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Analytical Methods

# A novel solid phase extraction – Ultra high performance liquid chromatography–tandem mass spectrometry method for the quantification of ochratoxin A in red wines

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ABSTRACT

A novel and advanced technology on solid phase extraction column prior to liquid chromatography coupled to tandem mass spectrometry has been used for the determination of ochratoxin A in red wine samples. Due to the need of a reliable and rugged method according to current regulations and with the aim of minimize heuristic efforts associated with analytical method development, the statistical design of experiment was employed. On other hand, the method validation according to European Commission 2002/657/EC was achieved. The values obtained for decision limit (CC$\alpha$), detection capability (CC$\beta$), limits of detection and quantification were 0.07 μg $L^{-1}$, 0.14 μg $L^{-1}$, 0.13 μg $L^{-1}$ and 0.41 μg $L^{-1}$, respectively. The recoveries values were ranged from 95.7% to 107.2%. These values were compatible with the 2.0 μg $L^{-1}$ maximum allowable concentration limit established by different international regulations.


## 1. Introduction

Ochratoxin A (OTA) is a toxic metabolite produced by some species of fungi belonging to the genus *Aspergillus*, such as *A. ochraceus*, *A. niger*, *A. carbonarius*, or *A. flavus*; *Penicillium* (*P. verrucosum*) or the genera *Petromyces* and *Neopetromyces* (Abarca, Bragulat, Castellá, & Cabañes, 1994; Frisvad & Samson, 2000), commonly found in red wine (Zimmerli & Dick, 1996) and other foodstuffs (el Khoury & Atoui, 2010). Although *P. verrucosum* and *A. ochraceus* are considered to be the main OTA producing species, there is strong evidence that *A. carbonarius* is the main contributor to OTA contamination in wine grapes (Bau, Bragulat, Abarca, Minguez, & Cabañes, 2005). OTA is one of the most important mycotoxins of concern for human health. This compound is a potent nephrotoxin (major toxic effect), which also exhibits immunosuppressive, teratogenic, neurotoxic, and carcinogenic properties (European Food Safety Authority, 2006). The International Agency for Research on Cancer (IARC) classified OTA as a possible human carcinogen (group 2B) (IARC, 1993). Several nephropathies affecting animals as well as humans have been attributed to OTA. Based on these and similar studies, OTA is frequently cited as the possible causative agent of endemic kidney disease observed in the Balkans (Balkan endemic nephropathy and related urinary tract tumors) (European Food Safety Authority, 2006).

Second to only cereal, wine is the major source of OTA in the diet. The consumption of OTA can represent up to 10% of the total European intake (European Commission., 2002a). The occurrence of OTA in wine is related to fungal growth on grapes, and the most important factors that influence OTA contamination of grapes and wine include: temperature and relative humidity in the month before harvesting the berries, the type of wine (maceration), and the percentage of damaged berries before vinification. The OTA mycotoxin is more frequently detected in red wines than in rosé and white wines (Quintela, Villarán, de Armentia, & Elejalde, 2013). Although levels are usually low in these samples, the concurrent intake of different contaminated food and drinks might provide a total amount of OTA near to the provisional tolerance for weekly intake set by the World Health Organization (WHO) at 100 ng $kg^{-1}$ body mass. In 1998, the Scientific Committee for Food of the European Commission considered that it would be prudent to reduce the tolerable daily intake to less than 5 ng $kg^{-1}$ body mass (European Commission., 1998), which indicates that OTA accumulation constitutes a considerable risk situation for consumers (Food & Agriculture Organization/World Health

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Organization, 1995, 2001). Moreover, OTA shows a high stability against degradation, possessing a high resistance to acidic conditions and high temperatures (el Khoury & Atoui, 2010). In addition, this toxin has shown to also be stable in blood, with a half-life of about 35 days in serum (Studer-Rohr, 1995).

Recent trends in regulatory programs for food safety have focused on the emerging threat of mycotoxins in foods. The International Organization of Vine and Wine (OIV) has recommended for wines, a maximum allowable concentration limit (MAL) of 2 μg $L^{-1}$, which is the same as the maximum permitted level established by the European Commission (European Commission, 2005).

Given the particular chemical properties of OTA and the fact that it can be present in wine at low concentrations, it is apparent that a reliable sample preparation and subsequent analytical testing method for wine samples constitutes an analytical challenge. The most commonly used analytical testing method for mycotoxins in food is high performance liquid chromatography (LC) coupled to a fluorescence detector (FL) (Tessini et al., 2010; González-Osnaya, Soriano, Moltó, & Mañes, 2008). This particular analytical protocol was established in a reference method for the determination of OTA in wine adopted by the European Standard EN 14133 (European Committee for Standardization (CEN), 2003). Several alternative detection methodologies, such as liquid chromatography with diode array detection (LC–DAD) (Soleas, Yan, & Goldberg, 2001) or liquid chromatography coupled to mass spectrometry (LC–MS or LC–MS/MS) (Zöllner & Mayer-Helm, 2006) have also been reported. In addition, thin layer chromatography (TLC) (Welke, Hoeltz, Dottori, & Noll, 2010), gas chromatography–mass spectrometry (GC–MS) (Olsson, Borjesson, Lundstedt, & Schnurer, 2002), enzyme (EIA) and fluorescence polarization immunoassays (Saha, Acharya, Roy, Shrestha, & Dhar, 2007; Zezza, Longobardi, Pascale, Eremin, & Visconti, 2009), and capillary electrophoresis (CE) with UV/Vis detection (Almeda, Arce, & Valcárcel, 2008; González-Peñas, Leache, López de Cerain, & Lizarraga 2006) have been reported for OTA's analysis.

Sample preparation is a very important step in the analysis of OTA in food matrices, like wine, due to the amount of endogenous interferences present, e.g., residual sugars, polyphenolic compounds, salts, and additives, that might interfere with an accurate analysis of the mycotoxin. In order to minimize matrix effects, different sample preparation-procedures have been reported for the extraction of OTA from food matrices. Most of the reported sample preparation procedures were based on the use of immunoaffinity columns (IACs), which contain specific antibodies for retaining OTA prior to washing away matrix interferences (Longobardi et al., 2013). However, the use of IACs is costly and the sample preparation procedure is tedious and time consuming. Other alternative sample preparation procedures like liquid–liquid extraction (LLE) in milk, wine, and beer (González-Osnaya et al., 2008; Sáez, Medina, Gimeno-Adelantado, Mateo, & Jiménez, 2004), and pressurized liquid extraction (PLE) in rice (González-Osnaya et al., 2008), have also been reported as successful for OTÁs removal/enrichment approaches. The stability of OTA when using PLE, microwave, ultrasound, and magnetic stirring-assisted extractions has been evaluated (Liazid, Palma, Brigui, & Barroso, 2007). Several published reviews have focused on the sample preparation trends related to the analysis of OTA and other mycotoxins and have compiled most of these methodologies (Cigić & Prosen, 2009; Turner, Subrahmanyam, & Piletsky, 2009). Recently, dispersive liquid–liquid microextraction (DLLME) has been used for the determination of OTA in wine by LC–MS (Campone, Piccinelli, & Rastrelli, 2011) or by laser-induced fluorescence detection (LIF) (Arroyo-Manzanares, Gámiz-Gracia, & García-Campaña, 2012).

Solid phase extraction (SPE)-based procedures have been reported for the removal of OTA from food matrices (Cao et al., 2013; Li et al., 2012; Sáez et al., 2004; Tamura, Takahashi, Uyama, & Mochizuki, 2012; Tessini et al., 2010; Yu & Lai, 2010) prior to different detection options. The number of publications in reference to the using of SPE for the analysis of OTA in wine has increased during the last decade. Solid phase extraction method development can be employed as a fast alternative for the sample preparation of OTA in wine. The method development process can be optimized by using a variety of experimental design techniques that afford a quick solution to identifying the best experimental strategy.

Approaches employing multivariate optimization have demonstrated to minimize heuristic efforts associated with analytical method development. The use of multivariate optimization would be of great importance for the qualitative and quantitative determination of OTA in complex samples. Among the alternatives, design of experiments (DOE) is a powerful technique that allows the minimization of the number of experiments and the understanding of the systems behavior and how the input variables (factors), and their interactions, affect the response (Aguilar & Rincón, 2007).

In the present work, full factorial designs were carried out to obtain the best SPE extraction and enrichment conditions of OTA from red wine samples. To the best of our knowledge, this is the first time that the new and novel SPE column technology known as the ISOLUTE® Myco cartridges has been applied towards the extraction of mycotoxins from a wine matrix. After the development of an optimized SPE methodology, a significant amount of wine matrix effect was substantially reduced, which translated in an improvement in the analytical detection sensitivity. The proposed SPE-UPLC–MS/MS methodology was validated according to the Commission Decision 657/2002/EC normative (Commission Decision, 2002b) and the obtained figures of merit were compatible with the maximum allowable limit established for OTA levels in wine samples.

## 2. Materials and methods

### 2.1. Reagents and Samples

Ochratoxin A, analytical standard, was obtained from Fluka (Steinheim, Germany). Acetonitrile (ACN), methanol (MeOH), and water Optima® LC–MS grade were purchased from Fisher Scientific (Fair Lawn, New Jersey, USA). Formic acid was obtained from Fisher Scientific (Loughborough, UK). Toluene HPLC grade was purchased from Fisher Scientific (Fair Lawn, New Jersey, USA). Working standard solutions in acetonitrile were prepared by stepwise dilution from a 10 mg $L^{-1}$ OTA stock standard solution immediately before use. Quantification was achieved by preparing spiked red wine samples with proper amounts of the analyte. The solutions were maintained at −4 °C, protected from light, and kept in amber flasks. Intermediate spiked samples of red wines without previously detected OTA were prepared. For the solid phase extraction step, 3 cc and 60 mg ISOLUTE® Myco cartridges (Biotage, USA) were used. Red wine samples (Malbec, Bonarda, Cabernet-Sauvignon, Syrah, and Torrontes varieties) were purchased from local liquor stores.

### 2.2. Mass spectrometry

Mass spectrometry analyses were performed on a Quattro Premier™ XE Micromass MS Technologies triple quadrupole mass spectrometer with a *Z*-Spray™ electrospray ionization source (Waters, Milford, USA). The source was operated in a positive (ES+) mode at a desolvation temperature 350 °C with $N_2$ as the nebulizer and the source temperature was kept at 120 °C. The capillary voltage was maintained at 3.5 kV and the extractor

voltage was set at 1.0 kV. Ultrapure nitrogen was used as desolvation gas with a flow of 800 L $h^{-1}$. Argon was used as collision gas at a flow of 0.18 mL $min^{-1}$. Detection was performed in multiple reaction monitoring (MRM) mode of selected ions at the first ($Q_1$) and third quadrupole ($Q_3$). To choose the fragmentation patterns of $m/z$ ($Q_1$) → $m/z$ ($Q_3$) for the analyte in MRM mode, direct infusion (via syringe pump) into the MS of OTA (1 mg $L^{-1}$) standard solution in acetonitrile was performed and the product ion scan mass spectra were recorded. The OTA quantification transition in MRM mode was ($m/z$) 404.1 → 239.2 produced at collision energy of 25 eV. The transitions used for confirmation purposes were ($m/z$) 404.1 → 341.1 and 404.1 → 358.2 produced at collision energy of 25 and 20 eV; respectively. The values optimized for the dwell time and cone voltage parameters were of 0.08 s and 20 V. The data were acquired using MassLynx Mass Spectrometry Software (Waters, Milford, USA).

### 2.3. Chromatography

An Acquity™ Ultra High Performance LC system (Waters, Milford) equipped with autosampler injection and pump systems (Waters, Milford) was used. The autosampler vial tray was maintained at 15 °C. The needle was washed with appropriate mixtures of acetonitrile and water. The separation was performed by injecting 25 μL sample onto an ACQUITY UPLC® BEH $C_{18}$ (Waters, Milford, USA) analytical column with 2.1 mm internal diameter × 50 mm length, and 1.7 μm particle size. The binary mobile phases consisted of water with 0.1% (v/v) of formic acid (A) and acetonitrile with 0.1% (v/v) of formic acid (B) delivered at 0.35 mL $min^{-1}$. The composition of the isocratic elution program was 30% A and 70% B. Under the mentioned conditions, OTA retention time was 0.71 min within a total chromatographic run time of 2.0 min. The column was held at a temperature of 30 °C. Under above conditions, no sample contamination or sample to sample carryover was observed.

### 2.4. First experimental design

The aim of this study was to evaluate the variables directly related to the efficiency of the solid phase extraction protocol. A two-level-three-factors ($2^3$) full factorial design consisting of 8 runs and three replicates of the central point was performed in order to determine the influence of the factors and their interactions in the optimization of the principal steps of the solid phase extraction procedure using ISOLUTE® Myco cartridges. All the experiments were carried out randomly and run in triplicates ($n$ = 3) with the purpose of minimizing the effects of uncontrolled factors. According to the experimental design, low, central, and high levels of the variables were designated as (−), (0), and (+); respectively. Since sample volume (SV; maximum and minimum levels: 3 and 7 mL; respectively, with 5 mL as the central point), solvent nature of both the interference wash (ISN; maximum and minimum levels: water and water:MeOH (90:10); respectively, with water:ACN (90:10) as the central point), and the elution step (ESN; maximum and minimum levels: toluene and 0.1% formic acid in MeOH; respectively, with 0.1% formic acid in ACN as the central point) would significantly influence extraction and cleanup efficiency, they were chosen as the critical variables to be optimized as a function of the analytical response (peak area of OTA on the chromatogram). The matrix design and the relative responses obtained are listed in Table 1. Statistical analysis of variance (ANOVA) and $p$-value (probability) were used to evaluate the statistical significance of the effects. The data analysis was performed using the software STATISTICA 8.0 (StatSoft Inc., OK, USA).

Main effects and their interactions for the first experimental design can be seen in the Pareto chart depicted in Fig. 1(a). This graphical representation demonstrated that the elution step was statistically significant at a 95% ($\alpha$ = 0.05) confidence level. As detailed, neither the interference wash nor the sample volume had a statistically significant effect on the response. On the other hand, OTA response was improved when 0.1% formic acid in methanol was used as solvent for the elution step.

### 2.5. Second experimental design

In order to improve the methods performance, a second experimental design strategy based on studying variables affecting the enrichment of the analyte was evaluated. Thus a two-level-three-factors full factorial design consisting of 11 experiments was performed. The studied variables were sample volume (SV), eluting solvent volume (EV, MeOH with 0.1% formic acid), and reconstitution volume (RV, MeOH). The factors in this second design were studied in the following ranges for SV: 4 (as the minimum point), 5 (as the central point), and 8 mL (as the maximum levels). In the case of EV, the minimum and maximum levels were 1 and 3 mL, with 2 mL as the central point. Finally, for RV, the values ranged between 0.25 and 0.55 mL, being 0.5 mL the central level. The experimental design matrix depicting the percentage recovery obtained for each experiment is detailed in Table 1.

Based on the obtained results (Fig. 1(b)), the reconstitution volume was the statistically important factor. As a consequence a reconstitution volume of 0.5 mL was chosen. Neither sample

**Table 1**
Full factorial design matrices.

| Experiment | First experimental design | | | | Second experimental design | | | |
|---|---|---|---|---|---|---|---|---|
| | SV | ISN | ESN | Relative response (%) (Peak area)[a,b] | SV | EV | RV | Recovery (%)[c] |
| 1 | + | + | + | 2.6 ± 0.2 | + | + | + | 98.3 ± 5.2 |
| 2 | + | + | − | 87.2 ± 3.9 | + | + | − | 75.1 ± 1.6 |
| 3 | + | − | + | 5.6 ± 2.2 | + | − | + | 81.3 ± 3.2 |
| 4 | + | − | − | 100.0 ± 7.3 | + | − | − | 50.1 ± 1.3 |
| 5 | − | + | + | 4.5 ± 3.4 | − | + | + | 100.0 ± 6.9 |
| 6 | − | + | − | 70.9 ± 4.6 | − | + | − | 81.3 ± 3.0 |
| 7 | − | − | + | 2.1 ± 0.7 | − | − | + | 89 ± 4.6 |
| 8 | − | − | − | 78.3 ± 1.8 | − | − | − | 63.2 ± 1.5 |
| 9 | 0 | 0 | 0 | 63.9 ± 1.6 | 0 | 0 | 0 | 90.2 ± 4.2 |
| 10 | 0 | 0 | 0 | 69.7 ± 1.9 | 0 | 0 | 0 | 82.3 ± 3.2 |
| 11 | 0 | 0 | 0 | 60.5 ± 2.4 | 0 | 0 | 0 | 85.5 ± 2.6 |

[a] Experiments' responses are shown as means ± standard deviation of three replicates ($n$ = 3).
[b] Response resulted from the OTAs chromatogram peak area measurements. Once these values were obtained, the highest one was considered the 100% and the others as a percentage of this maximum (relative response (%)).
[c] Experiments' responses are shown as means ± standard deviation of three replicates ($n$ = 3).

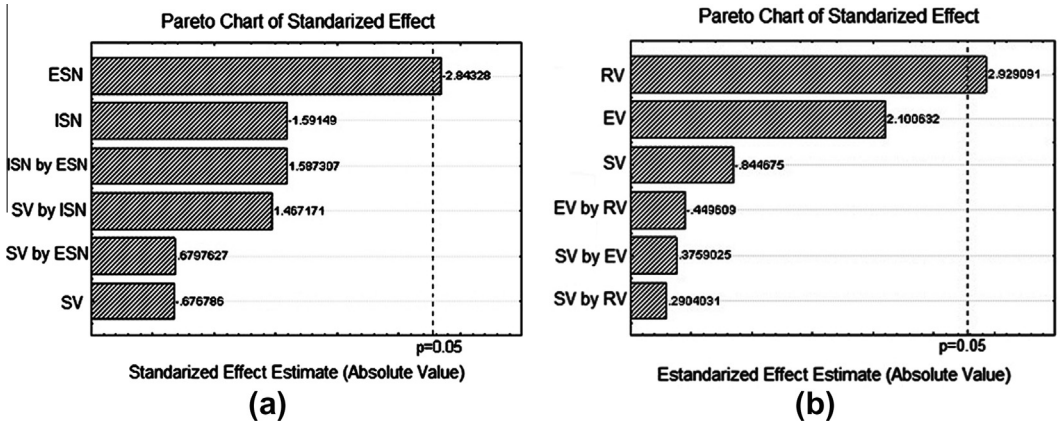


**Fig. 1.** (a) Pareto chart of standardized effects for the independent variables: sample volume (SV, mL), solvent nature of the interference wash (ISN), and solvent nature on the elution step (ESN); (b) Pareto chart of standardized effects for the independent variables sample volume (mL, SV), elution volume (mL, EV), and reconstitution volume (mL, RV).

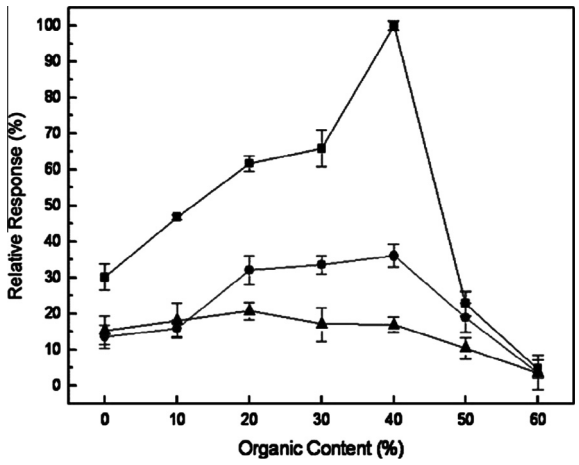


**Fig. 2.** Further optimization of the wash step. Red wine spiked samples with 10 μg $L^{-1}$ OTA concentration were used. Wash based on $H_2O$/ACN mixtures (●); wash based on $H_2O$/MeOH mixtures (▲); dual wash stage based on a water step prior $H_2O$/ACN mixtures at varied proportions (■).

volume nor elution volume or their interactions were statistically significant. Then, a sample volume of 5 mL, an elution volume of 2 mL, and a reconstitution volume of 0.5 mL were chosen as optimal for further experiments. An enrichment factor of 10-fold was achieved based on these above mentioned conditions.

### 2.6. Further interference wash optimization

Although solvent nature of the interference wash using water and mixtures of it with a 10% organic content was previously evaluated, a deleterious matrix effect was still observed. It follows that further optimization of the interference wash steps were required. Then an exhaustive assessment of the SPE interference wash was performed. Water mixtures with ACN and MeOH, at varied proportions, were studied.

As observed in Fig. 2, the OTA relative response (setting as 100% the maximum peak area obtained for a 10 μg $L^{-1}$ spiked red wine sample solution) increased as the ACN content increased up to 40%. Methanol was also evaluated at several proportions. In this case, OTA response increased as the methanol increased up to 20% (Fig. 2). As observed, better results were obtained with ACN. Thus a wash of interferences step based on a mixture $H_2O$/ACN (60:40) was employed in further experiments. Additionally, a water-based wash step prior to the $H_2O$/ACN one was assayed. As seen in the mentioned figure, the addition of this stage improved the response/background ratio by significantly diminishing the matrix effect previously observed. This fact was quantified by comparing the signal of the analyte with and without the SPE procedure (spiked samples) to the signal in pure solvent (ACN). Thus, calibration curves from spiked matrix and spiked pure solvent samples were created. The percentage of the quotient of the slopes ($b$) in the spiked and solvent samples was used as an indicator of the extent of the ion suppression or signal enhancement, which was calculated as: $100 - (b_{spiked}/b_{solvent} \times 100)$. No signal enhancement, but response reduction of approximately 95% due to matrix interference was observed for red wine without applying the SPE procedure, while only a 20% was observed after the SPE approach was carried out (see supplemental material).

A summary of the above optimized conditions for the SPE procedure is presented in Table 2.

**Table 2**
Optimized solid phase extraction procedure.

| Sample | Red wine |
|---|---|
| Condition | ACN (2 mL); 1 mL $min^{-1}$ |
| Equilibrate | $H_2O$ (2 mL); 1 mL $min^{-1}$ |
| Sample volume | 5 mL (gravity load) |
| Wash interference 1 | $H_2O$ (5 mL); 1 mL $min^{-1}$ |
| Wash interference 2 | $H_2O$/ACN (60:40) (5 mL); 1 mL $min^{-1}$ |
| Elute | 0.1% formic acid in MeOH (2 mL) |
| Evaporate | Drying under nitrogen stream (30° C) |
| Reconstitute | MeOH (0.5 mL); transfer to UPLC vials |

## 3. Results and discussion

### 3.1. Method validation

The method validation was performed using the concepts for the figures of merit according to the 2002/657/EC European Decision (Commission Decision, 2002b): decision limit (CCα) and detection capability (CCβ), detection and quantification limits, recovery percentage, trueness, precision, and linearity. According to the 2002/675/EC document, both the decision limit and the detection capability are important parameters to validate a method. The CCα was defined as "the limit at and above which can be concluded, with an error probability of α (α = 0.05), that a sample is non-compliant", and CCβ as "the smallest content of

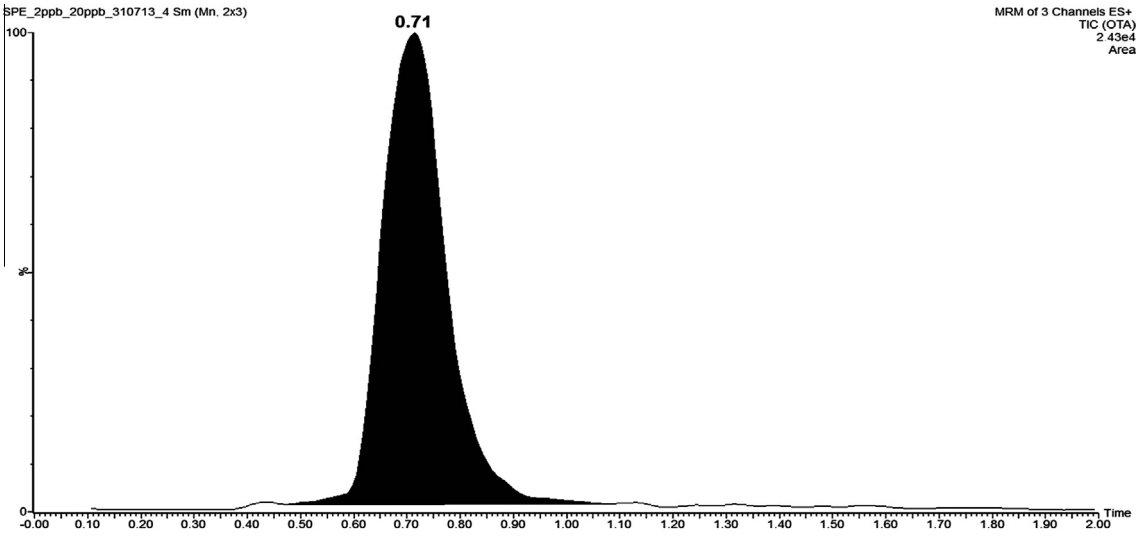


**Fig. 3.** Chromatogram of OTA in a red wine spiked sample at the MAL (2.0 μg $L^{-1}$).

the substance that may be detected, identified and/or quantified in a sample with an error probability of $\beta$ ($\beta = 0.05$)". Before the calculations, homoscedastic behavior, matrix effect, and the variability at each studied concentration level were evaluated. Hence, under linearity and homoscedasticity CC$\alpha$ and CC$\beta$ were given by:

$$CC\alpha = t_{df,1-\alpha}\frac{s_y}{b}\sqrt{1+\frac{1}{n}+\frac{\bar{x}^2}{J\sum_{i=0}^{I}(x_i-\bar{x})^2}} \quad (1)$$

$$CC\beta = \delta_{df,\alpha,\beta}\frac{s_y}{b}\sqrt{1+\frac{1}{IJ}+\frac{\bar{x}^2}{J\sum_{i=0}^{I}(x_i-\bar{x})^2}} \quad (2)$$

where $b$ is the slope of the regression curve, $\bar{x}$ the mean concentration; $t$ the associated $t$-values, $\delta$ the non-centrality parameter, $S_y$ the standard error of the estimate, $J$ the number of the replicates per concentration level of the spiked samples, and $I$ the number of concentration levels for the spiked samples: $i = 1, 2 \ldots I$. The non-centrality parameter can be approximated by $\delta_{\alpha,\beta} = t_{df,1-\alpha} + t_{df,1-\beta}$. For CC$\alpha$ and CC$\beta$ calculation, an approach based on spiked samples was developed. Thus, one red wine variety (Malbec) was evaluated. A total of 35 samples composed by five blank samples from red wines, 10 replicates at the MAL (2.0 μg $L^{-1}$), 5 replicates at 0.5 MAL (1 μg $L^{-1}$), 1.5 MAL (3.0 μg $L^{-1}$), 0.2 MAL (0.4 μg $L^{-1}$), and 2 MAL (4.0 μg $L^{-1}$) were used. Additionally, LoD and LoQ values were evaluated as $3.3S_y/b$ and $10S_y/b$ respectively. The values obtained for CC$\alpha$, CC$\beta$, LoD, and LoQ were 0.07 μg $L^{-1}$, 0.14 μg $L^{-1}$, 0.13 μg $L^{-1}$ and 0.41 μg $L^{-1}$, respectively. These values were compatible with the 2.0 μg $L^{-1}$ maximum allowable concentration limit established by different international regulations. In addition, the analytical performance of proposed methodology was in agreement or even better than many methods reported in the literature currently. A typical chromatogram of a red wine spiked sample is shown in Fig. 3. As can be seen, satisfactory shape and peak symmetry were obtained.

### 3.2. Precision, recovery, and trueness

In order to evaluate the methodology, precision, recovery, and trueness were calculated. Precision of the whole method was evaluated in terms of repeatability (intraday precision) and reproducibility (interday precision). Also, it is acceptable to study the trueness – expressed as bias (%) – of the measurements through recovery of additions of known amounts of the analyte to a blank matrix. Red wine spike samples composed by five blanks, 10 replicates at the MAL (2.0 μg $L^{-1}$), 5 replicates at 0.5 MAL (1 μg $L^{-1}$), 1.5 MAL (3.0 μg $L^{-1}$), 0.2 MAL (0.4 μg $L^{-1}$), and 2 MAL (4.0 μg $L^{-1}$) were analyzed under the conditions mentioned above. Reproducibility was evaluated with a similar procedure in three different weeks. The results are shown in Table 3. According to these results, low variability for the methodology was observed and the intra-day precision was in agreement with the current legislation (European Commission, 2006). On the other hand, the inter-day precision was 16.9% at a concentration level of 0.2MAL (0.4 μg $L^{-1}$), which was significantly lower than the indicative value, <23%, for concentrations lower than 100.0 μg $L^{-1}$, reported by the EU Decision (Commission Decision, 2002b). Moreover, recovery and trueness were in agreement with the same regulation, which establishes that for a concentration range $\leqslant$1 μg $L^{-1}$, an acceptable bias is between −50% and +20% and, for a concentration range >1 μg $L^{-1}$–10 μg $L^{-1}$, an acceptable bias is between −30% and +10%.

**Table 3**
Precision, Bias (%), and Recovery (%) for the Myco-based SPE-UPLC–MS/MS.

| Concentration Level (μg $L^{-1}$) | Intra-day RSD (%)[a] | Inter-day RSD (%)[a] | Recovery (%)[b] | Bias (%)[c] |
|---|---|---|---|---|
| 0.4 (0.2MAL) | 12.6 | 16.9 | 95.9 | 4.1 |
| 1.0 (0.5 MAL) | 14.3 | 15.8 | 107.2 | −7.2 |
| 2.0 (MAL) | 7.9 | 9.1 | 106.5 | −6.5 |
| 3.0 (1.5MAL) | 5.9 | 7.2 | 99.8 | 0.2 |
| 4.0 (2MAL) | 3.5 | 5.2 | 99.0 | 1 |

[a] RSD (%) = relative standard deviation.
[b] Recovery (%) = [(measured content/spiked level)×100].
[c] Bias(%) = [((measured content − spiked level)/spiked level) × 100].

### 3.3. Linearity

Linearity was evaluated from values closer to the CC$\alpha$ and the LoD up to approximately 200 μg $L^{-1}$. The linearity of the calibration curves for spiked red wine samples was satisfactory with determination coefficients ($R^2$) of 0.9976. The F-test demonstrated that linear regression was statistically acceptable in the working range and this model showed goodness of fit.

## 4. Conclusions

A Myco-based SPE sample clean-up/extraction/enrichment prior to UPLC–MS/MS determination of OTA in red wine at low

ppb levels has been developed. The critical variables influencing both the extraction and the enrichment procedures were evaluated by means multivariate strategies. Under optimized conditions, the initially observed matrix effects were substantially reduced and the obtained OTA quantification levels were below the maximum allowable limits. To the best of our knowledge, this is the first time that a study like the herein presented is proposed. The developed methodology allowed determining OTA with high efficiency, sensitivity, accuracy and an outstanding diminish of matrix effects, which are commonly observed when using mass spectrometry.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.foodchem.2014.09.094.

## References

Abarca, M. L., Bragulat, M. R., Castellá, G., & Cabañes, F. J. (1994). Ochratoxin A production by strains of *Aspergillus niger* var. *niger*. *Applied and Environmental Microbiology, 60*, 2650–2652.

Aguilar, M., & Rincón, F. (2007). Improving knowledge of garlic paste greening through the design of an experimental strategy. *Journal of Agricultural and Food Chemistry, 55*, 10266–10274.

Almeda, S., Arce, L., & Valcárcel, M. (2008). Combined use of supported liquid membrane and solid-phase extraction to enhance selectivity and sensitivity in capillary electrophoresis for the determination of ochratoxin A in wine. *Electrophoresis, 29*, 1573–1581.

Arroyo-Manzanares, N., Gámiz-Gracia, L., & García-Campaña, A. M. (2012). Determination of ochratoxin A in wines by capillary liquid chromatography with laser induced fluorescence detection using dispersive liquid–liquid microextraction. *Food Chemistry, 135*, 368–372.

Bau, M., Bragulat, M. R., Abarca, M. L., Minguez, S., & Cabañes, F. J. (2005). Ochratoxigenic species from Spanish wine grapes. *International Journal of Food Microbiology, 98*, 125–130.

Campone, L., Piccinelli, A. L., & Rastrelli, L. (2011). Dispersive liquid–liquid microextraction combined with high-performance liquid chromatography tandem mass spectrometry for the identification and the accurate quantification by isotope dilution assay of ochratoxin A in wine samples. *Analytical and Bioanalytical Chemistry, 399*, 1279–1286.

Cao, J., Kong, W., Zhou, S., Yin, L., Wan, L., & Yang, M. (2013). Molecularly imprinted polymer-based solid phase clean-up for analysis of ochratoxin A in beer, red wine, and grape juice. *Journal of Separation Science, 36*, 1291–1297.

Cigić, H., & Prosen, I. K. (2009). An overview of conventional and emerging analytical methods for the determination of mycotoxins. *International Journal of Molecular Sciences, 10*, 62–115.

el Khoury, A., & Atoui, A. (2010). Ochratoxin A: General overview and actual molecular status. *Toxins, 2*, 461–493.

European Commission (1998). Scientific Committee on Food Opinion on Ochratoxin A, CS/CNTM/MYC/14 final, Annex II to Document XXIV/2210/98 (pp. 28). Brussels.

European Commission. (2002a). Directorate-General Health and Consumer Protection. Reports on tasks for scientific cooperation. Report of experts participating in Task 3.2.7. Assessment of dietary intake of ochratoxin A by the population of EU member states. <http://ec.europa.eu/food/fs/scoop/3.2.7_en.pdf>.

Comission, European. (2002b). Commission Decision (EC) No. 2002/657/EC of 12 August 2002 implementing Council Directive 96/23/EC concerning the performance of analytical methods and the interpretation of results. *Official Journal of the European Union L, 221*, 8–36.

Commission, European. (2005). Commission regulation (EC) No.123/2005 of 26 January 2005 amending regulation (EC) No. 466/2001 as regards ochratoxin A. *Official Journal of the European Union L, 25*, 3–5.

Commission, European. (2006). Commission Regulation (EC) No. 401/2006 of 23 February 2006. Laying down the methods of sampling and analysis for the official control of the levels of mycotoxins in foodstuffs. *Official Journal of the European Union L, 70*, 12–34.

European Committee for Standardization (CEN) (2003). *Foodstuffs – determination of ochratoxin A in wine and beer – HPLC method with immunoaffinity column clean-up, EN 14133:2003*. Belgium: Brussels.

European Food Safety Authority (EFSA). (2006). Opinion of the scientific panel on contaminants in the food chain on a request from the commission related to ochratoxin A in food. *The EFSA Journal, 365*, 1–56.

Food and Agriculture Organisation/World Health Organisation (FAO/WHO). (1995). *Evaluation of certain food additives and contaminants (Fourty-fourth report of the Joint FAO/WHO Expert Committee on Food Additives)*. No: WHO Technical Report Series. 859.

Food and Agriculture Organisation/World Health Organisation (FAO/WHO). (2001). *Safety evaluation of certain mycotoxins in food, prepared by the fifty-sixth meeting of the Joint FAO/WHO Expert Committee on Food Additives (JECFA) – WHO Food Additives series 47 – FAO Food and Nutrition Paper – IPCS – International Programme on Chemical Safety*. Geneva: World Health Organisation.

Frisvad, J. C., & Samson, R. A. (2000). Neopetromyces gen. nov and an overview of teleomorphs of Aspergillus subgenus Circumdati. *Studies in Mycology, 45*, 201–207.

González-Osnaya, L., Soriano, J. M., Moltó, J. C., & Mañes, J. (2008). Simple liquid chromatography assay for analyzing ochratoxin A in bovine milk. *Food Chemistry, 108*, 272–276.

González-Peñas, E., Leache, C., López de Cerain, A., & Lizarraga, E. (2006). Comparison between capillary electrophoresis and HPLC-FL for ochratoxin A quantification in wine. *Food Chemistry, 97*, 349–354.

International Agency for Research on Cancer (IARC). (1993). *Some naturally occurring substances food items and constituents, heterocyclic aromatic amines and mycotoxin Monographs on the evaluation of carcinogenic risks to humans*. Lyon, France: IARC Science Publications, pp. 489–521.

Li, J., Liu, X., Han, S., Li, J., Xu, Q., Xu, H., et al. (2012). Analysis of ochratoxin a in wine by high-resolution UHPLC–MS. *Food Analytical Methods, 5*, 1506–1513.

Liazid, A., Palma, M., Brigui, J., & Barroso, C. G. (2007). Investigation on ochratoxin A stability using different extraction techniques. *Talanta, 71*, 976–980.

Longobardi, F., Iacovelli, V., Catucci, L., Panzarini, G., Pascale, M., Visconti, A., et al. (2013). Determination of ochratoxin A in wine by means of immunoaffinity and aminopropyl solid-phase column cleanup and fluorometric detection. *Journal of Agricultural and Food Chemistry, 61*, 1604–1608.

Olsson, J., Borjesson, T., Lundstedt, T., & Schnurer, J. (2002). Detection and quantification of ochratoxin A and deoxynivalenol in barley grains by GC–MS and electronic nose. *International Journal of Food Microbiology, 72*, 203–214.

Quintela, S., Villarán, M. C., de Armentia, L., & Elejalde, E. (2013). Ochratoxin A removal in wine: A review. *Food Control, 30*, 439–445.

Sáez, J. M., Medina, A., Gimeno-Adelantado, J. V., Mateo, R., & Jiménez, M. (2004). Comparison of different sample treatments for the analysis of ochratoxin A in must, wine and beer by liquid chromatography. *Journal of Chromatography, A, 1029*, 125–133.

Saha, D., Acharya, D., Roy, D., Shrestha, D., & Dhar, T. K. (2007). Simultaneous enzyme immunoassay for the screening of aflatoxin B1 and ochratoxin A in chili samples. *Analytica Chimica Acta, 584*, 343–349.

Soleas, G. J., Yan, J., & Goldberg, D. M. (2001). Assay of ochratoxin A in wine and beer by high-pressure liquid chromatography photodiode array and gas chromatography mass selective detection. *Journal of Agricultural and Food Chemistry, 49*, 2733–2740.

Studer-Rohr, I. (1995). Dissertation No. 11071, Swiss Federal Institute of Technology. Zürich.

Tamura, M., Takahashi, A., Uyama, A., & Mochizuki, N. (2012). A method for multiple mycotoxin analysis in wines by solid phase extraction and multifunctional cartridge purification, and ultra-high-performance liquid chromatography coupled to tandem mass spectrometry. *Toxins, 6*, 476–486.

Tessini, C., Mardones, C., von Baer, D., Vega, M., Herlitz, E., Saelzer, R., et al. (2010). Alternatives for sample pre-treatment and HPLC determination of ochratoxin A in red wine using fluorescence detection. *Analytica Chimica Acta, 660*, 119–126.

Turner, N. W., Subrahmanyam, S., & Piletsky, S. A. (2009). Analytical methods for determination of mycotoxins: A review. *Analytica Chimica Acta, 632*, 168–180.

Welke, J. E., Hoeltz, M., Dottori, H. A., & Noll, I. B. (2010). Determination of ochratoxin A in wine by high-performance thin-layer chromatography using charged coupled device. *Journal of the Brazilian Chemical Society, 21*, 441–446.

Yu, J. C. C., & Lai, E. P. C. (2010). Molecularly imprinted polymers for ochratoxin A extraction and analysis. *Toxins, 2*, 1536–1553.

Zezza, F., Longobardi, F., Pascale, M., Eremin, S. A., & Visconti, A. (2009). Fluorescence polarization immunoassay for rapid screening of ochratoxin A in red wine. *Analytical and Bioanalytical Chemistry, 395*, 1317–1323.

Zimmerli, B., & Dick, R. (1996). Ochratoxin A in table wine and grape-juice. Occurrence and risk assessment. *Food Additives and Contaminants, 13*, 655–668.

Zöllner, P., & Mayer-Helm, B. (2006). Trace mycotoxin analysis in complex biological and food matrices by liquid chromatography–atmospheric pressure ionization mass spectrometry. *Journal of Chromatography, A, 1136*, 123–169.