OIV-MA-AS315-10 Measuring ochratoxine A in wine after going through an immunoaffinity column and HLPC with fluorescence detection

Type II method

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.

3. Reagents

3.1. Reagents for separation of the OTA on an immunoaffinity column

The reagents listed below are examples. Suppliers of immunoaffinity columns may offer dilution solutions and eluents suitable for their products. If so, it is preferable to use these products.

3.1.1. Sodium hydrogen phosphate dihydrate($Na_2HPO_4.2H_2O$) CAS [10028-24-7]

3.1.2. Sodium dihydrogen phosphate monohydrate ($Nah_2PO_4.H_2O$) CAS [10049-21-5]

3.1.3. Sodium chloride (NaCl) CAS [7647-14-5]

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

3.1.5. Phosphate buffer (dilution solution)

Dissolve 60g of $Na_2HPO4.2H_2O$ (3.1.1) and 8.8g of $NaH_2PO_4.H_2O$ (3.1.2) in 950ml of water and add more water to make up to 1 litre.

3.1.6. Phosphate buffer saline (washing solution)

Dissolve 2.85g of $Na_2HPO_4.2H_2O$ (3.1.1), 0.55g of $NaH_2HPO_4.H_2O$ (3.1.2) and 8.7g of NaCl in 950ml of water and add more water to make up to 1 litre.

3.1.7. Methanol (CH_3OH) CAS [67-56-1]

3.2. Reagents for HPLC

3.2.1. Acetonitrile for HPLC (CH_3CN) CAS [75-05-8]

3.2.2. Glacial acetic acid (CH_3COOH) CAS [64-19-7]

3.2.3. Mobile phase: water: acetonitrile: glacial acetic acid, 99:99:2, v/v/v

Mix 990 ml of water with 990 ml of acetonitrile (3.2.2) and 20 ml of glacial acetic acid (3.2.3). In the presence of undissolved components, filter through a 0.45µm filter. Degas (with helium, for example) unless the HPLC equipment used includes a degassing step.

3.3. Reagents for the preparation of the OTA stock solution

3.3.1. Toluene ($C_6H_5CH_3$) CAS [108-88-3]

3.3.2. Mixture of solvents (toluene: glacial acetic acid, 99:1, v/v).

Mix 99 parts in volume of toluene (3.3.1) with one part volume of glacial acetic acid (3.2.2).

3.4. 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

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

▫ = optical pathway (cm)

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

3.5. 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.

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 □ 4.6 mm (i.d.) filled with a stationary phase C_{18} (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.

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 µlg/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 0.45 100 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.

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:

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.

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 (C_{OTA}) in ng/ml (equivalent to µg/l) by using the following formula:

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

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.

F: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	-	-	-	-
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, outlining critical points of the method are found in appendix III.

10. Participating 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.	FRANCE
Segalen, Bordeaux	FRANCE
Laboratoire de la D.G.C.C.R.F. de Bordeaux, Talence	SWEDEN
National Food Administration, Uppsala	SWEDEN
Systembolagets Laboratorium, Haninge	GERMANY
Chemisches Untersuchungsamt, Trier	CYPRUS
State General Laboratory, Nicosia	FINLAND
Finnish Customs Laboratory, Espoo	UNITED
Central Science Laboratory, York	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
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/Repeatability$_r$ (µg/l)	-	0.01	0.07	0.15	0.03
Relative spread-type (Variation percentage) /Repeatability RSD$_r$ (%)	-	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 s_R (µg/l)	-	0.01	0.14	0.23	0.04
Relative spread-type (variation percentage) /capacity of being reproduced RSD$_R$ (%)	-	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 excluded from the statistical 'evaluation due to high detection limit (= 0,2 µg/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.
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/Repeatability$_r$ (µg/l)	-	0.01	0.08	0.23	0.19
Relative spread-type (Variation percentage) /Repeatability RSD$_r$ (%)	-	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 s_R (µg/l)	-	0.02	0.10	0.34	0.23
Relative spread-type (variation percentage) /capacity of being reproduced RSD$_R$ (%)	-	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

Appendix III

Guide to the critical points of the method of measuring ochratoxin A by immunoaffinity column, type II.

The critical points to observe are listed below for information purposes only and are a guide to applying the method. Numbering refers to paragraphs of the resolution.

1. Field of application

For information purposes only the method can be applied to grape musts, partially fermented grape musts, and new wines still under fermentation. The validation parameters concern wines only.

2. Principle

The method is broken down into two steps. The first step involves purification and concentration of the OTA in the wine or the must by capture on an immunoaffinity column followed by elution. The second step involves quantification of the eluate by HPLC using fluorescence detection.

3. Reagents

3.1. OTA stock solution

The use of OTA in solid form in not recommended; it is recommended to use a standard solution of OTA (point 3.5)

3.2. Standard OTA solution

Use of a commercial solution of standard concentration (around 50 µg/ml) with an analysis certificate stating the reference value and uncertainty of the concentration.

In theory the volume of these solutions is not certified, and they must be sampled with certified pipettes to constitute stock solutions from 0.25 to 1 mg/l in pure ethanol or in the mobile phase of the HPLC method (see 3.2.3).

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

4. Equipment

4.1. Recommendations for assessment of the performance of immunoaffinity columns (optional)

The step of concentration on an immunoaffinity column is a major source of inaccuracy in the analysis method. Experience shows that the various columns offered on the market could have recovery rates of between 70 and 100%.

It is therefore recommended to check the performance of a batch of columns before

use. This step is recommended where there has been a change in supplier or column references.

4.2. Characterisation of the batch of columns (measure of recovery rate):

Select around 10 columns representative of the types of column routinely used in the laboratory, and all from different batch numbers. Prepare the same number of wines representing different matrices, with zero OTA concentrations, with known additions x_i of between 0.5 and

2 µg·kg^{-1}. After the known additions quickly analyse these n samples with the batch of selected columns. Let y_i be the values found.

The recovery rate data are calculateed, the rate being the measured quantity in relation to the known added quantity.

$$t_i = \frac{y_i}{x_i}$$ Recovery rate with column

$$T = \frac{\sum t_i}{n}$$ Average recovery rate

$$S_t = \sqrt{\frac{\sum (t_i - T)^2}{n-1}}$$ Standard deviation of the recovery rate

The standard deviation of the recovery rate calculated in this way represents not only the variability of the recovery rate of the columns, but also the standard uncertainty of the measurement system used after use of the columns (HPLC). It is nevertheless possible to establish a reasonable estimate of the standard deviation of the recovery rate of the columns by deducting the standard uncertainty of the HPLC system from the calculated recovery error:

- Estimate the standard uncertainty S_V (expressed as the standard deviation) of the measurement system in the strict sense of the word (without considering the the immunoaffinity column step).

For this it is possible to use a fidelity study on the OTA solutions.

The standard deviation of the recovery rate Sp is estimated as follows:

$$S_P = \sqrt{S_t^2 - S_v^2}$$

For a fairly wide concentration range, it is preferable to express this value as the coefficient of variation of the standard deviation (RSDR).

$$CV\% = Sp.100/concentration\ of\ the\ addition$$

5. Procedure

The procedure outlined in point 5 is an example. The composition of dilution and washing solutions may differ from one column manufacturer to another. Likewise, the concentration of the diluted wine sample may be adjusted as needed.

6. Quantification of ochratoxine A (OTA)

6.1. Calibration curve

Prepare a calibration curve daily or each time that the chromatographic conditions change. Prepare the curve using solutions produced by diluting the stock solution in the mobile phase (see 3.2.3). The values chosen must provide the working range taking into account the concentration factor of the wine.