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Comparison of different sample treatments for the analysis of ochratoxin A in must, wine and beer by liquid chromatography☆

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Abstract

Ochratoxin A (OTA) is a mycotoxin produced by some species of *Aspergillus* and *Penicillium verrucosum*. It has been found in foods and feed all over the world. There is a great concern about OTA because it is nephrotoxic and probably, carcinogenic to humans. Most of analytical methods developed for OTA in wine, beer and other products are based on LC with fluorescence detection (LC–FLD). In the present work, various procedures for extraction and/or clean-up for determination of OTA in musts, wine and beer by LC–FLD were compared: (1) dilution with polyethylen glycol 8000 and $NaHCO_3$ solution and clean-up an on immunoaffinity column (IAC); (2) extraction with chloroform and IAC clean-up; solid-phase extraction (SPE) on (3) reversed-phase (RP) C_{18}; (4) RP phenylsilane and (5) Oasis HLB cartridges. SPE on phenylsilane and Oasis HLB have not been reported for OTA analysis in beverages. The same LC–FLD conditions and concentration ratio were used. The former procedure was simple, rapid and provided flat baselines, free from most impurity peaks, high OTA recoveries and quite repeatable results. RP C_{18} using methanol–acetic acid (99.5:0.5) as elution solvent provided good recoveries and precision, thus becoming a cheaper but interesting alternative at 0.1–1 ng/ml spiking levels. Oasis HLB cartridges were usually better than phenylsilane. Possible binding of OTA to proteins or other components was tested by acid treatment before extraction but no significant differences with controls appeared.

Keywords: Extraction methods; Wine; Beer; Must; Food analysis; Ochratoxin A; Mycotoxins

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*, the genus *Penicillium* (*P. verrucosum*) or the genera *Petromyces* and *Neopetromyces* [1–4]. OTA is widely distributed and its occurrence has been reported in cereals [5–7], coffee [8–11], beans, soya, cacao, nuts, dried fruits, milk [12], wine [13–17], beer [18–24], meat, and human blood serum [25,26]. This mycotoxin has been shown to be nephrotoxic, hepatotoxic, teratogenic and immunotoxic to animals. It causes kidney and liver tumours in rats and mice [27,28]. Supposedly, OTA might be the causal agent of an endemic fatal disease in south-eastern Europe (Balkan Endemic Nephropathy) [1]. In 1993, the International Agency for Research on Cancer (IARC) classified OTA as possible carcinogenic for humans (group 2B) [29]. Therefore, there is a great concern on this metabolite at present because it can be taken from different food sources. Although levels are usually low in each commodity, the concurrent intake of different contaminated food and drinks might provide a total amount of OTA near the provisional tolerable weekly intake set by the World Health Organization (WHO) at 100 ng/kg body mass [30]. OTA is relatively stable in human blood and its half-life may reach about 35 days in serum [31]. 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 body mass [32], which indicates that OTA accumulation constitutes a risk situation for consumers.

Some authors have reported on the presence of this mycotoxin in wine and beer at low but variable levels (from

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below 0.1 μg/l to more than 1 μg/l) [13–21]. It has been also found in musts and grape juices. The data about the proportion of contaminated wine samples and the level of OTA in these beverages are rather inconsistent. It also happens with beer. In a survey on beers consumed in Spain, Legarda and Burdaspal have found OTA in 100 and 97.4% of imported and national beers, respectively (the overall OTA range of positive samples was 0.005–0.121 ng/ml) [21]. Maximum allowable limit (MAL) for OTA in wine should be set to 2 ng/ml, according to the OIV proposal [33]. However, different MAL for OTA in wine and beer have been laid down in various countries such as The Netherlands (0.3 ng/ml) or Finland (0.5 ng/ml) [34]. Therefore, it is necessary to use highly sensitive and accurate analytical methods for this toxin.

Liquid chromatography with fluorescence detection (LC–FLD) is the most widely used technique for analysis of OTA. LC using other detection methods, such as photodiode array [34] or mass spectrometry (LC–MS–MS) [22,35–37] has also been reported. These two detection methods are less sensitive than FLD but can aid as confirmative tools. Moreover, TLC, GC–MS of the trimethylsilyl derivative [34], electrophoresis with laser-induced fluorescence [26] and enzyme immunoassay (EIA) have been also employed. The GC–MS method was reported to have poor sensitivity, recovery and precision [34]. EIA methods usually provided higher values than chromatography.

There are various extraction and clean-up protocols for OTA in wines. Zimmerli and Dick [13,14] used extraction with chloroform after addition of NaCl and acidification followed by clean-up on an immunoaffinity column (IAC). Addition of $NaHCO_3$ and polyethylene glycol (PEG) 8000 to sample followed by IAC clean-up has been reported [16,38]. Zöllner et al. [35] used solid-phase extraction (SPE) on previously activated C_{18} cartridges before separation by LC–MS–MS. Jornet et al. [39] used a slight modification of this procedure for OTA analysis in wine but LC–FLD was applied. Satisfactory results using ion-exchange cartridges for clean-up were not found. Purification on silica gel SPE cartridges has been reported in wine [40] or beer [22,37]. Recently, new molecularly imprinted polymers prepared with OTA mimics have been synthesized to bind specifically this mycotoxin [41]. However, no applications of this material have been reported.

In beer, the extraction has been done by using $NaHCO_3$ and NaCl, after degasification of samples [21], followed by IAC clean-up. A collaborative assay in which dilution with PEG 8000–$NaHCO_3$, IAC clean-up and analysis by LC–FLD were applied to red and white wine, and beer has been performed, being promoted as official first action by the AOAC [38].

With regard to LC conditions, reversed-phase column employing acetonitrile–water–acetic acid [16] or methanol–water–acetic acid [13,14] mixtures as mobile phases has been used in most papers. A gradient of methanol (9%) aqueous acetic acid solution has been applied [17,21]. Post-column addition of ammonia to acidic mobile phase has been reported to increase fluorescence yield [13,14,21]. Sodium acetate-acetic acid buffer mixed with acetonitrile has also been used [39,42,43].

In this work, we have made a comparative study on the sample preparation (extraction/clean-up) procedures for determination of OTA by LC–FLD in must, wine and beer. Our aim was to find an optimised methodology that could be applied to all these matrices in order to provide high recovery and precision and low limit of detection (LOD) of the toxin. Some of the clean-up SPE procedures (phenylsilane, Oasis HLB) have not been previously reported for determination of OTA in these matrices. A test to assess for possible binding OTA-matrix proteins or other components was also undertaken.

2. Experimental

2.1. Samples

Samples of red wine, and beer and must were purchased in the retail market. The samples were stored in their original bottles or containers in fridge at 4–5 °C until analysis. In the case of beer, bottles were opened the day before analysis and still kept in fridge. All samples were analysed to find OTA original level. Those showing the lowest OTA levels were selected for spiking experiments. OTA levels in these samples were: 0.16 ng/ml in red wine, 0.08 ng/ml in beer and 0.19 ng/ml in must. The samples were spiked with known amounts of OTA solutions at three levels (0.1, 0.5 and 1.0 ng/ml). An aliquot of standard solution (as low as possible) was added to flask, the solvent was evaporated at 40 °C under N_2 stream and an appropriate volume of sample was added to the dry residue. After through mixing, three aliquots of each spiked sample were taken. They underwent the different treatments (extraction/clean-up procedures) in the same day. Triplicate sample controls (no added OTA) were always run in parallel with each of the spiked samples; they underwent the same treatment and their responses were used to correct for initial OTA level. Wine and beer spiked samples at 0.1 and 1 ng/ml levels were kept in sealed glass flasks at 4–5 °C for 20 days and tested for possible binding of OTA to proteins or other materials as described below.

2.2. Chemicals and materials

The OTA standard was purchased from Sigma (Sigma–Aldrich, Alcobendas, Spain). A stock solution (approximately 500 mg/l) was prepared by solving 1 mg of OTA in 2 ml of toluene–acetic acid (99:1, v/v). A series of working standards from 0.2 to 100 ng OTA/ml was prepared by evaporation of known volumes of the stock solution under N_2 stream, followed by dissolution in LC mobile phase filtered trough 0.2-μm filter. They were used to calibrate the LC detector response. The concentration of the stock solu-

tion was determined by measuring the absorbance at 333 nm of a diluted solution (20–30 mg/l) of OTA in toluene–acetic acid (99:1, v/v) [34].

Acetonitrile, chloroform, acetic acid and methanol (LC grade) were from J.T. Baker (Deventer, The Netherlands). Pure water was obtained from a Milli-Q apparatus (Millipore, Milford, MA, USA) and was used when water was required. Phosphate-buffered saline (PBS) was prepared in the laboratory (0.13 M NaCl, 7 mM Na_2HPO_4, 3 mM NaH_2PO_4) and its pH was adjusted to 7.4. Phosphoric acid (85%), sodium hydrogencarbonate, sodium monohydrogenphosphate, sodium dihydrogenphosphate dihydrate, and sodium chloride were analytical grade from Panreac (Barcelona, Spain) and polyethylene glycol (PEG) 8000 was from Aldrich (Sigma–Aldrich).

The following SPE columns were used: C_{18} 200 mg of sorbent (Waters, Milford, MA, USA), Bakerbond phenylsilane of 500 mg sorbent (J.T. Baker), hydrophilic–lipophilic balanced Oasis HLB 200 mg of sorbent (Waters) and OchraTest immunoaffinity columns (Vicam Science Technology, Watertown, MA, USA).

2.3. Instruments

The LC system consisted on a Waters 600 pump, an automatic injector Waters 717 and a Waters 474 scanning fluorescence detector. Millennium 32 software version 2.0 (Waters) was used to control the chromatograph and process the signals. Separation was performed on a stainless steel LiChrospher 100 C_{18} reversed-phase column (250 mm × 4 mm, 5 μm particle size) connected to a guard column (4 mm × 4 mm, 5 μm particle size) filled with the same phase. The column was kept at 30 °C. The mobile phase was acetonitrile–water–acetic acid (99:99:2, v/v/v) at a flow-rate of 1.0 ml/min. The mobile phase was degassed by passing through an on-line degassing device supplied by Waters. The excitation and emission wavelengths were 333 and 460 nm, respectively. One hundred microlitres of solution was injected into the chromatograph. Two injections were performed for each sample. Confirmation of the identity of OTA peak in samples was done by LC–FLD of the methyl ester derivative as previously described [17,21].

2.4. Extraction

Different extraction procedures of OTA in beverages were tested. Partially degasified beer samples were always completely degasified in ultrasonic bath before further treatment.

(a) Dilution with a solution containing $NaHCO_3$ and PEG 8000 [16]. This procedure is not an extraction 'sensu stricto' because many components of the sample matrix are present before clean-up. Twenty millilitres of sample was thoroughly mixed with 20 ml of an aqueous solution containing 5% $NaHCO_3$ and 1% PEG 8000. The pH was adjusted to 8.5 with 1 M solution of NaOH. The resulting solution was filtered through Whatman glass microfibre filter to remove any present solid.

(b) Acidification with H_3PO_4 and addition of NaCl followed by liquid–liquid extraction with chloroform. Ten ml of wine, beer or must was mixed with 20 ml of an aqueous solution containing 3.4% of phosphoric acid (85%) and 11.8% of NaCl. The mixture was shaken for 5 min. Then it was intensively mixed by hand with 5 ml of chloroform in separatory funnel. The organic phase was separated by centrifugation (2500 × *g*, 4 °C, 5 min) and the aqueous phase was extracted twice with 5 ml of chloroform for two more times. The organic extracts were reunified and evaporated in rotary evaporator under controlled vacuum (Büchi) at 40 °C. The residue was dissolved in 10 ml of PBS solution containing 10% (v/v) ethanol and cleaned-up using IAC.

(c) SPE using reversed-phase C_{18} cartridges. In our experiences, 10 ml of tested sample was passed through a C_{18} cartridge, previously conditioned with 2 ml of acetonitrile and 2 ml of water. After air drying elution of OTA was carried out with 2 ml of acetonitrile. Alternatively, 2 ml of methanol–acetic acid (95.5:0.5, v/v) was used as elution solvent to test for differences in OTA extracting capacity. The solvent was evaporated to dryness under N_2 stream at 50 °C. The residue was dissolved in 250 μl of mobile phase for injection into the liquid chromatograph. The solution can be kept in tightly closed vials at 4 °C until injection. The following modification of the procedure was tried: 10 ml of sample was mixed with 10 ml of PEG–$NaHCO_3$ solution and treated as described in (a). The filtrate was acidified with 3.4% H_3PO_4 aqueous solution and the whole volume was loaded into the C_{18} cartridge. The preceding protocol was followed and methanol–acetic acid (95.5:0.5, v/v) was used as elution solvent.

(d) SPE on reversed-phase phenylsilane cartridges. This is more polar phase than C_{18} and has been used to clean-up roasted coffee extracts before using IAC clean-up [44]. We included it in our study to test its behaviour against other SPE cartridges. The usage protocol was different because no further clean-up by IAC was carried out and no application to OTA analysis in beverages was found in available literature. The cartridge was conditioned with 10 ml of methanol and 5 ml of water but taking care not to let the column run dry. Then, 10 ml of sample was passed through the column. The column was washed with 5 ml of water, air-dried and finally, OTA was eluted with 5 ml of methanol–acetic acid (99.5:0.5, v/v). The solvent was evaporated to dryness at 50 °C under N_2 stream and the residue was solved in 250 μl of mobile phase.

(e) SPE on Oasis HLB cartridges. Guidelines from the recommended generic procedure given in the supplier's brochure (Waters Catalogue, 2001–2002) were followed because no specific protocol for OTA was found. The cartridge was conditioned with 5 ml methanol and 5 ml

water. Then, 10 ml of sample was passed through the cartridge. Higher sample volumes can be extracted if desired but we used always 10 ml of sample. After a washing step with 5 ml of water–methanol (95:5, v/v), the toxin was eluted with 2 ml of methanol. The solvent was evaporated to dryness at 50 °C under N_2 stream and the residue was solved in 250 μl of mobile phase.

2.5. Clean-up with IAC

After the sample dilution performed as described (a) in Section 2.4, the procedure described by Visconti et al. [16] was performed, but using double sample volume. Briefly, 20 ml of filtrate (equivalent to 10 ml of sample) was passed through the OchraTest column at 1 drop/s flow-rate. The column was washed with 5 ml of a solution containing NaCl (2.5%) and $NaHCO_3$ (0.5%) and afterwards, with 5 ml of water. OTA was eluted with 2 ml of methanol. The solvent was evaporated to dryness at 50 °C under N_2 stream and the residue was solved in 250 μl of mobile phase. Double sample volume was used in order to compare all the procedures working with the same sample amount. However, the maximum OTA capacity of the sorbent (about 160 ng) [16] was not surpassed in any case.

The samples extracted were purified according to the following procedure: the residue solved in 10 ml of PBS–ethanol (see (b) in Section 2.4) was loaded into the OchraTest column, which had been conditioned with 20 ml of PBS solution. Then, it was washed with 10 ml of water. After drying with air, OTA was eluted with 3 ml of methanol–acetic acid (98:2, v/v). The elution solvent was evaporated to dryness at 50 °C under N_2 and the residue solved in 250 μl of mobile phase [36].

2.6. Acidic treatment with phosphoric acid

Twenty days after spiking, the 0.1 and 1 ng/ml spiked samples of wine and beer were taken from the fridge and analysed again in duplicate. One control set of two 20-ml aliquots was analysed following the procedure (a) in Section 2.4. Another set of two 10-ml aliquots was acidified with 10 ml of a 3.4% aqueous solution of H_3PO_4 and heated at 50 °C for 30 min. The whole volume (20 ml) was neutralised with 0.1 M NaOH, 20 ml of PEG 8000–$NaHCO_3$ solution was added and the pH was adjusted to 8.5. After filtration through glass microfibre filter, the whole volume was cleaned up on OchraTest column, concentrated as described (a) in Section 2.4 and analysed in under the same LC–FLD conditions. Controls of non-spiked samples were run in parallel.

3. Results and discussion

Quantification of OTA in samples of red wine, beer and must by LC–FLD was performed using an external calibration curve obtained by diluting appropriate aliquots of OTA stock solution in the filtered mobile phase. The experimental points fit well to a straight line in the range 0.4–100 ng/ml ($r^2 = 0.9964$), which was equivalent to 0.01–2.5 ng/ml of sample due to 40:1 concentration ratio attained in sample preparation. Determination of OTA in samples was made using this calibration graph, assuming that the OTA peak is free from matrix interfering substances that may remain in the extract after clean-up. According to Leitner et al. [36], this assumption is true using IAC clean-up, because of its selectivity. When less selective clean-up procedures were used (SPE), the peak area was obtained by manual integration. A sensitive confirmation technique such as LC–FLD of OTA methyl ester, which elutes at different time [21] or LC–MS–MS is necessary to assure peak purity at these low levels [22,37]. The OTA peak was always visible when 100 μl of the 0.4 ng/ml standard solution was injected (40 pg of OTA) but at this level the standard deviation was high and quantification was not reliable.

The red wine sample used for recovery experiments contained 0.16 ng of OTA/ml due to natural contamination (determined using PEG–$NaHCO_3$ dilution and IAC clean-up). Therefore, average peak area of OTA in non-spiked controls run in parallel was always subtracted from the area of OTA peak in each chromatogram. The results of recovery experiments are shown in Table 1.

The two first procedures ((a) and (b) in Section 2.4 plus IAC clean-up) offered the cleanest LC–FLD chromatograms (flat baseline, practical lack of impurity peaks) for all the studied matrices due to IAC selectivity, as can be seen in Fig. 1 for a must sample. The chromatograms obtained with the second procedure (chloroform extraction/IAC clean-up) were very similar to Fig. 1. SPE procedures provided more complex chromatograms with irregular descending baselines and impurity peaks at the beginning of the plots (Figs. 2–4). The recoveries in red wine using method (a) in Section 2.4 were 75.4–107% (mean 91.9%) in the spiking range. The precision, estimated by the R.S.D. of the recovery, was also high (0.9–3%). The recoveries using method (b) in Section 2.4 were 65.9–73.4% (mean 69.6%) in the same range. These recoveries are lower than those that were obtained with the previous procedure in agreement with other reports [16,36], but R.S.D. were a bit lower (0.15–3.1%). Substitution of $CHCl_3$ by CH_2Cl_2 as extraction solvent in method (b) in Section 2.4 was tried, but the results were very disappointing because clean phase separation was not achieved and very low recoveries were obtained (results not shown).

Recoveries using RP C_{18} with acetonitrile as elution solvent were 65.8–82.9% (mean 76.3%) in the 0.1–1 ng of OTA/ml spiking range, and precision was comparable to the preceding methods, except at 0.1 ng/ml spiking level (R.S.D. = 22%). The use of methanol–acetic acid (99.5:0.5, v/v) increased the average recovery although more matrix pigments were also extracted from the cartridge. However, acetonitrile was considered suitable for analysis of OTA in wine with 0.5–1 g C_{18} cartridges [39]. The experience of

Table 1
Recovery of OTA in samples of red wine, beer and must recently fortified with 0.1 to 1 ng toxin/ml ($n = 3$)

Extraction/clean-up procedure	Matrix	Spiking level (ng/ml)					
		0.1		0.5		1.0	
		Mean recovery (%)	R.S.D. (%)	Mean recovery (%)	R.S.D. (%)	Mean recovery (%)	R.S.D. (%)
(a) Dilution with PEG–$NaHCO_3$ + IAC clean-up	Red wine	107	3.0	75.4	0.9	93.2	1.0
	Beer	61.0	6.9	63.7	4.8	75.2	19.2
	Must	80.1	7.8	82.2	3.2	89.5	1.9
(b) Extraction with $CHCl_3$ + IAC clean-up	Red wine	65.9	3.1	69.5	0.55	73.4	0.15
	Beer	33.0	5.1	38.0	1.2	35.4	0.12
	Must	47.1	0.32	52.0	3.7	55.3	4.1
(c1) SPE/C_{18} (MeOH–HAc, 99.5:0.5, v/v)	Red wine	78.0	13	95.0	0.3	99.2	0.4
	Beer	92.0	65	102	3.7	95.1	1.9
	Must	75.3	4.3	89.2	1.5	81.1	2.2
(c2) SPE/C_{18} (acetonitrile)	Red wine	65.8	22	80.1	0.16	82.9	1.9
	Beer	21	7.0	21	4.2	33	3.4
	Must	53	17	17.3	2.0	11.4	8.2
(c3) SPE/C_{18} (PEG–$NaHCO_3$ + MeOH–HAc)	Red wine	95.0	4.1	97.2	1.1	101	0.3
(d) SPE/phenylsilane	Red wine	87	13	97.6	3.8	84.7	2.4
	Beer	–	–	20.6	5.3	37.0	7.6
	Must	34.3	10	55.8	14	78.1	4.9
(e) SPE/Oasis HLB	Red wine	47.3	2.9	51.1	2.5	41.1	1.9
	Beer	59.1	3.4	57.2	2.6	70.6	1.1
	Must	80.9	12	64.2	4.2	76.4	4.7

Concentration ratio: 40:1. Separation and detection were performed by LC–FLD under the same conditions (see Section 2).

combining treatments (a) and (c) in Section 2.4 (dilution with PEG–$NaHCO_3$ and filtration plus acidification of the filtrate before SPE on C_{18} with methanol–acetic acid as elution solvent) was successful and very promising because recoveries reached 95–101% (mean 97.7%) in the 0.1–1 ng of OTA/ml spiking range. Very high recoveries and low dispersion of results (mean R.S.D. 1.8%) compensates for more time consumption. Moreover, as considerable amount of wine pigments are retained in glass microfibre filter, chromatograms were cleaner than those recorded when PEG–$NaHCO_3$ treatment was avoided. This may suppose a substantial improvement in OTA analytical methodology for sample

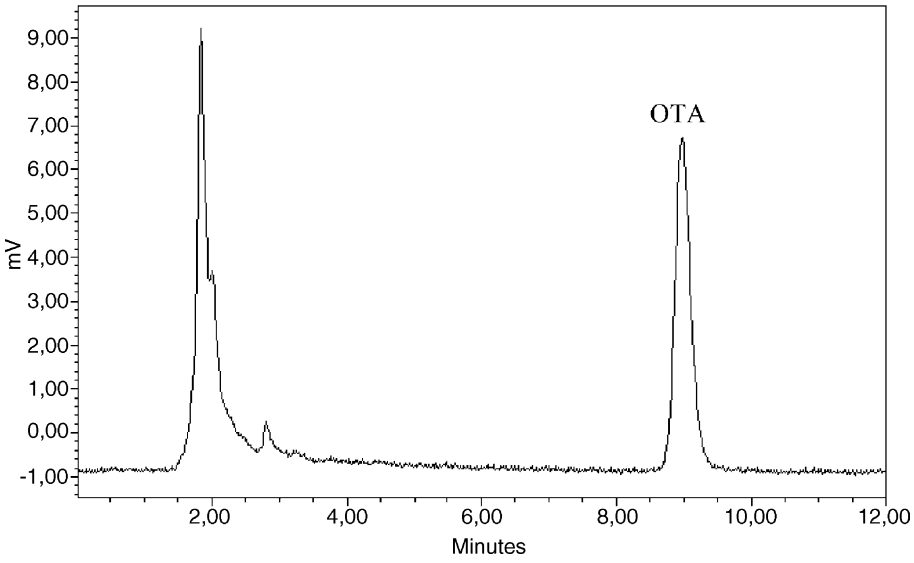

Fig. 1. LC–FLD chromatogram obtained by dilution with a 1% PEG 8000 + 5% $NaHCO_3$ solution, filtration and IAC clean-up of must sample naturally contaminated with 0.19 ng ochratoxin A (OTA)/ml spiked with 0.5 ng OTA standard/ml. Chromatographic conditions: column, LiChrospher 100 C_{18} reversed-phase column (250 mm × 4 mm, 5 μm particle size); column temperature, 30 °C; mobile phase, acetonitrile–water–acetic acid (99:99:2, v/v/v); flow-rate, 1.0 ml/min; injection volume, 100 μl. Excitation wavelength, 333 nm; emission wavelength, 460 nm.

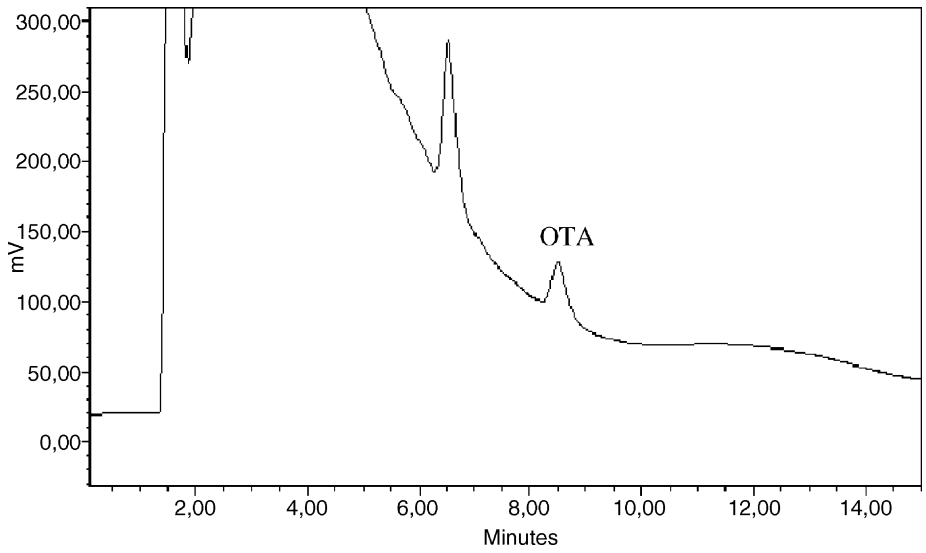

Fig. 2. LC–FLD chromatogram obtained by SPE on RP C_{18} cartridge using methanol–acetic acid (99.5:0.5, v/v) as elution solvent of beer sample naturally contaminated 0.08 ng OTA/ml with spiked with 1.0 ng OTA standard/ml. Chromatographic conditions as in Fig. 1.

treatment because expensive non-reusable immunoaffinity columns might be avoided. This extraction method can be very useful for laboratories analysing OTA in wines after validation by further extensive application study and collaborative analysis.

SPE on phenylsilane provided recoveries ranging from 84.7 to 97.6% (mean 89.8%) in wine, which were high and comparable to those provided by clean-up on C_{18} or by liquid–liquid extraction/IAC. The precision was acceptable in the 0.5–1 ng/ml spiking level (R.S.D. about 3%); however, this parameter was 13% when 0.1 ng OTA/ml was added.

The recoveries using SPE on Oasis HLB with the generic protocol were quite low (41.1–51.1%) at 0.1–1 ng/ml spiking levels.

The controls showed that the beer sample used for experiments was also contaminated with OTA at low level (0.08 ng/ml), which was considered to calculate recoveries. As can be seen in Table 1, the procedure that provided the best recoveries in beer was SPE on C_{18} using methanol–acetic acid (99.5:0.5, v/v) as elution solvent instead of acetonitrile. This last solvent provided very dirty extracts and poor recoveries. Method (a) in Section 2.4 provided recoveries ranging from 61.0 to 75.2% (mean 66.6%), which are lower than expected, according to the results found in the red wine sample. However, 3 out of 18 of the collaborators involved in the AOAC collaborative assay reported some problems working in beer with this procedure, and some of the remain-

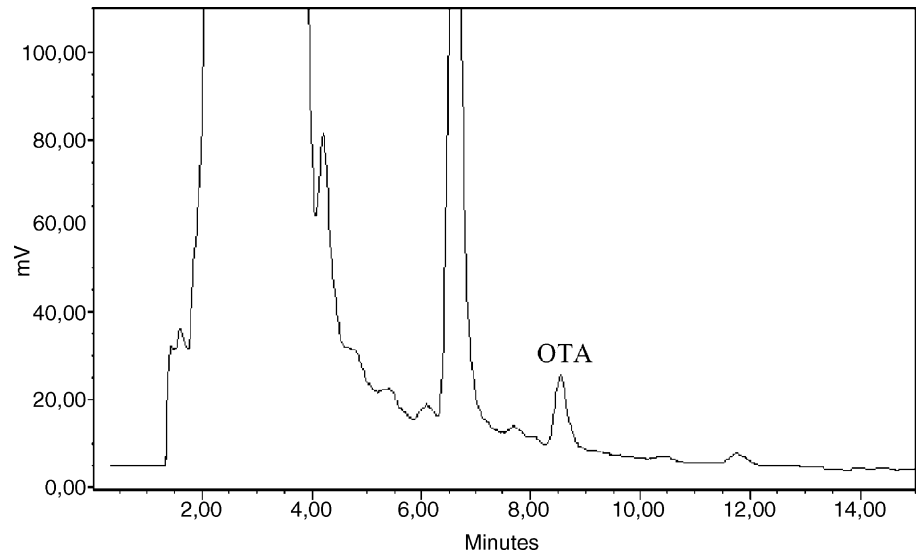

Fig. 3. LC–FLD chromatogram obtained by SPE on RP phenylsilane cartridge of a must sample naturally contaminated with 0.19 ng OTA/ml and spiked with 1.0 ng OTA standard/ml. Chromatographic conditions as in Fig. 1.

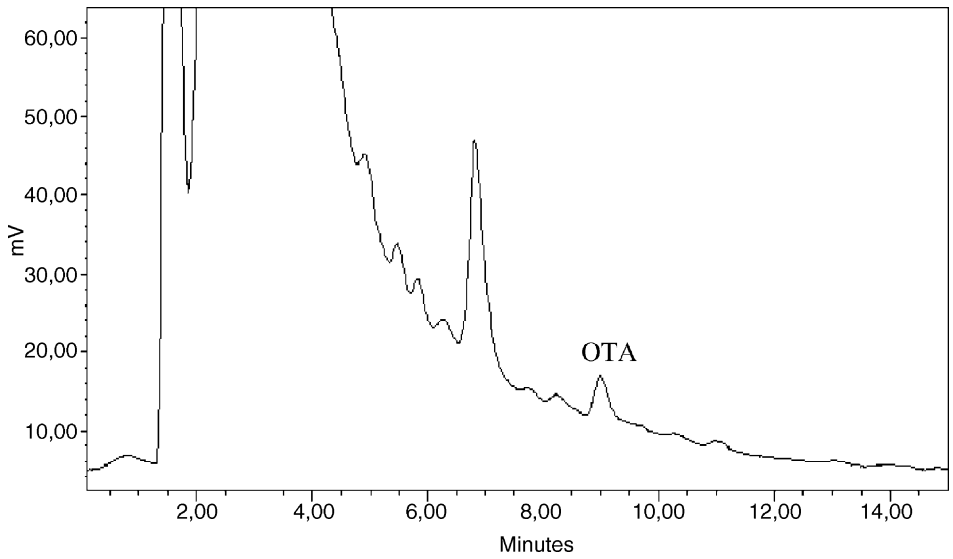

Fig. 4. LC–FLD chromatogram obtained by SPE on hydrophilic–lipophilic balanced Oasis HLB cartridge of red wine sample naturally contaminated with 0.16 ng OTA/ml and spiked with 1.0 ng OTA standard/ml. Chromatographic conditions as in Fig. 1.

ing laboratories obtained recoveries far from 85 to 90% [38].

The method (b) in Section 2.4 also provided unexpected lower recoveries (33.0–38.0%, mean 35.5%) in beer than in wine and emulsions always occurred while they did not appear in wine. More extraction steps seem to be necessary to extract most OTA from beer but the procedure would be very lengthy.

SPE on C_{18} (method (c) in Section 2.4) using methanol–acetic acid (99.5:0.5, v/v) as elution solvent provided the best recovery results in beer. The range was 92–102% (mean 96.3%) with acceptable precision (except at 0.1 ng/ml level). When acetonitrile was used as elution solvent the recoveries decreased significantly to 21 and 33% for 0.5 and 1 ng/ml levels, respectively, and the eluate was very dirty. Perhaps fatty material was co-eluted.

The recovery ranges, working with phenylsilane and Oasis HLB cartridges, were 20.6–37.0% (mean 28.8%) and 57.2–70.6% (mean 62.3%), respectively. The former was unable to quantify OTA at 0.1 ng/ml spiking level. Therefore, Oasis HLB can be applied to this kind of beverage and further optimisation of elution solvents can lead to higher recoveries. This optimisation can be also applied to wines. In any case, the chromatograms obtained by using C_{18}, phenylsilane and Oasis HLB cartridges (Figs. 2–4) show the OTA peak on a descending baseline and a great peak appears before the OTA peak (it does not interfere, however) in contrast with the chromatograms obtained using IAC clean-up (Fig. 1). This problem makes the integration of the OTA peak more difficult.

The must sample used for the study was naturally contaminated with OTA (0.19 ng/ml). Data concerning the recovery of OTA in spiked must sample appear in Table 1. The dilution with PEG–$NaHCO_3$ and IAC clean-up worked better than in beer because recoveries ranged from 80.1 to 89.5% (mean 83.9%) in the spiking interval 0.1–1 ng of OTA/ml. The liquid–liquid extraction/IAC method provided low recoveries (47.1–55.3%, mean 51.5%) but higher than those achieved in beer. Phase separation was not as difficult as in the case of beer but more extraction steps seem also to be necessary. Fifteen percent losses of OTA are probably due to water washing during IAC clean-up [14].

The use of SPE on C_{18} using acetonitrile as elution solvent did no provide good recoveries in must (11.4–53.0%, mean 27.3%), and the lowest value corresponded to the highest spiking level. On the basis of our experience, acetonitrile should not be used as elution solvent for OTA in must (and beer) working with C_{18} cartridges we used. Methanol slightly acidified with acetic acid is a good choice working with this matrix because recoveries were 81.1–89.2%.

SPE on phenylsilane provided relatively low recoveries (mean 56.1%) in the 0.1–1 ng/ml spiking range. Oasis HLB sorbent provided better recovery values (64.2–80.9%) than procedures (b) and (d) in Section 2.4. However, these results can be improved by further optimisation.

Concerning the test for possible binding of OTA to matrix proteins or other components by analysis of red wine and beer samples fortified at 0.1 and 1 ng/ml spiking levels carried out 20 days after the spiking day, the recoveries and their standard deviations are shown in Table 2. Although the recovery values were usually higher when acid treatment as described in 2.6 was achieved, no significant differences between mean recoveries were detected (*t*-test, $P = 0.95$). So, possible linkage to proteins or other components was not proved in this experience. Other acid treatments can be assessed to complete the assay but they must avoid hydrolysis of the OTA molecule.

Table 2
Effect of acidification with phosphoric acid (50 °C, 30 min) on the recovery of OTA in wine and beer spiked with OTA after 2 weeks

Matrix	Spiking level (ng/ml)	Acidic treatment	Mean recovery (%) ± S (%)
Red wine	0.1	Yes	102 ± 2.8
	0.1	No	97 ± 8.5
	1	Yes	88 ± 4.2
	1	No	80 ± 2.2
Beer	0.1	Yes	70 ± 1.5
	0.1	No	62 ± 3.7
	1	Yes	75.0 ± 0.5
	1	No	74.8 ± 0.5

Extraction/clean-up procedure was PEG–$NaHCO_3$/IAC.

4. Conclusions

The procedure that uses PEG–$NaHCO_3$ and IAC clean-up was rapid, straightforward and provided high OTA recoveries with acceptable precision in wine and must and something lower recoveries in beer. The IAC clean-up provides clean chromatograms and flat baselines thus allowing for good integration of the OTA peak. This procedure can be considered very satisfactory in terms of selectivity and general performance. The disadvantages are the high cost and lack of reusability of IAC columns (according to manufacturer instructions), which pose a real problem for control in developing countries.

Partition with chloroform followed by IAC clean-up provided very clean chromatograms and worked better with wine. More extraction steps are necessary to increase performance but time expenses will also increase. The main drawbacks are the following: recoveries were lower than those achieved with PEG–$NaHCO_3$/IAC or most SPE procedures, it was time-consuming, a thorough phase separation is difficult, especially in the case of beer because of the emulsions, and IAC columns are expensive. Chloroform should also be avoided due to its possible impact on human health and environmental pollution.

General advantages of SPE cartridges with respect to preceding IAC procedures are speed, relatively low-cost and common use in laboratories. General disadvantages are lack of selectivity and that chromatograms seem rather similar so that OTA peak appears in a descending baseline, which may suppose a problem for correct integration, especially at low toxin levels.

SPE on RP C_{18} cartridges using methanol–acetic acid (99.5:0.5, v/v) as elution solvent afforded for good recoveries, comparable or better than the former using PEG–$NaHCO_3$/IAC, especially for beer. However, when acetonitrile was used as elution solvent, low recoveries were obtained with this cartridge in beer and must. Great improvement in chromatogram appearance and recovery (very near 100%) for wine was noticed when dilution with PEG 8000–$NaHCO_3$ and filtration followed by acidification of the filtrate preceded SPE treatment. It is worth to improve recoveries in this way, which is a very interesting alternative to expensive IAC methods, and this treatment should be studied and optimised in the future for wine and other matrices.

SPE phenylsilane cartridges showed relatively high recoveries for wine (comparable to C_{18} or PEG–$NaHCO_3$/IAC) but not for beer or must. They worked worse than C_{18} cartridges.

Oasis HLB cartridges provided high recoveries working with must. A further optimization of the eluting solvent may increase recovery values, especially with other matrices.

Possible binding of OTA to matrix components of beer or wine was not evidenced.

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