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Determination of ochratoxin A in wine using liquid-phase microextraction combined with liquid chromatography with fluorescence detection

E. González-Peñas [a,*], C. Leache [a], M. Viscarret [a], A. Pérez de Obanos [b], C. Araguás [a], A. López de Cerain [b]

[a] *Organic and Pharmaceutical Chemistry Department, Faculty of Pharmacy, University of Navarra, C/Irunlarrea s/n, 31008 Pamplona, Spain*
[b] *Food Science and Toxicology Department, Faculty of Pharmacy, University of Navarra, C/Irunlarrea s/n, 31008 Pamplona, Spain*

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Abstract

A liquid–liquid microextraction technique (LPME) has been applied to the extraction of ochratoxin A (OTA) from wine prior to its quantification by HPLC-fluorescence detection. OTA was extracted from wine, through 1-octanol immobilized in the pores of a porous hollow fiber, and introduced into 1-octanol inside the fiber. Recovery was 77%. The method was adequate for quantification of OTA in wine at levels within the range 0.25–10 ng/ml with a LOD of 0.2 ng/ml, and can be a simple and inexpensive alternative to the use of inmunoaffinity columns in order to quantify OTA levels in wine.

Keywords: Wine; Liquid-phase microextraction; Mycotoxins; Ochratoxin A

1. Introduction

Ochratoxin A (OTA) is a mycotoxin produced by several fungal species from *Aspergillus* and *Penicillium* genera. These fungi grow spontaneously in a wide range of commodities, most commonly in cereals, but also in beans, coffee, dried fruits, wine, etc. The presence of OTA in wine was reported for the first time in 1995 [1]. Since then, OTA contamination of wine has been reported by several authors [2–6]. Wine is a product widely consumed in developed and developing countries and a major source of daily OTA intake for the population, second only to cereals [4].

OTA is known to have nephrotoxic, immunotoxic, teratogenic and carcinogenic effects [7]. Recently the European Commission has fixed maximum limits for OTA in cereals, raw cereal grains, products derived from cereals, and dried wine fruits; in addition, a maximum limit for OTA in green and roasted coffee and coffee products, wine, beer, grape juice, cocoa and cocoa products and spices is expected to be introduced in 2003 [8].

Methods for mycotoxins are needed for compliance with tolerances and guidelines, for monitoring and survey work, and for research in areas such as epidemiology, mycology, metabolism, pharmacokinetics, food processing and decontamination. For more than 30 years, considerable research has been devoted to developing methods for detecting and determining mycotoxins in foods, feed, and biological fluids [9]. In the case of OTA, the technique used in most studies is HPLC with fluorescence detection, mostly because a very low detection limit can be reached, due to the fact that OTA has natural fluorescence [10].

Sample extract clean-up is an essential part in analytical methods for micotoxins. Generally, it is necessary to obtain low detection limits and to protect the HPLC column. The methods most frequently used in the extract clean-up are liquid–liquid and solid–liquid extraction; however, in the case of mycotoxins, the most important development in the field of clean-up methods until now is the use of immunoaffinity columns (IAC) [10]. The extract is poured onto a column filled with immobilized antibodies against the specific mycotoxin. Other compounds in the sample are washed off by water or aqueous buffer and the toxin is eluted by methanol or methanol-buffer. Many commercial IACs are available for ochratoxin A and most of the current analytical methods for the determination of OTA in wine use IACs

* Corresponding author. Tel.: +34-948-425653; fax: +34-948-425652.
E-mail address: mgpenas@unav.es (E. González-Peñas).

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as a sample clean-up tool. Some of these methods maintain a previous process of liquid–liquid extraction with organic solvents as chloroform [11]. However, some authors have reported the direct application of the wine diluted with phosphate buffer onto the immunoaffinity column [4,5] in order to reduce or eliminate toxic solvents in the analytical process.

IACs have the advantage that OTA is bound specifically to the antibody, thereby permitting almost total removal of the matrix. The more important disadvantage of the method is the high cost of the columns. Zimmerly and Dick [12] tried to solve this problem by reusing the columns, but our experience showed that reused columns give some problems in the reproducibility of results. Another problem with IACs is that they may not be completely specific because the ethyl ester of ochratoxin A is also retained, and yet another problem is the possibility that they may contain ochratoxin A as contaminant [9].

In 1999, Pedersen-Bjergaard and Rasmussen [13] developed a microextraction technique based on liquid-phase extraction with a porous hollow fiber. This technique is simple and inexpensive, with the advantage being that the fiber is disposable after use due to its low cost. The deionized analytes in an aqueous solution were extracted from the sample solution, entering into the organic solvent included in the pores of the hollow fiber, and further into the inside of the hollow fiber which holds a small volume of an acceptor solution. The technique provides very clean extracts. This extraction procedure has been applied to the analysis of some acidic and basic drugs, pesticides, aminoalcohols, chiral drugs and other analytes [14–21], but until now we have not heard of its application to OTA analysis.

In this work, we investigated the application of liquid–liquid microextraction technique (LPME) combined with HPLC-fluorescence detection to determine OTA in wine samples. Parameters affecting the extraction efficiency as solvent selection, extraction time, and composition of donor and acceptor solutions are studied. A comparative study has been carried out using IACs.

2. Experimental

2.1. Chemicals, reagents and wine samples

Ochratoxin A was purchased from Sigma (St. Louis, MD, USA). All reagents were pro-analysis grade. Methyl sulfoxide, monobasic potassium phosphate, potassium chloride, di-basic natrium phosphate, natrium hydrogencarbonate and hydrochloric acid 35% were purchased from Panreac (Barcelona, Spain). Diisopropyl ether and 1-octanol 99.5% were obtained from Riedel de Häen (Seelze, Germany), while dichloromethane, ethyl acetate, acetonitrile and methanol HPLC grade were obtained from Riedel de Häen (Seelze, Germany). Ochratest immunoaffinity columns were purchased from Vicam Inc. (Watertown, MA. USA). Millipore type I water was used to prepare all of the aqueous solutions. Needles BD Microbalance^{TM} 3 of 0.8 mm × 25 mm came from Becton Dickinson (Huesca, Spain). Vials with a screw top septum and polypropylene screw cap, with a hole of 13 mm, for 4 ml vials were from Supelco (Madrid, Spain). Accurel PP Q3/2 hollow fiber came from Membrana GMBH (Wuppertal, Germany) (inner diameter: 600 μm, pore size: 0.2 μm and wall thickness: 200 μm). Wines (red, white and rosé) were purchased at the local market.

2.2. Preparation of standards

A stock solution of 100 μg/ml of OTA in methanol was prepared and the concentration was verified spectrophotometrically (Mr: 403.8, $\varepsilon_{333\,nm}$5500 M^{-1} cm^{-1}) [22]. Sample spiking solutions and standard curve solutions were prepared from appropriate dilutions of the stock solution with methanol. All of the solutions were stored at −20 °C. Prior to the HPLC analysis, 200 μl of the standard curve solutions were evaporated under a stream of N_2 and dissolved in 200 μl of mobile phase, in the same way as the extracts from wine samples were prepared.

2.3. Extraction of OTA from wine samples and immunoaffinity clean-up of the extracts

The method used in the extraction of OTA from wine samples and immunoaffinity clean-up was as follows: 5 ml of wine diluted with 45 ml of phosphate buffered saline (PBS) adjusted to pH 7.00 in a polypropylene tube was directly applied onto an immunoaffinity column that had been pre-conditioned with 10 ml of PBS. Next, the IAC was washed with 10 ml of water and dried by passing air with the use of a syringe for 10 s. OTA was eluted with four 1 ml portions of methanol at a flow rate of 20–30 drops/min. The eluate was evaporated to dryness under a stream of nitrogen at 40 °C, and the residue was redissolved in 200 μl of mobile phase. This method presents a recovery of 95% and a uncertainty of 14% ($K = 2.3$).

2.4. Extraction of OTA from wine samples and LPME clean-up of the extracts

The LPME extraction device is very simple. Four milliliters of wine (donor phase) was poured into a vial with a screw top septum. One hollow fiber was placed in the vial while two conventional 0.8 mm O.D. medical syringe needles were inserted through the silicon septum in the screw top and connected to the extremes of the hollow fiber in order to introduce the acceptor solution into the hollow fiber prior to extraction and in order to support the hollow fiber inside the vial. The extraction process was carried out in a magnetic stirrer (Labortechik Telemodul) at 1000 rpm, because stirring of the sample is expected to enhance extraction and reduce the time of extraction required to reach equilibrium between the donor and acceptor phases

[18]. Each piece of fiber was used for a single extraction only.

The preconcentration factor in the LPME extraction process is defined as the ratio between the final OTA concentration in the acceptor phase and the initial concentration of OTA in wine; and recovery (R) has been calculated as the quotient between the OTA amount in the acceptor solution and the initial ng OTA in wine:

$$\mathrm{PF} = \frac{\text{ng/ml OTA acceptor solution}}{\text{ng/ml OTA donor solution}},$$

$$R\,(\%) = \frac{\text{ng OTA acceptor phase}}{\text{ng OTA donor phase}} \times 100 = \frac{V_a}{V_d} \times \mathrm{PF} \times 100$$

where V_a and V_d are the volumes of acceptor and donor solutions, respectively.

2.5. Chromatographic conditions for OTA analysis

HPLC analysis was performed in an Agilent technologies 1100 high-performance liquid chromatograph coupled to a fluorescence detector (model G1321A) and controlled by Chemstation 3D software. The chromatographic conditions, based on these used in the method of Lopez de Cerain et al. [11] were column: Tracer Extrasil ODS-2, 5 μm, 25 cm × 0.4 cm, Teknokroma (Barcelona, Spain) at 40 °C, mobile phase: 29:29:42 (v/v/v) methanol–acetonitrile–sodium acetate 5 mM (pH 2.2 with phosphoric acid) at 1.5 ml/min. Volume of injection: 100 μl; fluorescence detection: λ_{ex} = 225 nm; λ_{em} = 461 nm and t_R = 4.9 min.

2.6. Validation of the method

The selectivity of the method was studied in red, white, and rosé wine samples. Linearity was assessed in the range of 0.25–10 ng/ml; and 0.2 ml of six samples of OTA in methanol (3–200 ng/ml) equivalent to 0.15–10 ng OTA/ml of wine, were evaporated, resuspended in 0.2 ml of mobile phase and chromatographied. The LOD was calculated using the following relation:

$$\mathrm{LOD} = \frac{Y_{bl} + (K \times S_{bl})}{b}$$

Being Y_{bl} (area of the blank) and b the respective intercept and the slope of a curve made by analyzing samples of fortified rosé wine at levels of 0.2–1 ng/ml; K is a factor of 3. S_{bl} (standard deviation of the blank) is the intercept of the curve obtained, representing the standard deviations for each concentration level versus the concentration. LOQ was calculated as the lowest concentration for which acceptable data of recovery and precision were obtained. Within-day and between-day precision and recovery of the method have been studied at the lowest, medium and highest OTA levels of the range (0.25, 1 and 10 ng/ml) in spiked white, rosé and red wine; for each concentration level, three replicates have been analyzed in 1 day and another three replicates have been assayed on three different days. The recovery has been determined by comparing the peak area of OTA obtained from the wine spiked samples and from the calibration standards. Where relevant, the measured OTA levels have been corrected for any natural contamination, as indicated by the analysis of the nonspiked material.

2.7. Uncertainty estimation

Uncertainty has been estimated taking into account the following uncertainty sources: the calibration curve (u^2_M), the determination of the concentration of the OTA dissolution used in the fortification of wine (u^2_{OTA}), the volumetric equipment used ($\sum u^2_{v_i}$) and the estimation of the recovery value ($u^2_{Recovery}$).

$$u^2_{wine} = u^2_M + u^2_{OTA} + \sum u^2_{v_i} + u^2_{Recovery}$$

The degrees of freedom have been estimated from the Welch–Scatterthwaite formula, and assuming that in the case of the uncertainty from a type B evaluation, the degrees of freedom may be considered as ∞:

$$v_{ef} = \frac{u^4 y}{\sum_{i=1}^{N} (u^4_{x_i}(y)/v_i)}$$

3. Results and discussion

3.1. Selection of solvents for the impregnation of the hollow fiber and composition of the donor and acceptor phases

Solvents used as donor and acceptor phases should be selected taking into account the physicochemical characteristics of the analyte. OTA has weak acid characteristics with two pK_a values due to its molecular characteristics (7-carboxy-5-chloro-8-hydroxy-3,4-dihydro-3R-methylisocumarin linked to L-β-phenylalanine by an amide bond) (Fig. 2). The pK_a value for the carboxyl group of the phenylalanine part is 4.4, whereas the pK_a of the phenolic hydroxyl group is 7.3–7.05 [10]. It is slightly soluble in aqueous solutions and soluble in both organic solvents and diluted solutions of natrium hydrogencarbonate at basic pH. Therefore, pH of the donor solution (wine) should be adjusted to within the acidic range in order to deionize the molecule and to promote its extraction.

Different solvents were assayed as the acceptor phase: diluted solution of natrium hydrogencarbonate, 2-dichloromethane, di-isopropyl ether, ethyl acetate, DMSO and 1-octanol. After 24 h of extraction, no acceptor solution was recovered when 1,2-dichloromethane and di-isopropyl ether were used; this was most likely due to their volatility. The preconcentration factors obtained with natrium hydrogencarbonate, ethyl acetate and DMSO were very low (3.3, 2.4 and 0.6, respectively). 1-Octanol showed the biggest preconcentration factor: 34 (R.S.D. = 16%, n = 4) when 25 μl were

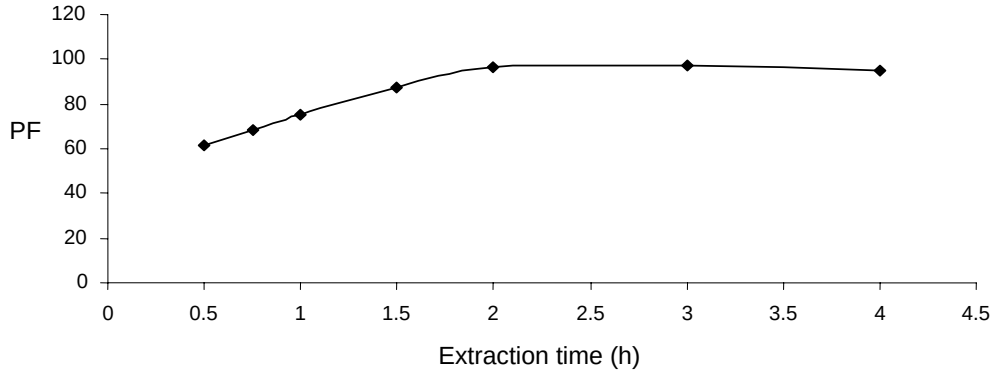

Fig. 1. Preconcentration factor versus extraction time.

put into the fiber as the acceptor phase. This value increased to 58 (R.S.D. = 11%, $n = 4$) when 15 μl were used, and to 90 (R.S.D. = 11%, $n = 6$) when working with wine fortified at 2.5 ng/ml of OTA; this could indicate that a saturation process occurred when high levels of OTA in wine were assayed.

Selection of the solvent for impregnation of the fiber is one of the critical steps in LPME. It should meet certain requirements: unmiscible with wine, nonvolatile, easily immobilized in the pores, and the solubility of OTA should be higher in the solvent than in wine. 1-Octanol, ethyl acetate and methyl sulfoxide (DMSO) were assayed. The empty hollow fiber was dipped in the organic solvent for 5 s in order to fill the pores, and the excess was removed by sonication during 30 s in a waterbath. Only 1-octanol has provided good results.

3.2. Extraction time

Three fortified wine samples (2.5 ng/ml) were extracted during 0.5–4 h at room temperature with a constant stirring speed of 1000 rpm. Two hours was sufficient time for extracting the maximum amount of OTA. Longer extraction times did not result in a better preconcentration factor (Fig. 1).

3.3. OTA level in wine

Six fortified samples of wine (1–54 ng/ml) were extracted for 2 h. The preconcentration factor was maintained between 80 and 90 in the range of 1–20 ng/ml, and slows down close to 58 at the 54 ng/ml level. This proves that a saturation process is occurring in the 1-octanol phase.

3.4. Recovery

In order to increase the recovery of the extraction process, the influence of certain factors has been studied.

No substantial improvement in the recovery factor was observed after adding NaCl to wine nor when two 4 cm fibers were introduced into the vial, simultaneously, in a first attempt and sequentially in a second experience. The extracts from both fibers were collected, mixed, evaporated and reconstituted in 200 μl of mobile phase. The recovery factor enlarged slightly (38), especially when two fibers were used sequentially (41). However, they were not considered adequate.

The recovery experimented an important increase, reaching close to 80%, when the 1-octanol fixed within the pores of the fiber was collected and mixed with the acceptor phase. In order to do this, the fiber was rinsed with water after extraction, transferred into a conical tube with 3 ml of methanol and sonicated for 5 min. The polypropylene fiber was discarded, the solution was evaporated to dryness in a nitrogen stream, and the residue was redissolved in 200 μl of mobile phase before being analyzed.

3.5. Final extraction conditions

Based on the experiments previously discussed, the optimal extraction efficiency of OTA from wine was obtained by using a 4.0 cm long porous fiber, wine acidified to pH 1.05 with HCl as the donor phase, and 15 μl of 1-octanol inside the fiber as the acceptor phase. 1-Octanol has also been used as solvent for impregnation of the pores of the fibre. The extraction time fixed was 2 h, with a stirring speed of 1000 rpm. 1-Octanol (inside the fiber and immobilized in the pores of the fiber) was collected by sonication in methanol. The extract was evaporated and resuspended in 200 μl of mobile phase before analysis by HPLC. In Fig. 2, a chromatogram obtained from a rosé wine sample fortified with 1 ng/ml is shown.

3.6. Validation of the method

No peaks appeared at the OTA retention time (4.9 min) that can interfere in their quantification. All of the criteria used to verify linearity have been matched in the concentration range studied: curve equation: $y = 0.77x + 0.15$, $r = 0.9999$, R.S.D. among response factors 3.9 (<5%), slope interval ($P = 95\%$) not include zero (0.76–0.77) and intercept interval ($P = 95\%$) include zero (−0.62 to 0.93). The analysis of 3, 30 and 200 ng OTA/ml methanol during 3 days showed adequate values of precision (R.S.D. (%))

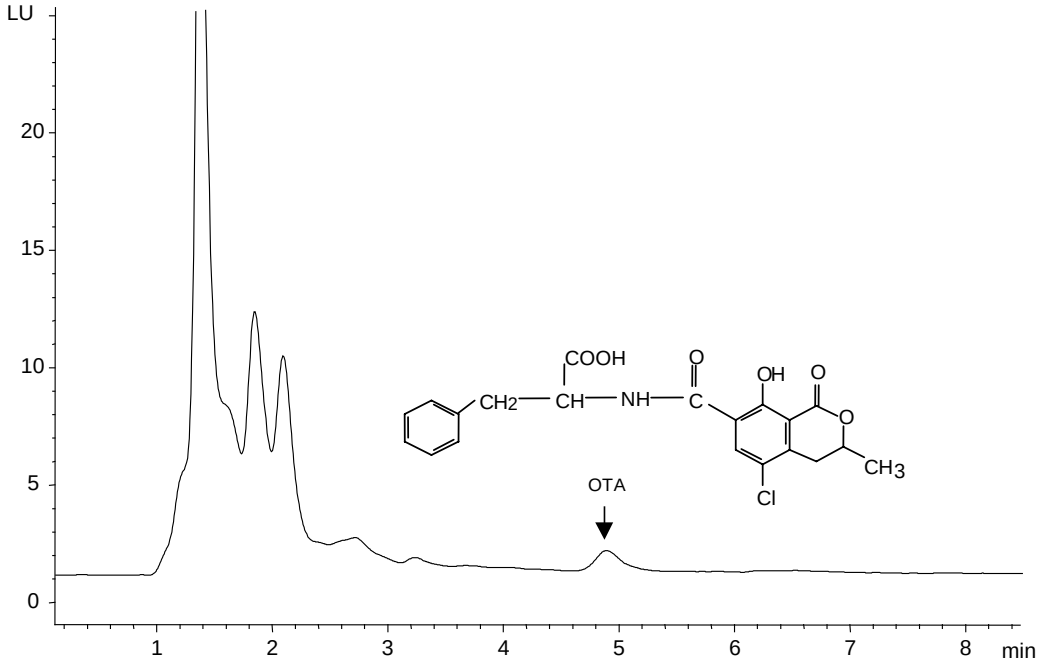

Fig. 2. Chemical structure of OTA and chromatogram obtained from a sample of rosé wine fortified at 1 ng OTA/ml.

and accuracy (as standard error of the mean) of the lineal curve (less than 10%).

The estimated LOD and LOQ values were 0.20 ng OTA/ml and 0.25 ng/ml of wine, respectively. The LOQ obtained was acceptable for analyzing OTA in wine because the maximum level most likely allowed in future legislation is between 0.5 ng/ml [23] and 1–2 ng/ml [1].

The recovery values obtained are homogeneous in the three types of wine (R.S.D. = 1.8%), and among the OTA concentrations assayed (R.S.D. = 0.3%) (Table 1). The ANOVA test did not show any significant difference among data obtained with different types of wines ($n = 3$, $F = 0.028$, significance: 0.972) or with different concentrations ($n = 3$, $F = 0.827$, significance: 0.450). Finally, the same percentage of recovery was achieved in both between-day and 1-day recovery experiments 77%. This value is acceptable for quantification of OTA in wine. The Commission Directive 2002/26/EC [24] established that the analytical methods used for the control of the ochratoxin A levels in foodstuffs should have a recovery value between 70 and 110% in the 1–10 ng/ml levels, and between 50 and 120% in the <1 ng/ml levels.

The estimated uncertainty of the method was 19% ($K = 2.28$).

4. Comparison with IAC

Nine samples (three samples of each type of wine) of fortified wine in the range of 0.4–3 ng/ml, and one sample of white wine from a interlaboratory study, were assayed and quantified by LPME and IAC procedures. The results obtained for both processes are similar (Table 2). Moreover, a lineal relationship is obtained when representing the results obtained from the IAC process versus those obtained from the LPME process; this is evidenced by the good

Table 1
Precision and recovery of the method

ng OTA/ml	White wine X (%); S.D.[a]	Rosé wine X (%); S.D.	Red wine X (%); S.D.	Mean (%); S.D.	R.S.D. (%)
Precision and recovery intra-day					
0.25	78; 7	79; 3	75; 2	77; 4	4
1	78; 3	74; 3	79; 14	77; 6	8
10	79; 3	75; 2	79; 6	78; 3	4
	First day X (%); S.D.	Second day X (%); S.D.	Third day X (%); S.D.		
Precision and recovery between days (rosé wine)					
0.25	78; 2	76; 5	77; 1	77; 3	4
1	74; 3	79; 1	79; 4	77; 3	4
10	75; 1	77; 2	77; 1	76; 2	2

[a] Standard deviation.

Table 2
Comparison between IAC and LPME

Wines	Sample	LPME (ng/ml)	IAC (ng/ml)
White	1	0.38 ± 0.07	0.40 ± 0.06
	2	0.79 ± 0.15	0.55 ± 0.08
	3	1.1 ± 0.2	0.94 ± 0.14
Rosé	1	1.2 ± 0.2	1.2 ± 0.2
	2	1.4 ± 0.3	1.4 ± 0.2
	3	1.7 ± 0.3	1.9 ± 0.3
Red	1	2.4 ± 0.5	2.2 ± 0.3
	2	2.8 ± 0.5	2.6 ± 0.4
	3	3.2 ± 0.7	na[a]
Interlaboratory sample	1	1.9 ± 0.4	1.8 ± 0.3

Levels measured (±uncertainty) (ng/ml) by both techniques in the fortified samples.
[a] Not available.

correlation coefficient obtained ($r = 0.99$), a slope close to 1 (0.98) (confidence interval 95%: 0.85–1.12) and an intercept value near 0 (−0.04 ng/ml) (confidence interval 95%: 0.26–0.18 ng/ml).

5. Conclusions

Wine contaminated with OTA continues to be a problem and future EU legislation fixing a maximum level of tolerance in wine is expected. Therefore, validated methods to determine OTA in wine are needed for both surveillance and research. A simple, rapid and economic extraction procedure of OTA from wine has been presented in this paper. The mycotoxin was extracted from 4 ml of wine acidifed to pH 1.05 to an acceptor phase of 1-octanol. The organic solvent is located inside and in the pores of a hollow fiber immersed in the donor phase. Extraction and clean-up occur in this two liquid-phase system. In spite of the fact that extraction time is 2 h, parallel extraction of several samples can be carried out simultaneously in order to compensate for this disadvantage.

The price of each extraction unit is low, much lower than that of an IAC cartridge and the instrumentation required is inexpensive and very easy to use. LPME requires 15 μl of solvent for extraction; therefore, a large reduction in solvent consumption is achieved in comparison with the IAC method. In addition, OTA extraction and purification of the extract are achieved in one step. The method meets all of the pre-established validation parameters and the analytical performance is fully satisfactory. Therefore, the method is suitable for determining the OTA content in wine.

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