second. Evaporate the eluate to dryness at 50° C with nitrogen. Dissolve again immediately in 250 µl of the HPLC mobile phase (3.10) and keep at 4° C until the HPLC analysis.

5.3. HPLC analysis

Using the injection loop, inject 100 µl of reconstituted extract (equivalent to 2 ml of wine) in the chromatography.

Operating conditions

Flow: 1 ml /min.

Mobile phase: acetonitrile: water: glacial acetic acid (99:99:2, v/v/v)

Fluorescence detector: Excitation wavelength = 333 nm

Emitting wavelength = 460 nm

Volume of injection: 100 µl

6. QUANTIFICATION OF OCHRATOXINE A (OTA)

The quantification of OTA should be calculated by measuring the area or the height of the peaks at the OTA retention time and compared to the calibration curve

6.1. Calibration curve

Prepare a calibration curve dayly and every time chromatographical conditions change. Measure out 0.5 ml of the standard OTA solution (3.14) at 2 µg/ml in a glass flask and evaporate the solvent using nitrogen.

Dissolve again in 10 ml in the HPLC mobile phase (3.10) which was previously filtered using a 0.45 µm filter. This produces an OTA of 100 ng/ml solution.

Prepare 5 HPLC calibration solutions in five 5 ml graduated flasks following Table 1.

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Complete each 5 ml standard solution with HPLC mobile phase. (3.10).

Inject 100 µl of each solution in the HPLC.

Table 1

	Std 1	Std 2	Std 3	Std 4	Std 5
µl of mobile phase filtered HPLC (3.10)	4970	4900	4700	4000	2000
µl of OTA solution at 100 ng/ml	30	100	300	1000	3000
OTA concentration (ng/ml)	0.6	2.0	6.0	20	60
Injected OTA (ng)	0.06	0.20	0.60	2.00	6.00

NOTE:

1. If the quantity of OTA in the samples is outside the calibration range, an appropriate dilution should occur or smaller volumes should be injected. In these cases, the final (7) should be reviewed on a case by case basis.
2. Due to the great variations in concentrations, it is recommended to pass the linear calibration by zero in order to obtain an exact quantification for low concentrations of OTA. (less than 0.1 µg/l)

7. CALCULATIONS

Calculate the quantity of OTA in the aliquot of the solution testes and injected in the HPLC column.

Calculate the concentration of OTA (COTA) in ng/ml (equivalent to µg/l) by using the following formula:

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$$C_{OTA} = M_A \times 2/V_1 \times V_3/V_2$$

Where:

M_A is the volume of ochratoxin A (in ng) in the aliquot part of the template injected on the column and evaluated from the calibration curve.

2 is the dilution factor

V_1 is the sample volume to be analysed (10 ml)

V_2 the volume of the solution tested and injected in the column (100 μl)

V_3 is the volume of solution used to dissolve the dry eluate (250 μl)

8. PERFORMANCES USING THIS METHOD IN LABORATORIES

Table 2 regroups performances of the method applied to white, rosé and red wines in laboratories participating in the validation of this method.

Table 2. Recovery of ochratoxin A from wines overweighted with different concentrations of added ochratoxin A

	Red wine		Rosé wine		White wine	
Addition (µg/l)	Yield ± SD* (%)	RSD# (%)	Yield ± SD* (%)	RSD# (%)	Yield ± SD* (%)	RSD# (%)
0.04	96.7 ± 2.2	2.3	94.1 ± 6.1	6.5	91.6 ± 8.9	9.7
0.1	90.8 ± 2.6	2.9	89.9 ± 1.0	1.1	88.4 ± 0.2	0.2
0.2	91.3 ± 0.6	0.7	88.9 ± 2.1	2.4	95.1 ± 2.4	2.5
0.5	92.3 ± 0.4	0.5	91.6 ± 0.4	0.4	93.0 ± 0.2	0.2
1.0	97.8 ± 2.6	2.6	100.6 ± .,5	2.5	100.7 ± 1.0	1.0
2.0	96.5 ± 1.6	1.7	98.6 ± 1.8	1.8	98.0 ± 1.5	1.5
5.0	88.1 ± 1.3	1.5	-	-	-	-
10,0	88,9 ± 0,6	0,7	-	-	-	-

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Average of averages	92.8 ± 3.5	3.8	94.5 ± 5.2	5.5	94.5 ± 4.1	4.3

* SD = Spread type (Standard deviation) (n = 3 replicates) ;

RSD = Relative spread type (Variation percentage).

9. GROUP WORK

The method was validated by a group study with the participation of 16 laboratories in 8 countries, following the protocol recommendations harmonised for validating the analysis methods. [2]. Each participant analysed 10 white wines, 10 red wines, representing 5 random duplicate wines; naturally contaminated or with OTA added. The performances of the method which resulted from this work are found in appendixes I and II.

10. PARTICPATING LABORATORIES

Unione Italiana Vini, Verona	ITALY
Istituto Sperimentale per l'Enologia, Asti	ITALY
Istituto Tecnico Agraria, S. Michele all'Adige (TN)	ITALY
Università Cattolica, Piacenza	ITALY
Institute for Health and Consumer Protection, JRC – Ispra	ITALY
Neotron s.r.l., S. Maria di Mugnano (MO)	ITALY
Chemical Control s.r.l., Madonna dell'Olmo (CN)	ITALY
Laboratoire Toxicologie Hygiène Appliquée, Université V. Segalen, Bordeaux	FRANCE

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Laboratoire de la D.G.C.C.R.F. de Bordeaux, Talence	FRANCE
National Food Administration, Uppsala	SWEDEN
Systembolagets Laboratorium, Haninge	SWEDEN
Chemisches Untersuchungsamt, Trier	GERMANY
State General Laboratory, Nicosia	CYPRUS
Finnish Customs Laboratory, Espoo	FINLAND
Central Science Laboratory, York	UNITED KINGDOM
E.T.S. Laboratories, St. Helena, CA	UNITED STATES

11. REFERENCES

1. A. Visconti, M. Pascale, G. Centonze. Determination of ochratoxin A in wine by means of immunoaffinity column clean-up and high-performance liquid chromatography. Journal of Chromatography A, 864 (1999) 89-101
2. AOAC International 1995, AOAC Official Methods Program, p. 23-51

APPENDIX I

The following data was obtained in inter-laboratory tests, according to harmonised protocol recommendations for joint studies in view of validating an analysis method.

WHITE WINE		Added OTA (µg/l)			
Sample	White	0.100	1.100	2.000	n.c.
Inter-laboratory test year	1999	1999	1999	1999	1999

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Number of laboratories	16	16	16	16	16
Number of laboratories retained after eliminating absurd findings	14*	13*	14	14	15
Number of eliminated laboratories	-	1	2	2	1
Number of accepted results	28	26	28	28	30
Average value (µg/l)	<0,01	0,102	1,000	1,768	0,283
Spread-type/Repeatabilityr (µg/l)	-	0.01	0.07	0.15	0.03
Relative spread-type (Variation percentage) /Repeatability RSDr (%)	-	10.0	6.6	8.5	10.6
Repeatability limit r (µg/l)	-	0.028	0.196	0.420	0.084
Spread-type/capacity of being reproduced sR (µg/l)	-	0.01	0.14	0.23	0.04
Relative spread-type (variation percentage) /capacity of being reproduced RSDR (%)	-	14.0	13.6	13.3	14.9
Capacity of being reproduced limit R (µg/l)	-	0.028	0.392	0.644	0.112
Extraction yield %	-	101.7	90.9	88.4	-

*2 laboratories were exclued from the statistical evaluation due to high detection limit (= 0,2 μ/l

n.c.=sample naturally contaminated

APPENDIX II

The following data was obtained in inter-laboratory tests, according to harmonised protocol recommendations for joint studies in view of validating an analysis method.

RED WINE		Added OTA (µg/l)			
samples	White	0.200	0.900	3.000	n.c.

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Inter-laboratory test year	1999	1999	1999	1999	1999
Number of laboratories	15	15	15	15	15
Number of laboratories retained after eliminating absurd findings	14*	12*	14	15	14
Number of eliminated laboratories	-	2	1	-	1
Number of accepted results	28	24	28	30	28
Average value (µg/l)	<0.01	0.187	0.814	2.537	1.693
Spread-type/Repeatabilityr (µg/l)	-	0.01	0.08	0.23	0.19
Relative spread-type (Variation percentage) /Repeatability RSDr (%)	-	5.5	9.9	8.9	10.9
Repeatability limit r (µg/l	-	0.028	0.224	0.644	0.532
Spread-type/capacity of being reproduced sR (µg/l)	-	0.02	0.10	0.34	0.23
Relative spread-type (variation percentage) /capacity of being reproduced RSDR (%)	-	9.9	12.5	13.4	13.4
Capacity of being reproduced limit R (µg/l)	-	0.056	0.280	0.952	0.644
Extraction yield %	-	93.4	90.4	84.6	-

* 1 laboratory was excluded from the statistical evaluation because of high detection limits (= 0,2 µg/l).

n.c. = naturally contaminated sample

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