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Determination of ochratoxin A and T-2 toxin in alcoholic beverages by hollow fiber liquid phase microextraction and ultra high-pressure liquid chromatography coupled to tandem mass spectrometry

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ABSTRACT

A new method for the determination of ochratoxin A and T-2 toxin in alcoholic beverages (wine and beer) by hollow fiber liquid microextraction was optimized. The extraction step was followed by ultra high-pressure liquid chromatography coupled to tandem mass spectrometry (UHPLC–MS/MS). The extraction procedure was based on the extraction of mycotoxins from the sample to the organic solvent (1-octanol) immobilized in the fiber, and afterwards, they were desorbed in a mixture of acetonitrile/water (80:20, v/v) at pH 7 prior to chromatographic determination. Different variables affecting the extraction process such as organic solvent, salt content, extraction time and desorption solution were studied. The developed method was validated in wine and beer, using white wine and alcoholic beer as representative matrices for both types of samples. Relative recoveries higher than 70% were obtained for the selected mycotoxins. Good linearity ($R^2 > 0.993$) was obtained and quantification limits (0.02–0.09 $\mu g\,L^{-1}$) below European regulatory levels were achieved. Repeatability, expressed as relative standard deviation, was always lower than 12%, whereas interday precision was lower than 21%. The proposed method was applied to the analysis of several types of wines and beers and ochratoxin A was detected in a rosé wine at 1.1 $\mu g\,L^{-1}$.

1. Introduction

Mycotoxins are secondary metabolites produced by many species of fungi and they are considered one of the major contaminants of agricultural products [1]. Ochratoxin A is among the most important mycotoxins found in several food commodities. It has been detected in a variety of foodstuffs such as cereals, coffee beans, wheat, barley, maize and beverages such as wine, beer and grape juice [2,3] and the International Agency for Research on Cancer (IARC) has classified ochratoxin A as possible carcinogen to humans (Group B) [4]. Another important group of mycotoxins are trichothecenes. These are responsible for a wide range of disorders in animals, including feed refusal, weight loss and vomiting [5]. Although the number of characterized trichothecenes is large, only a few of them have been detected [6], and among them, T-2 toxin is a highly toxic compound that mainly occurs in grains such as barley, corn and cereal-based products [7].

These mycotoxins are commonly present in alcoholic beverages such as beer and wine, and they may increase the risk on human health in high beer and wine consuming countries. For instance and in relation to ochratoxin A, an EU report [8] indicates that the contribution of various food commodities are estimated as 50% for cereals, 13% for wine, 10% for coffee, 8% for species and 5% for beer, highlighting that alcoholic beverages consumption could contribute significantly to human intake of this mycotoxin. Furthermore, Codex Alimentarious has indicated that 15% of total intake of ochratoxin A in humans is due to wine consumption [9].

Because their toxicity, many countries have set up regulations for their control in food. Thus, European legislation has established maximum levels of ochratoxin A in several matrices, recommending a tolerance level lower than 2.0 $\mu g\,kg^{-1}$ for all types of wine [10], whereas no legislation has been established for ochratoxin A in beer and T-2 toxin in wine and beer so far, although for HT-2 and T-2 a combined temporary daily maximum intake of 0.06 $\mu g\,kg^{-1}$ body weight has been proposed [10].

In order to ensure compliance with current or future legislation, it is necessary to provide reliable and accurate mycotoxin analytical methods which allow unambiguous identification and confirmation at trace levels. Traditionally, mycotoxins were mainly determined by immunochemical methods, based on enzyme-linked immunosorbent assay (ELISA) [11,12], due to speed, ease

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of operation, sensitivity and high throughput [13]. However, they can provide false positives because cross-reaction, and nowadays chromatographic methods are mainly used. In this sense, thin-layer chromatography (TLC) [14], gas chromatography (GC) [15] and liquid chromatography (LC) [16] have been commonly applied, although in the last few years, LC coupled to mass spectrometry (MS) has become the most suitable technique for the analysis of these compounds because it allows the determination of several type of mycotoxins and it can also be applied for confirmation purposes [17,18].

However, before chromatographic step, sample preparation that implies extraction, purification and concentration of the extract must be carried out to remove the major part of interferences present in the sample and to preconcentrate the analytes, in order to reach the concentrations established by legislation. Traditionally, the extraction of mycotoxins from liquid samples such as wine and beer has been based on solid phase extraction (SPE) using immunoaffinity columns (IAC). Basically this procedure is based on percolating the sample or the extract onto a column filled with immobilized antibodies against the specific mycotoxin. The sample is washed off by water or aqueous buffer and the toxin is eluted. In fact, for the determination of ochratoxin A in wine, an official method based on dilution with hydrogen carbonate and polyethylene glycol (PEG) followed by IAC has been proposed [19], taking advantage that the ochratoxin A is bound specifically to the antibody, allowing total removal of the matrix. However these columns present several problems such as the high cost of the columns, which cannot be reused, and the complex matrices contain thousands of compounds, and some of them can interfere with the antibodies as it happens with ochratoxin C that can produce cross-reaction when ochratoxin A must be determined [20].

In order to reduce the cost of the analysis, several alternatives to IAC have been checked. For instance, other sorbents such as C18 [21,22], OASIS HLB [23] and ion exchangers [24] have been proposed. However, most of these procedures are laborious and employ large amount of organic solvents, and microextraction techniques can be used as alternative procedures because they eliminate or minimize the use of organic solvents. In this sense, several procedures have been proposed for the extraction of ochratoxin A from beer and wine using solid phase microextraction (SPME) [25,26], although it has several disadvantages such as high cost, sample carry-over and a decline in performance with time. In this sense, other alternatives such as hollow fiber liquid phase microextraction (HF-LPME) can be used, bearing in mind that it is effective, simple, low cost, uses microliters of organic solvents, allows the concentration of the analytes and provides an excellent sample clean-up ability. Although this technique has been applied for the extraction of several organic compounds from different matrices [27,28], including alcoholic beverages [29], applications of HF-LPME in the field of mycotoxins are substantially lacking, and to the best of our knowledge, there are only two examples, which both describe determination of ochratoxin A in wine. The first one uses hollow fibers to immobilize the organic solvent (1-octanol) used to extract the mycotoxin and fluorescence detection [30], whereas the second one applies a combined procedure, immobilizing the organic solvent (also 1-octanol) in a Teflon membrane and a second extraction by SPE prior capillary electrophoresis (EC) separation and detection by UV [31].

In this work, a simple, sensitive and cost effective HF-LPME procedure has been developed for the extraction of ochratoxin A and T-2 toxin from wine and beer samples prior chromatographic determination, using ultra high-pressure liquid chromatography coupled to tandem mass spectrometry (UHPLC–MS/MS). The potential of the method has been demonstrated through the analysis of a number of different samples.

2. Experimental

2.1. Chemicals and materials

Ochratoxin A and T-2 toxin were obtained from Biopure (Tulln, Austria). Stock standard solutions of individual compounds (concentration of 200 mg L^{-1}) were prepared by careful weighing of the powder and dissolution in 50 mL of acetonitrile (J.T. Baker, Deventer, The Netherlands). Then, a multicompound working solution at a concentration of 2 mg L^{-1} was prepared by appropriate dilution of the stock solutions with acetonitrile. These solutions were kept at 4 °C and renewed weekly.

Acetic acid (>97%), formic acid (>98%) and ammonium formate were obtained from Panreac (Barcelona, Spain). HPLC-grade methanol was supplied by Sigma (Madrid, Spain). Hydrochloric, orthophosphoric and boric acid were supplied by Riedel-de-Haën (Seelze-Hannover, Germany). Sodium hydroxide and sodium chloride were purchased from Panreac. Other reagents were 1-octanol (99.5%, Fluka, Neu-Ulm, Germany), dihexyl ether (≥97%; Fluka) and *n*-undecane (99%, Sigma). Ultrapure water was obtained from a Milli-Q Gradient water system (Millipore, Bedford, MA, USA).

Q3/2 Accurel PP hydrophobic polypropylene hollow fiber tubing (200 μm wall thickness, 600 μm i.d., and 0.2 μm pore size) was obtained from Membrana GmbH (Wuppertal, Germany). 10-μL syringe plungers were provided by Hamilton (Bonaduz, Switzerland).

2.2. Apparatus and software

Chromatographic analyses were performed with an Acquity UPLC system (Waters, Milford, MA, USA), using an Acquity UPLC BEH C18 column (100 mm × 2.1 mm, with 1.7 μm particle size), from Waters. MS/MS detection was performed using an Acquity TQD tandem quadrupole mass spectrometer (Waters, Manchester, UK). The instrument was operated using an electrospray (ESI) source in positive mode. Data acquisition was performed using MassLynx 4.0 software with QuanLynx software (Waters). A Vortex mixer Heidolph, model Reax 2000 (Heidolph, Schwabach, Germany) and an analytical AB204-S balance (Mettler Toledo, Greinfesee, Switzerland) were also used. A Reax-2 rotary agitator from Heidolph was used for sample extraction. An ultrasound bath from JP Selecta (Barcelona, Spain) was used for beer degasification.

2.3. UHPLC–MS/MS analysis

Chromatographic analyses were carried out using a gradient elution with eluent A being methanol and eluent B consisting of an aqueous solution of ammonium formate (5 mM). The analysis started with 25% of eluent A, which was increased linearly up to 100% in 3.75 min. This composition was held for further 1.25 min before being returned to 25% of eluent A in 0.50 min, followed by a re-equilibration time of 1 min, to give a total run time of 6.5 min. The flow rate was set at 0.35 mL min^{-1} and column temperature was maintained at 30 °C. Aliquots of 5 μL of sample extract were injected into the chromatographic system.

The mycotoxins were detected using ESI in positive mode. The ionization source parameters were: capillary voltage 3.5 kV, extractor voltage 3 V, source temperature 120 °C, desolvation temperature 150 °C, cone gas flow 50 L h^{-1} and desolvation gas flow 650 L h^{-1} (both gases were nitrogen). Collision-induced dissociation was performed using argon as collision gas at a pressure of 4×10^{-3} mbar in the collision cell. The multiple reaction monitoring (MRM) transitions and the applied cone voltages and collision energies are summarized in Table 1.

Table 1
Logarithm of octanol-water partition coefficient ($\log K_{ow}$), retention time windows (RTWs) and MS/MS conditions.

Mycotoxin	$\log K_{ow}$	RTW (min)	Cone voltage (V)	Quantification transition[a]	Confirmation transition[a]
Ochratoxin A	4.74	3.32–3.43	25	404.2 > 239.2 (20)	404.2 > 358.2 (15)
T-2 toxin	2.25	3.77–3.92	25	484.7 > 215.3 (20)	484.7 > 245.4 (15)

[a] Collision energies (eV) are given in brackets.

2.4. Sample preparation

Ochratoxin A and T-2 toxin were extracted from samples using HF-LPME procedure, based on a setup previously described [29,32]. Briefly, the procedure was as follows: 12 mL of wine or degassed beer and 3 mL of an aqueous solution of hydrochloric acid (0.01 M) were introduced in a 15 mL test tube. After that, 1.5 g of sodium chloride was added to the mixture and the tube was vortexed for 1 min. The hollow fibers were cut into 2-cm pieces, and a 10-μL syringe plunger was inserted into the hollow fibers. After that, the fiber was impregnated with 1-octanol during 1 min, and then it was placed into the 15-mL test tube containing the sample. The tube was capped and put into a rack in the rotary agitator for 4 h at 90 rpm. After the extraction, the fiber was withdrawn from the plunger, and it was put into a 2-mL vial containing 1.5 mL of a mixture of acetonitrile and buffer phosphate at pH 7 (80:20, v/v). This vial was placed into a rack and then in the rotary agitator for 10 min at 30 rpm in order to perform desorption of the analytes from the fiber to the solvent. Finally, the fiber was removed from the vial with tweezers and 5 μL was injected into the chromatographic system.

2.5. Samples

Wine samples were purchased from local supermarkets in Almeria (Spain) and from home-made productions. Bottled and canned beer samples were also purchased from local supermarkets. Nine wine samples (six red, two white and one rosé) and eight beer samples (including three non-alcoholic beers) were analyzed. Samples were stored at 4–5 °C until analysis.

3. Results and discussion

3.1. Optimization of the HF-LPME procedure

First, the optimization of the HF-LPME conditions for the extraction of both mycotoxins from alcoholic beverages was evaluated. For that purpose, blank white wine was spiked with 5 μg L^{-1} of each mycotoxin and the following parameters were optimized: composition of the organic phase, composition of donor and desorption solution and extraction time. Peak area was selected as analytical response during the optimization process.

In HF-LPME the organic phase selection is based on proper immobilization in the pores of the fiber, stability (non-volatility), immiscibility with the sample and extraction efficiency of analytes. 1-octanol, dihexyl ether and *n*-undecane were evaluated, showing in Fig. 1A the obtained results. It can be observed that 1-octanol provided higher peak areas for both mycotoxins than dihexyl ether, whereas *n*-undecane did not allow the extraction of the selected compounds. Furthermore, good repeatability values were obtained when 1-octanol was used, and therefore, it was selected for further experiments.

Once the organic phase had been optimized, the conditions of the donor phase were evaluated. Because ochratoxin A is a weak acid, having pK_a values of 4.4 and 7.5 for the carboxylic and phenolic groups respectively, pH of the donor solution was studied to improve the extraction of the selected compounds. For that purpose, pH of the donor solution was studied from 1 to 13, and Fig. 1B shows the obtained results. It can be noted that the extraction of both compounds decreased considerable when basic pHs (higher than 7) were used. Furthermore, it can be observed that from pH 1 to 4, the peak area was kept constant. Therefore, further experiments were carried out at pH 2, which is in accordance to previous results obtained for other extraction techniques [26].

The ionic strength was also evaluated, considering that increasing the ionic strength of the sample solution can improve the extraction of organic compounds from aqueous solutions. The effect of ionic strength was investigated by varying the concentration of NaCl in the range of 0–25% (w/v) in the donor. Fig. 1C shows the effect of salt concentration on the peak area and it can be indicated that ochratoxin A was better extracted when 10% of NaCl was used, whereas for T-2 toxin, higher peak area was obtained at 20%. However, no significant differences were observed between 10% and 20% (see Figure 1C) and bearing in mind that repeatability was better at 10% of NaCl, it was selected as optimum value.

Desorption solution was also studied. pH and the ratio of acetonitrile/water was evaluated, considering that mycotoxins are usually extracted when a mixture of acetonitrile/water (80:20, v/v)

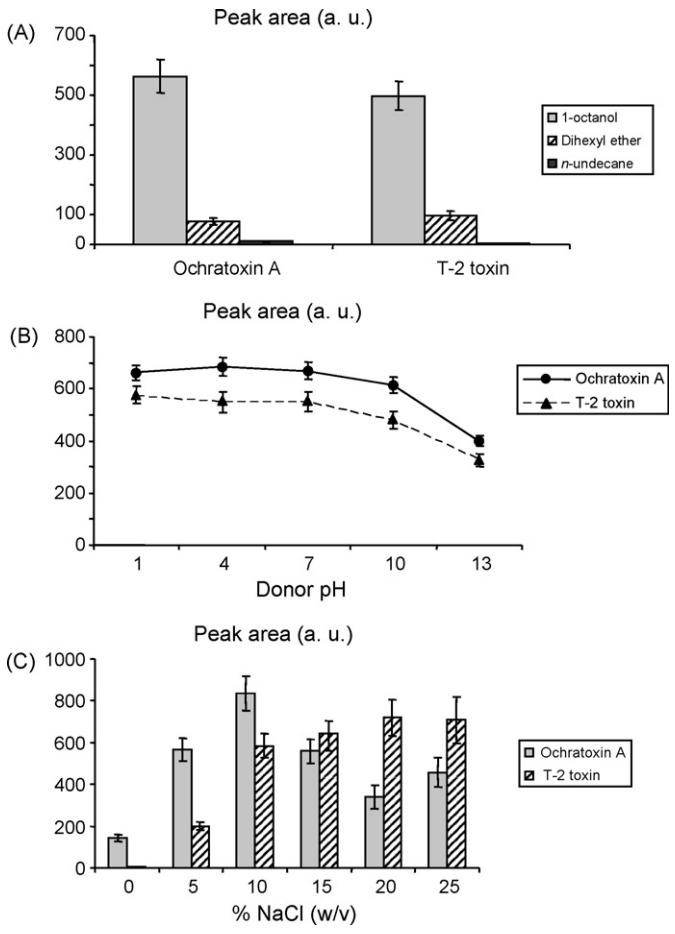

Fig. 1. Effect of: (A) organic solvent; (B) donor pH and (C) ionic strength on the peak area of the selected mycotoxins when a blank white wine sample was spiked at 5 μg L^{-1}. Error bars indicated the standard deviation (n = 3). Extraction conditions: extraction time: 2 h; desorption solvent: acetonitrile; desorption time: 5 min.

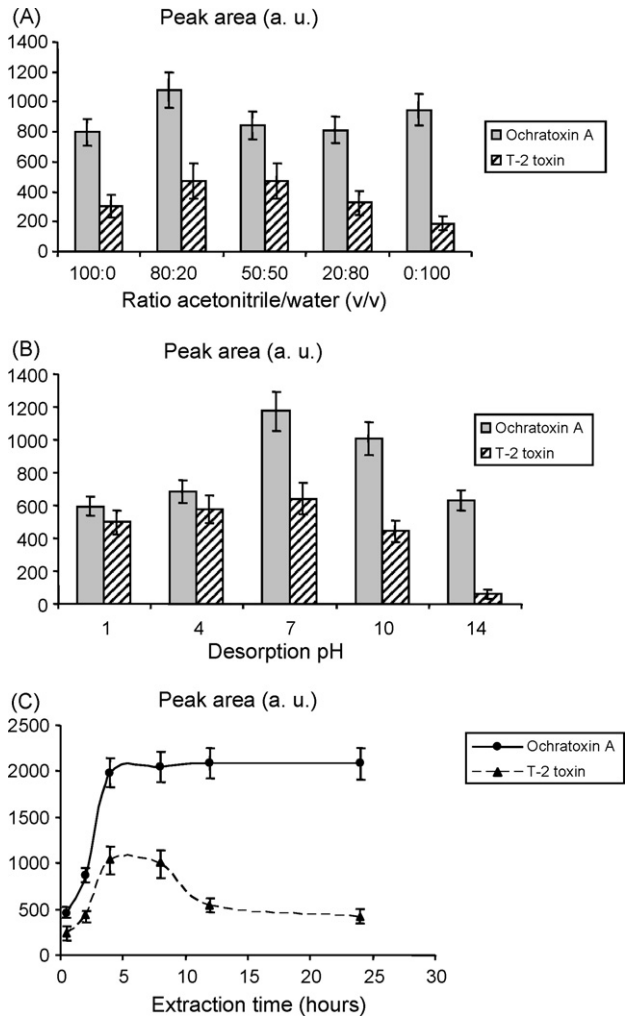

Fig. 2. Effect of: (A) ratio of acetonitrile/water in the desorption solvent; (B) desorption pH and (C) extraction time on the peak area of the selected mycotoxins when a blank white wine sample was spiked at 5 μg L^{-1}. Error bars indicated the standard deviation (n = 3). Extraction conditions: organic solvent: 1-octanol; donor pH: 2; ionic strength: 10% NaCl (w/v); desorption time: 5 min.

is used [33], and this mixture is LC-compatible. Fig. 2A shows the results when different ratios of acetonitrile/water were evaluated, whereas Fig. 2B shows the results when the pH of desorption solution ranged from 1 to 14. It can be observed that better results were obtained when the 80:20 (v/v) acetonitrile/water mixture was used. When the percentage of water increases lower peak areas were obtained. On the other hand, higher peaks were achieved at pH 7 in the desorption solution, obtaining worse results at acidic or basic pHs.

Extraction time was studied from 0.5 to 24 h, with a constant stirring speed at 90 rpm. Fig. 2C shows the effect of extraction time on the peak area of the compounds. The signal of ochratoxin A increased with longer extraction time up to 4 h, and after that, the signal kept constant, indicating that the equilibrium was reached. However, for T-2 toxin, peak area reached a maximum at 4 h, and after that decreased, indicating the desorption of this mycotoxin could occur. After 8 h, peak area was kept constant. Bearing in mind the obtained results, 4 h was selected as optimum extraction time. Although the extraction time was relatively long, a large number of different samples can be extracted simultaneously, increasing sample throughput.

Ethanol can influence the extraction of mycotoxins from alcoholic beverages, and the dilution of the sample was evaluated.

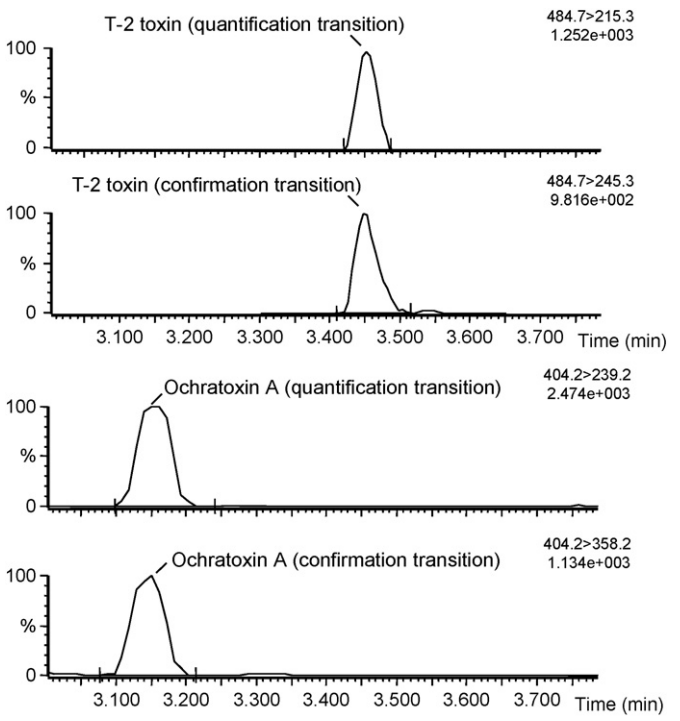

Fig. 3. UHPLC–MS/MS chromatograms from a white wine spiked with ochratoxin A and T-2 toxin at 2 μg L^{-1}.

15 mL of extraction volume was fixed, and several volumes of wine (from 2 to 12 mL) were checked, adding water to 12 mL. Additionally, 3 mL of the HCl solution 0.01 M was used to fix the pH. No differences were observed (data not shown) and no dilution of sample was carried out in order to minimize sample handling.

Finally other parameters such as agitation rate of the sample, desorption agitation rate and desorption time were fixed at 90 rpm, 30 rpm and 5 min, respectively.

Fig. 3 shows an UHPLC–MS/MS chromatogram of a spiked blank white wine (2 μg L^{-1}) extracted using the optimized HF-LPME procedure, obtaining a clean chromatogram without interferences.

3.2. Validation of the proposed method

To evaluate the applicability of the proposed method, linearity, trueness, repeatability and quantification limits were investigated in wine and beer

First, the influence of matrix was evaluated, studying the extraction of ochratoxin A and T-2 toxin from different types of wine (red, rosé and white) and from different types of beers (alcoholic and non-alcoholic). For that purpose, enrichment factor and relative recoveries were evaluated spiking the selected matrices with 5 μg L^{-1} of the targeted compounds. White wine was used as representative matrix for the analysis of wine samples and alcoholic beers for the two types of selected beers. Table 2 shows the obtained results, and it can be observed that for ochratoxin A, similar enrichment factor was obtained for both types of matrices, whereas T-2 toxin showed different results. Furthermore, relative recoveries ranged from 92% to 101% for ochratoxin A in wines and from 79% to 96% in beers, whereas for T-2 toxin, relative recoveries ranged from 87% to 105% (wine samples) and from 89% to 93% (beer samples). The obtained values indicated the suitability of the method for the analysis of these mycotoxins in wine and beer.

Linearity was evaluated by spiking blank white wine and alcoholic beer, as representative matrices, with different amounts of mycotoxins to obtain a final concentration ranging from 0.1 to

Table 2
Enrichment factor and relative recoveries of mycotoxins in different wines and beers at 5 μg L^{-1}.

Mycotoxin	Wine				Beer		
	Ee[a]	Relative recovery (%)[b]			Ee[a]	Relative recovery (%)[b]	
		Red	Rosé	White		Alcoholic	Non-alcoholic
Ochratoxin A	8.3 (11.7)	92.3 (19.6)	101.0 (9.7)	98.1 (6.6)	7.7 (7.1)	96.2 (7.7)	79.1 (12.8)
T-2 toxin	4.1 (9.3)	87.0 (12.1)	105.0 (9.8)	98.3 (7.9)	5.6 (8.8)	92.9 (9.8)	89.1 (11.2)

[a] Mean enrichment factor. Relative standard deviation is given in brackets (n = 6).
[b] Relative standard deviation is given in brackets (n = 5).

25 μg L^{-1}, before the extraction procedure was applied. Peak area was selected as analytical response and the calibration curves were linear in the range studied with linear determination coefficients higher than 0.993 (see Table 3).

Trueness was estimated through recovery studies, obtaining satisfactory results with recoveries higher than 70% for both matrices and mycotoxins (see Table 3).

Repeatability was studied by running five extractions of white wine and alcoholic beer spiked at 1 and 10 μg L^{-1} of the two mycotoxins. The relative standard deviations (RSDs) ranged from 10.3% to 14.2% for ochratoxin A, whereas for T-2 toxin, RSDs ranged from 9.8% to 16.0% (see Table 3). The interday precision was evaluated at 1 μg L^{-1}, analyzing two samples for a period of 4 days. RSDs were lower than 20% for the two mycotoxins in both matrices, except T-2 toxin in wine, which has values slightly higher than 20% (20.1%), indicating the good precision of the extraction method.

Quantification limits were determined as the lowest concentrations of the analytes that produce chromatographic peaks at signal to noise ratio of 10. As it can be observed in Table 3, quantification limits were lower than 0.1 μg L^{-1} for the two mycotoxins evaluated in both matrices. It must be highlighted that for ochratoxin A, the obtained values were lower than the tolerance levels established for EU legislation in wine (2 μg L^{-1}) [10] and other published methods based on HF-LPME [30].

Finally, the selectivity of the method was studied by running control blank samples. The absence of any signal at the same retention time as the selected mycotoxins indicated that no matrix interferences or chemical compounds are extracted and give a false positive signal.

3.3. Application to real samples

The developed method was applied for the simultaneous determination of ochratoxin A and T-2 toxin in nine wine samples and eight beers, including three non-alcoholic samples. To assure the quality of the results, an internal quality control was carried out in every batch of samples. This quality control implies the extraction of a spiked blank sample (wine and beer) at 2 μg L^{-1} in order to check the reliability of the proposed method, a calibration curve and a blank reagent.

Table 3
Validation parameters of the optimized HF-LPME procedure.

Matrix	Parameter	Ochratoxin A	T-2 toxin
Wine	R^2	0.9957	0.9936
	Relative recovery[a] (1 μg L^{-1})	95.0 (14.2)	88.4 (15.4)
	Relative recovery[a] (10 μg L^{-1})	81.3 (11.5)	87.9 (10.8)
	Interday precision[b] (1 μg L^{-1})	17.4	20.1
	Quantification limit (μg L^{-1})	0.02	0.09
Beer	R^2	0.9969	0.9979
	Relative recovery[a] (1 μg L^{-1})	82.9 (12.4)	79.2 (16.0)
	Relative recovery[a] (10 μg L^{-1})	90.9 (10.3)	85.2 (9.8)
	Interday precision[b] (1 μg L^{-1})	13.9	19.6
	Quantification limit (μg L^{-1})	0.06	0.07

[a] Repeatability values, expressed as relative standard deviation, are given in brackets (n = 5).
[b] Relative standard deviation (n = 4).

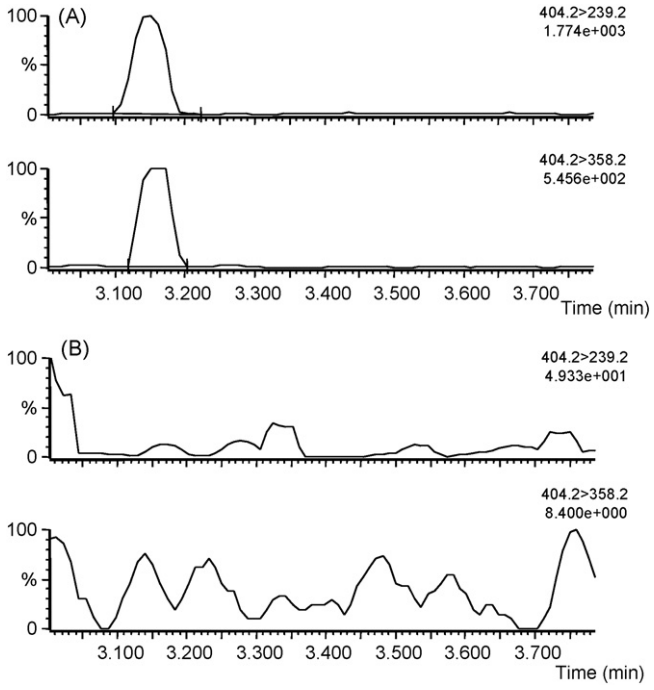

Fig. 4. UHPLC–MS/MS chromatograms obtained from: (A) a wine sample containing ochratoxin A at 1.1 μg L^{-1} and (B) a blank red wine sample.

Ochratoxin A was only detected in a wine sample (rosé wine) at 1.1 μg L^{-1}, which was below the tolerance level established by European legislation [10], whereas neither ochratoxin A nor T-2 toxin were detected in beer samples.

Fig. 4 shows the UHPLC–MS/MS chromatograms for the positive sample of ochratoxin A at 1.1 μg L^{-1} in a wine sample as well as a blank sample, observing that no interfering peaks appear on the chromatogram, indicating the high selectivity of the HF-LPME procedure coupled to UHPLC–MS/MS.

4. Conclusions

A new method for the determination of ochratoxin A and T-2 toxin in alcoholic beverages has been developed using HF-LPME. This method is simpler and cheaper compared to the most widely adopted clean-up procedures based on immunoaffinity columns. The method can be considered environmentally friendly because small amount of organic solvent is used. The proposed method eliminates the interferences present in wine and beer samples, increasing the sensitivity of the UHPLC–MS/MS. Good performance characteristics (linearity, trueness, repeatability, interday precision and quantification limits) were obtained, determining the assayed mycotoxins below the values established by European legislation. For instance quantification limits were always lower than

0.1 $\mu g\,L^{-1}$, allowing the determination of ochratoxin A in wines at 0.02 $\mu g\,L^{-1}$, which were ten times lower than previous results using HF-LPME and LC-fluorescence detection [30]. Furthermore, the proposed methodology was applied to several matrices (wine and beer), increasing the applicability of HF-LPME.

Two drawbacks that may be overcome are that the method is not fully automated and reproducibility could be influenced by the operator skill, and extraction time is too long. However, this last problem can be solved considering that a large number of samples can be extracted simultaneously.

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