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Rapid and automatable determination of ochratoxin A in wine based on microextraction by packed sorbent followed by HPLC-FLD

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ABSTRACT

The development of miniaturized and automatized analytical methods for OTA determination, requiring a reduced use of solvents and a limited involvement of expert operators, is highly desirable. Therefore, a rapid and automatable method for the determination of OTA in wine using a microextraction by packed C18 sorbent followed by high performance liquid chromatography with fluorescence detection was developed and validated for a successful application in the context of wine production. Important experimental parameters, such as sample and eluent volumes, extraction mode, draw and dispense speeds, number of eluent passes up and down through the stationary phase, were optimized. The validation included the comparison of the sensitivities related to solvent-matched, matrix-matched and standard addition calibrations and the participation to a proficiency test in a inter-laboratory circuit. Matrix effects were also investigated. Accuracies relevant to real samples were estimated to range between 76 and 100%, at 0.2 μg/L, and between 84 and 108%, at 1.0 μg/L, in compliance with the EU Regulation 401/2006; the limits of detection and quantification were of 0.08 and 0.24 μg/L, respectively, i.e. much lower than the maximum level currently permitted for OTA in the European Union (2.0 μg/kg, corresponding to ca 2.0 μg/L). 60 different wines produced in the Foggia (Italy) area were analyzed for their OTA content using the developed method and none of them was found to overcome the maximum permitted limit.

1. Introduction

The mycotoxin Ochratoxin A (OTA), chemically known as N-{[(3R)-5-chloro-8-hydroxy-3-methyl-1-oxo-7-isochromanyl]-carbonyl}-3-phenyl-L-alanine, was classified in 1993 as a possible human carcinogen, in the group 2B, by International Agency for Research on Cancer (IARC, 1993). Its immunosuppressive, teratogenic, carcinogenic and mutagenic properties were widely reported by the European Food Safety Authority (EFSA) in 2006; in particular, the EFSA Scientific Panel on Contaminants in the Food Chain established an OTA Tolerable Weekly Intake of 120 ng/kg body weight (EFSA, 2006).

OTA is present in several food products, such as cereals, beans, spices, groundnuts, milk, coffee, wine and beer (Duarte, Pena, & Lino, 2010; Bertuzzi, Rastelli, Mulazzi, & Donadini, 2011; Bellver Soto, Fernandez-Franzon, Ruiz, & Juan-García, 2014; Gil-Serna, Patiño, Cortes, Gonzalez-Jaen, & Vazquez, 2015); after cereals, wine represents the second source of OTA in the European diet (Miraglia & Brera, 2002). In particular, the highest OTA levels in wines are usually found in the Mediterranean area, frequently in Spain, southern France and Italy (Otteneder & Majerus, 2000; Battiliani, Magan, & Logrieco, 2006; Brera et al., 2008). The presence of OTA in wine grapes is generally attributed to ***Aspergillus carbonarius*** and ***Aspergillus niger*** (Bau, Bragulat, Abarca, Minguez, & Cabañes, 2005), although ***Penicillium verrucosum*** and ***Aspergillus ochraceus*** are recognized to be the main OTA producing species in food (Covarelli, Beccari, Marini, & Tosi, 2012). OTA occurrence in wines is due both to the fungal growth on grapes and to extraction during winemaking, therefore its concentration depends on various factors, such as climatic conditions, mycoflora composition, grape cultivation and winemaking techniques (Delage, d'Harlingue, Colonna Ceccaldi, & Bompeix, 2003). A maximum limit of 2.0 μg/kg in wine is recommended by the European Commission (EC) for a safe intake, according to the Regulation No 1881/2006 (EC, 2006a).

The main analytical methods for OTA determination in wine are

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based on reversed-phased high performance liquid chromatography (RP-HPLC) combined with fluorescence detection (FLD) (Battilani et al., 2004; Aresta, Vatinno, Palmisano, & Zambonin, 2006), often following a clean-up step, such as solid-phase extraction (SPE) or immunoaffinity clean-up (IAC) (Visconti, Pascale, & Centonze, 1999; Hernández, García-Moreni, Durán, Guillén, & Barroso, 2006). The latter method is recommended by Resolution OENO 16/2001 of the Official International Organization of Vine and Wine OIV, 2001). Due to the complexity of such procedures, usually time-consuming and requiring expert operators, especially for sample preparation, the development of miniaturized and automatized analytical methods, hopefully requiring a reduced use of solvents and a limited involvement of expert operators, would be highly desirable for a high-throughput analysis of wines by analytical laboratories, including those directly related to wineries.

Microextraction by packed sorbent (MEPS) can be defined as a miniaturization of the conventional solid phase extraction (SPE), using reduced sample and solvent volumes (μL volumes) and easily interfaced to LC and GC systems to provide a completely automated method (Altun, Abdel-Rehim, & Blomberg, 2004; Abdel-Rehim, 2010). MEPS combines sample preparation by SPE with syringe-based sample injection; indeed, the MEPS sorbent bed is integrated into a syringe needle, allowing manipulations of low void volumes either manually or automatically by means of laboratory robotics. The time to prepare and inject samples is reduced from hours to minutes; additionally, the cartridge can be reused about 100 times. MEPS applications have been initially developed for the analysis in biological matrices, such as in human plasma, urine and blood (Abdel-Rehim et al., 2005; Saracino et al., 2014). A few applications to food analysis have been reported so far, including the analysis of polycyclic aromatic hydrocarbons in water (El-Beqqali, Kussak, & Abdel-Rehim, 2006) and of phenolic compounds in wine (Gonçalves, Mendes, Silva, & Câmara, 2012). Although a method based on the extraction by a molecularly imprinted polymer packed into a syringe needle has been reported for the analysis of ochratoxin A in red wine (Wei, Longhui, Yu, & Lai, 2007), a MEPS approach based on commercially available products for the analysis of this mycotoxin in wine has been never explored so far.

Therefore, the aim of the present study was to develop and validate a new, simple, fast and accurate method for the determination of OTA in wine using a MEPS extraction combined with HPLC-FLD detection. Besides the parameters generally considered for method validation, such as linearity, LOD, LOQ, precision and accuracy, the method performance was evaluated also in terms of easiness and rapidity, i.e., highly desirable parameters for a successful application in the context of wine production.

2. Materials and methods

2.1. Materials

The OTA standard was purchased from Sigma (Sigma–Aldrich, St. Louis, MO, USA). A stock solution (5 g/L) was prepared in HPLC gradient grade methanol (Sigma–Aldrich); intermediate standard solutions (500 μg/L, 100 μg/L and 50 μg/L) were obtained by diluting the stock one in HPLC gradient grade methanol; all standards were stored at −20 °C in the dark. Seven working standard solutions (0.1–3.0 μg/L) were prepared daily, in duplicate, by dilution in 2% aqueous acetic acid/ethanol (88:12, v/v). Water used in this work was purified using a Milli-Q system (Millipore, Bedford, MA, USA). Acetonitrile and methanol (HPLC gradient grade), acetic acid (analytical quality), ethanol (99% purity), sodium chloride (NaCl), polyethylene glycol (PEG 8000) and sodium hydrogencarbonate ($NaHCO_3$) were obtained from Sigma–Aldrich.

2.2. Wine samples

Sixty different wines, with alcoholic grade ranging between 11% and 14%, elaborated from grapes Montepulciano, Merlot, Cabernet, Syrah, Nero di Troia, Chardonnay, Falanghina, Bombino, Fiano, cultivated in Foggia territory (Italy) and provided by Teanum (San Severo, Foggia, Italy) and La Marchesa (Lucera, Foggia, Italy), were analyzed during this study. Among them only a rosé wine was found to be virtually free of OTA (i.e., it contained OTA levels well below the limits of detections of the applied method) and was then used as a blank sample. The OTA reference material, having an assigned concentration value of 3.35 μg/kg and a $-2<z<2$ z score range corresponding to a 1.88–4.82 μg/kg interval (RM, T17128QC), and the proficiency test material, with an assigned concentration value of 2.34 μg/kg and a range for the $-2<z<2$ z score corresponding to a 1.31–3.37 μg/kg interval (PTM, 17143), were obtained from Fapas (Fera Science Ltd, York, UK). Both the RM and the PTM were white wines.

2.3. Optimization of the MEPS-based method on standard OTA solutions

During the present investigation MEPS was performed using the Barrel Insert and Needle Assembly (BIN) provided by SGE Analytical Science (Milton Keynes, UK), characterized by a 8 μL barrel volume, packed with 4 mg of C18 sorbent material (particle size 45 μm, pore size 60 Å). The BIN was always mounted on a 100 μL eVol® MEPS™ hand-held automated analytical syringe, also manufactured by SGE. Before each extraction the sorbent phase was conditioned using 50 μL of acetonitrile, 50 μL of methanol and 50 μL of a 2% aqueous acetic acid/ethanol mixture (88:12, v/v). The sample volume subjected to the loading procedure (V_s), the eluent volume (V_e) and the influence of OTA concentration were evaluated with the aim of maximizing the OTA recovery, changing one variable at a time. Multiple 50 μL aliquots were drawn through the BIN when sample volumes higher than 50 μL were loaded. Additionally, the two different loading approaches available with the described MEPS device were compared during this study, namely the “draw-eject” mode, consisting in a sequence of aspirations and injections cycles in the same sample vial, and the “extract-discard” mode, consisting in a similar cycle sequence, the only difference being that the drawn sample is discarded into the waste each time, in the second case. Besides the loading mode, the speed adopted during the extract/discard or draw/inject procedures, for which three values were available (level-1, 3.33 μL/s; level-2, 7.14 μL/s; level-3, 16.67 μL/s), was optimized preliminarily on a OTA standard solution (concentration 0.5 μg/L). Further details on the optimization procedure and on the application of the MEPS-based method to wine samples will be provided in the Results and Discussion section.

2.4. Comparative experiments on wine samples: sample preparation by solid-phase extraction (SPE), immunoaffinity cleanup (IAC) and MEPS

For the sake of comparison the OTA concentration was determined in a naturally OTA-containing wine sample using a SPE, a IAC or a MEPS procedure for the extraction, all followed by HPLC-FLD analysis under the same conditions. A standard addition approach was adopted for calibration purposes in all cases; in particular, wine aliquots (50 mL) were spiked with OTA at different concentration levels, ranging from 0 to 3.0 μg/L, with two replicates for each level. Standard addition volumes were such to leave the wine sample volume virtually unchanged.

2.4.1. SPE purification

OTA extraction was performed using Bond Elut C18 (500 mg) cartridges (Varian, Harbor City, USA) and a vacuum manifold (Varian), as reported and validated by Hernàndez et al. (2006), with some modifications. The cartridge was first conditioned with 4 mL of acetonitrile and 4 mL of methanol, then it was equilibrated with 4 mL of 2% aqueous acetic acid/ethanol (88:12, v/v). 10 mL of spiked wine, diluted with 10 mL of 2% aqueous acetic acid, were passed through the C18 cartridge. The cartridge was then washed with 2 mL of 2% aqueous acetic acid and 2 mL of methanol/2% aqueous acetic acid (40/60, v/v), before being air-dried. Finally, OTA elution was carried out with 2 mL of acetonitrile. The eluted extract was injected into the HPLC system.

2.4.2. IAC purification

OTA was extracted according to the method reported by Visconti et al. (1999), which has become the official method adopted by OIV, as well as by the Association of Official Analytical Chemists (AOAC International). In particular, a 10 mL volume of spiked wine was diluted with 10 mL of a water solution containing PEG (1%) and $NaHCO_3$ (5%), mixed and filtered through a cellulose filter Whatman grade-1 (Maidstone, England). A 10 mL volume of diluted and filtered wine (equivalent to 5 mL of the original wine) was cleaned up through an OTA CLEAN™ (LCtech GmbH, Dorfen, Germany) immunoaffinity column (3 mL volume, wide bore). The column was washed with 5 mL of a solution containing NaCl (2.5%) and $NaHCO_3$ (0.5%), followed by 5 mL milliQ water. OTA was eluted with 2 mL methanol and collected in a clean glass vial.

2.4.3. MEPS purification

Each sample of spiked wine was divided into two sample subsets: diluted 1:4 and 1:2 (v/v) with 2% aqueous acetic acid; they were then subjected to the optimized MEPS procedure, as described in the Results and discussion section.

All the extracts were analyzed by the HPLC-FLD method described in the following section.

2.5. HPLC-FLD analysis

Chromatographic analysis was performed by an Agilent (Palo Alto, USA) chromatographic system, including a model G1311A pump, a model G1329B autosampler, a Zorbax SB-C18 column (100 mm × 4.6 mm i.d., 1.8 μm packing) and a model G1321A fluorescence detector. The excitation and emission wavelengths adopted for fluorescence detection were 333 and 460 nm, respectively. The elution was carried out at a flow rate of 0.6 mL/min using a binary gradient based on water containing 2% acetic acid (solvent A) and acetonitrile (solvent B). The gradient was run at ambient temperature as follows: (1) from 50% to 75% B in 7 min, followed by washing and re-equilibrating the column. The injection volume was 20 μL. Under these conditions OTA was eluted after 5.3–5.5 min.

2.6. Method validation

Method validation for OTA quantification in wines implied the assessment of selectivity and linearity and the determination of LOD and LOQ, precision (expressed as relative standard deviation - RSD), accuracy, matrix effect (expressed as signal suppression/enhancement - SSE%). The performance characteristics on wines were established using a blank wine spiked with OTA, the RM and the PTM.

Selectivity was assessed by the analysis of several fortified wines, to ensure the absence of chromatographic interferences. Linearity and linear range were first evaluated in standard solutions, through a calibration curve constructed by plotting OTA peak area vs OTA concentrations, ranging from 0.02 to 3.0 μg/L. The analysis at each concentration was performed in triplicate. Detection and quantification limits (LOD and LOQ respectively) in standard solutions were calculated using the regression line parameters, as follows: LOD = 3.3 σ/b and LOQ = 10 σ/b, where σ is the intercept standard deviation and b the slope.

In order to evaluate matrix effects, a matrix-matched calibration was performed using aliquots of the already cited OTA-free rosé wine purposely spiked with different OTA concentrations. As a result, linearity was found to occur between 0.02 and 3.0 μg/L (correlation coefficient 0.9988). Once the slopes relevant to standard and matrix-matched calibration lines were known, the signal suppression/enhancement (SSE%) was calculated as $SSE\% = (slope_{spiked\ wine}/slope_{standard\ solution}) \times 100$. The precision of the whole method was evaluated in terms of repeatability (intra-day precision) and reproducibility (inter-day precision), expressed as percent relative standard deviation (% RSD), both for standard solutions and for spiked wine samples. Repeatability was assessed by the application of the whole procedure to the same sample, on the same day and by the same analyst (eight experimental replicates performed on a 0.5 μg/L standard solution or on the OTA-free rosé wine spiked at 0.5 μg/L, adopted as representative of a real sample). Inter-day precision was evaluated with a similar procedure, by analyzing the same wine sample on different days (eight experimental replicates in eight days).

3. Results and discussion

3.1. Optimization of the MEPS procedure on OTA standard solutions

In the first stage of MEPS method development some parameters were evaluated with the aim of maximizing the recovery. The recovery (R) was calculated using the following formula: $Area_{MEPS}/(F_{conc} \times Area_{HPLC\text{-}FLD})$, where $Area_{MEPS}$ represents the peak area for OTA as obtained by HPLC-FLD analysis after the MEPS procedure, $Area_{HPLC\text{-}FLD}$ is the peak area obtained using HPLC-FLD directly on the OTA standard solution and F_{conc} is the concentration factor, expressed as the V_s to V_e ratio. The influence of three key factors, namely, the sample (V_s) and eluent (V_e) volumes and the OTA concentration (C_{OTA}) was evaluated changing one variable at a time and the main results are shown in Fig. 1. At this stage, the "extract-discard" mode, operated at 3.33 μL/min, was used, since a previous investigation had suggested this to be the most efficient approach (Quinto et al., 2014).

As MEPS is the miniaturization of SPE, we started from typical SPE conditions as the initial parameters to be scaled down. Thus, a V_s of 100 μL and a V_e of 20 μL (concentration factor as for SPE) were first adopted for a 1.0 μg/L OTA solution and a 75% recovery was obtained (see Fig. 1a), likely because the elution volume was a limiting factor. Indeed, the recovery was increased to 92% upon increasing V_e to 50 μL, whereas no significant variation was observed after a further increase of V_e to 80 μL (see Fig. 1a). Since the best concentration factor obtained with the described V_s and V_e values (F_{conc} 2) could be not suitable for wines containing very low OTA concentrations, an increase of V_s was attempted, keeping V_e at 50 μL, to reach good recoveries for higher F_{conc} values. As shown in Fig. 1b, a recovery higher than 90% was obtained also for V_s = 350 μL and Ve = 50 μL, thus for $F_{conc} = 7$; on the other hand, a further increase of the sample volume, up to 600 μL, corresponding to $F_{conc} = 12$, led to a significant recovery decrease. This result can be explained with the combination of two phenomena: the saturation of the extraction phase in the BIN and a partial elution of OTA extracted in the first stage of sample loading, due to the prolonged withdrawal of sample.

After fixing V_s as 350 μL, the influence of the elution volume was

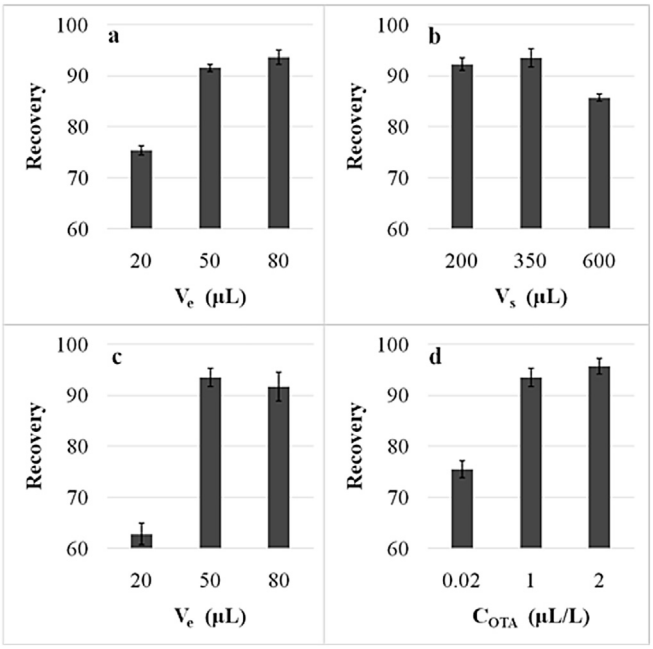

Fig. 1. Effect of elution volume (V_e), sample volume (V_s) and OTA concentration (C_{OTA}) on the OTA recovery provided by the MEPS procedure. a)-c) V_e at constant V_s (a, $V_s = 100$ µL; c, $V_s = 350$ µL) and at $C_{OTA} = 1$ µg/L; b) V_s, at $V_e = 50$ µL and $C_{OTA} = 1$ µg/L; d) C_{OTA} at $V_s = 350$ µL and $V_e = 50$ µL.

checked again, using two further values for V_e, namely 20 and 80 µL (Fig. 1c). A $V_e = 50$ µL was found to be already able to provide a good recovery. Finally, after choosing 350 and 50 µL, respectively, as the best values for V_s and V_e, the evolution of the recovery with OTA concentration was investigated by considering two further values, namely 0.02 and 2.0 µg/L; although the recovery was significantly lower for the lowest concentration, as shown in Fig. 1d, the values retrieved for the recovery were generally satisfactory over the investigated concentration range, as required by the European Commission (EC) Regulation No 401/2006 (EC, 2006b).

Among further experimental factors related to the MEPS procedure, those defined as "draw speed" and "dispense speed" were evaluated on the 1.0 µg/L OTA standard solution and the best recovery was achieved by keeping both speeds at their lowest value (3.33 µL/s). This result is likely related to the longer time available for the interaction between OTA and the sorbent phase when lower drawing and dispense speeds are adopted. The "extract-discard" mode was also compared to the "draw-eject" during a specific test and was found to provide a better recovery (88 vs 64%, expressed as mean values obtained from three replicates), in accordance with Quinto et al. (2014), thus it was adopted during the subsequent steps of method optimization.

Finally, a slight improvement (5%) was observed by increasing the number of eluent passes up and down through the BIN from 1 to 2, thus two elution cycles were adopted when the method was applied.

3.2. Application of the MEPS-based method to wine samples: evaluation of matrix interference

Starting from the parameter values optimized on OTA standard solutions the MEPS-based method was applied to OTA-containing wine samples. In this case, after preliminary experiments based on the cited C18 BIN mounted on an eVol® autosampler (SGE), the method was transferred to the MEPS sample preparative workstation HT4000A (HTA Scientific Instruments, Brescia, Italy), in order to achieve automation of the analysis.

As described in Fig. 2, after washing and conditioning the BIN, wine analysis was started by loading 350 µL (7 × 50 µL) of each sample through the syringe and the C18 sorbent phase at a speed of 3.33 µL/s (level-1 speed). The sorbent bed was then washed first with 20 µL of 2% aqueous acetic acid and then with the same volume of a 2% aqueous acetic acid/methanol mixture (60/40 v/v), to remove eventual interferences, and dried. The adsorbed analyte was subsequently eluted with 50 µL (2 × 25 µL) of acetonitrile/2% aqueous acetic acid (90/10, v/v), which was pulled/pushed through the syringe twice, at the speed of 3.33 µL/s. In view of subsequent analyses, the BIN was washed with 50 µL-acetonitrile/2% aqueous acetic acid (90/10, v/v) for three times after each extraction. To control memory effects blank samples were also randomly extracted on a previously washed BIN and the eluent was analyzed by HPLC-FLD, under the same conditions adopted for real samples. As a result, no significant memory effect was observed. Indeed, the same sorbent could be used reliably for more than 100 subsequent wine extractions during the present work.

Before undertaking the systematic application of the MEPS-based method to wine samples an evaluation of eventual interference effects due to the wine matrix was performed. At this aim the only wine found to be virtually free of OTA (a rosé wine, see the Experimental section) was used as a blank matrix and was spiked with 0.5 µg/L OTA, thus obtaining a matrix-matched standard solution of the micotoxyn. An aliquot of the spiked wine was first injected directly, without any dilution, into the HPLC-FLD system. The resulting OTA peak, shown in Fig. 3 (trace a), was found to be

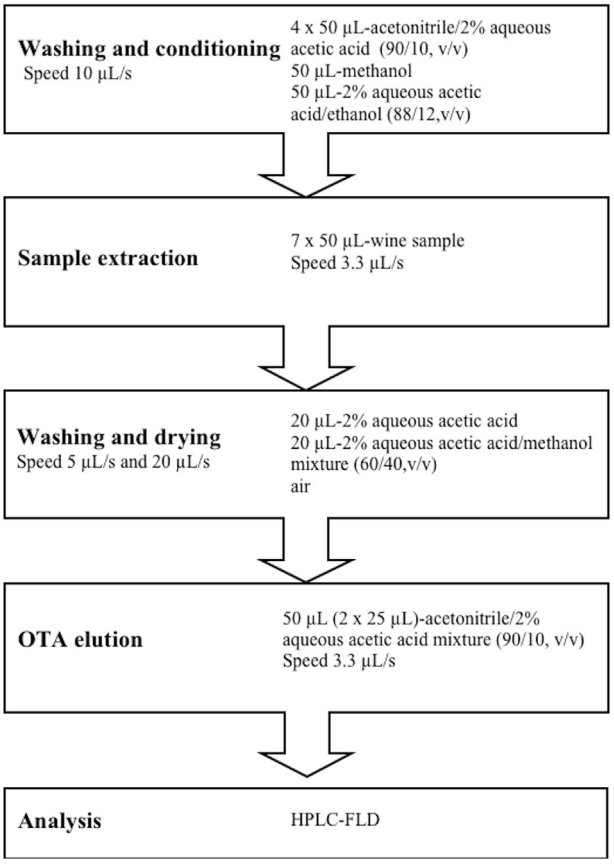

Fig. 2. Schematic representation of the MEPS-based method developed for OTA determination in wine.

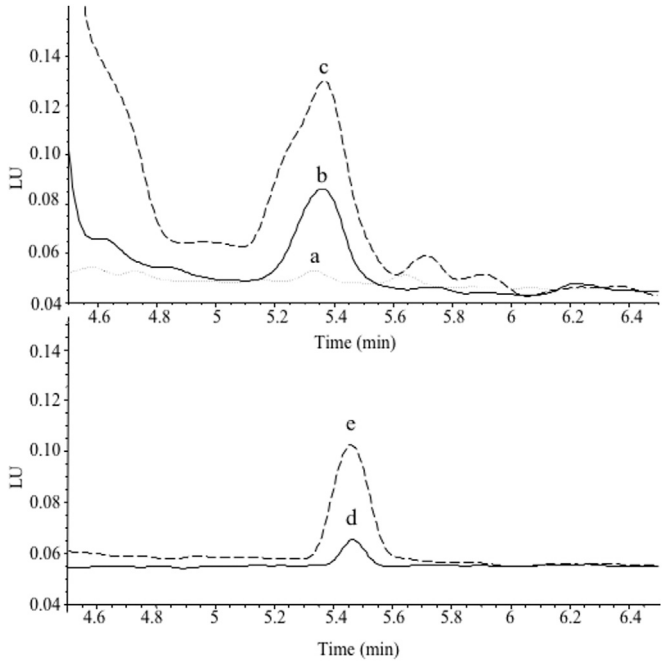

Fig. 3. Effects of wine matrix and of the MEPS procedure on the characteristics of the OTA chromatographic peak. a) Undiluted 0.5 μg L^{-1} spiked wine without previous MEPS extraction; b) MEPS extract on the same wine after 1:2 dilution or c) undiluted; a 0.2 μg L^{-1} standard solution d) without and e) after MEPS extraction.

almost symmetric (symmetry, S, 0.88), with a full width at half height peak (FWHH) equal to 0.094 min. On the other hand, the low peak height (H, 4.6×10^{-3}) suggested the presence of suppression effects due to interfering compounds, although it is not possible to establish if such effects arose from a fluorescence quenching, a chemical interference or both. Another aliquot of the same OTA-spiked blank wine was subjected, undiluted, to MEPS extraction followed by HPLC-FLD analysis, as described before. The resulting OTA peak (see trace c in Fig. 3), although significantly higher, as expected, due to the preconcentration associated to the MEPS procedure, was found to be asymmetrical and wide (S 1.43, FWHH 0.23 min, H 7.4×10^{-2}). When the extract obtained from the MEPS procedure performed on the same wine previously diluted 1:2 with 2% aqueous acetic acid/ethanol (88/12, v/v) was analyzed by HPLC-FLD the OTA peak (see trace b in Fig. 3) appeared symmetrical but still significantly larger than the peak obtained after wine direct analysis (S 1.09, FWHH 0.18 min). It is worth noting that the OTA peak enlargement seems to be related to the MEPS procedure itself, rather than to an effect of wine matrix; indeed, the enlargement occurred also when OTA standard solutions were involved, as clearly inferred by the comparison of traces d and e in Fig. 3. The phenomenon could then be due to the higher amount of OTA injected into the HPLC column when the MEPS procedure is performed.

As far as peak height is concerned, a value higher by almost an order of magnitude, compared to that retrieved for OTA after direct HPLC-FLD analysis of the wine sample, was observed in trace b (H 3.9×10^{-2}). Since the final preconcentration factor inherent to the optimized MEPS procedure on a 1:2 diluted wine is actually equal to 3.5 (i.e., the ratio between the MEPS preconcentration factor and the wine dilution factor), the almost ten-fold improvement observed in peak height, with respect to direct injection of OTA, might be related to an enhancement in OTA fluorescence, achieved by reducing the incidence of matrix interferences. Consequently, the drawback of peak enlargement is clearly overcome by the advantage in terms of sensitivity provided by the MEPS procedure. A final feature observed in Fig. 3 deserves a comment. Indeed, the retention time observed for OTA when a wine sample was involved was systematically, although only slightly, lower than that observed on standard solutions of the mycotoxin. This peculiar effect could be due to interactions of the OTA molecule with one, or more, wine matrix components, a process that does not seem to impair the fluorescence yield but is able to influence the interaction of OTA with the C18 stationary phase.

As a result of the experiments now described, a 1:2 (v/v) dilution of the wine samples seemed to provide the best compromise between fluorescence signal intensity and peak width. Actually, the peak enlargement due to the MEPS procedure did not represent a relevant problem during the analysis of wine samples; indeed, a comparison of the chromatograms obtained for unspiked and OTA-spiked wines, carried out for ten different wine samples, showed no interfering peaks apparently overlapping with the OTA one.

3.3. Study of method reliability. Comparison of the results obtained using SPE, IAC and MEPS for the OTA extraction from a red wine sample

The reliability of MEPS extraction was evaluated by comparison with the well-established SPE (Hernàndez et al., 2006) and IAC techniques (Visconti et al., 1999), the latter being also recommended by the International Organization of Vine and Wine (OIV). In particular, OTA concentration was determined in a naturally OTA-containing red wine sample by SPE-HPLC/FLD, IAC-HPLC/FLD and MEPS-HPLC/FLD, using a standard addition method, in order to account for matrix effects. It is worth noting that two dilution factors (1:2, 1:4) were adopted in the case of the MEPS-HPLC/FLD method, for the sake of performance comparison. Indeed, as the positive effect of wine dilution was assessed during the experiments described in the 3.2 section, a 1:4 dilution was also considered to evaluate the occurrence of eventual signal improvements (in spite of the higher dilution of the matrix). The extrapolated OTA concentrations, along with standard deviations and 95% confidence interval widths, are reported in Table 1. According to *t*-test results (95% confidence level), the OTA concentration values obtained by the MEPS procedure on the differently diluted wines were not statistically different and were comparable with those resulting from the SPE and IAC procedures. As far as precision is concerned, the MEPS procedure appeared similar to the IAC one, especially when the 1:4 diluted wine was considered, whereas SPE was clearly characterized by a worse reproducibility. The 1:4 dilution of wine before MEPS extraction might then be useful to guarantee a good precision also in the case of wines whose OTA content is relatively high (thus enabling the use of a higher dilution factor), yet the preliminary 1:2 dilution of wine was considered as the usual approach during the present study, thus it was introduced in the automatized MEPS procedure in all cases.

It is worth noting that the comparison with the well-established

Table 1
Comparison between the results obtained during a standard addition-based determination of OTA in a test wine sample using different clean-up methods. x_E is the OTA concentration, retrieved as intercept of the standard addition line on the axis reporting added concentrations; s_{xE} and $s_{xE} \times t_{(0.975)}$ represent its standard deviation and the width of its 95% confidence interval, respectively.

	x_E (μg/L)	s_{xE} (μg/L)	$s_{xE} \times t_{(0.975)}$ (μg/L)
SPE-HPLC/FLD	0.64	0.11	0.31
IAC-HPLC/FLD	0.66	0.03	0.09
MEPS (1:4)-HPLC/FLD	0.64	0.05	0.14
MEPS (1:2)-HPLC/FLD	0.63	0.08	0.21

SPE and IAC procedures was done using a red wine sample to understand if the MEPS procedure could be applied also to wine matrices much more complex than those represented by white wines, especially due to the presence of pigments. Moreover, the choice of a naturally OTA-contaminated red wine for the test was due to the fact that neither a red wine-based reference material nor a OTA-free red wine (that could be subsequently spiked to generate a real sample with a known OTA concentration) were available. Nonetheless, the successful comparison obtained with respect to SPE and IAC approaches, whose accuracy is well established, suggested that the MEPS-based one has a good accuracy even when red wine matrices are concerned. The accuracy of the MEPS-based standard addition approach, following a 1:2 dilution of the original wine sample, could be directly assessed on a white wine using the reference material (RM) cited in the experimental section. Indeed, the OTA concentration in the RM was found to be 3.22 ± 0.12 μg/L (95% confidence interval), a value in accordance with the certified one (3.35 μg/kg, corresponding to 3.33 μg/L considering a wine density of 0.9946 g/mL).

3.4. 4Validation of MEPS-HPLC/FLD method for OTA determination: comparison of the use of different calibration curves

Quantitative data obtained for OTA-spiked wine samples during the comparison test described in Section 3.3 were very promising in terms of linearity of the developed MEPS-based method, yet they were obtained using a standard addition approach, that it is certainly complex and time-consuming, thus it is not the most practical one, especially if several real samples have to be analyzed at a time. Further tests were then made to verify whether an external calibration could be used reliably for quantitation purposes.

In particular, the MEPS-HPLC/FLD method was applied, under identical conditions, to eight OTA standard solutions in 2% aqueous acetic acid/ethanol (88:12, v/v), with concentrations ranging between 0.02 and 3.0 μg/L, and to as many samples obtained from the already cited OTA-free rosé wine spiked with OTA at the same concentrations. The solutions were analyzed in triplicate and the corresponding average responses were plotted against OTA concentrations, thus enabling a direct comparison between a solvent-matched and a matrix-matched calibration. The comparison provided excellent results, as emphasized in Table 2, where the main calibration parameters, namely, linear range, linearity (R), regression equation, LOD and LOQ were reported. In particular, the 95% confidence intervals of the respective slopes: 0.81 ± 0.03 and 0.78 ± 0.04 LU min L/μg (where LU represents the luminescence units) were clearly overlapped, indicating no significant signal suppression or enhancement, i.e., a SSE% close to 100%. Moreover, the intercepts of the regression lines were not statistically different from zero (at a 95% confidence level) in both cases, thus indicating the absence of a response due to an interferent eventually present either in the solvent or in the wine matrix. The method showed also promising quantitative performances, as both LOQs were remarkably lower than the maximum level permitted in the European Union (2.0 μg/kg, which corresponds to as many μg/L, if a wine density closed to unity is assumed) for the OTA concentration in wines.

Interestingly, the SSE% was evaluated also after comparing the calibrations lines obtained for the same set of solvent- and matrix-matched standards but without applying the MEPS procedure as a preliminary step. The resulting value, 20%, was dramatically low, thus confirming the precious role of MEPS in removing wine matrix interferents that can lead to a significant suppression of the OTA response.

Turning back to the calibrations involving the MEPS step, one could argue that a single successful comparison between solvent- and matrix-matched calibrations does not guarantee that the solvent-matched calibration can be used as a general approach to the quantification of OTA in every possible wine, since wines could be potentially very different in terms of matrix interference. Since further wines virtually free from OTA were difficult to find, the evaluation of matrix effects could be extended only by using standard addition calibrations, which were applied to ten wines (two for each of the following varieties: Nero di Troia, Cabernet, Merlot, Syrah and Montepulciano) naturally containing OTA levels detectable by the MEPS-based method. As a result, a good method linearity was always found over the explored concentration range, i.e. up to 1.2 μg/L (correlation coefficients of linear regressions ranging in the interval 0.985–0.999). Moreover, ***t***-tests showed nine and seven slopes to be not significantly different from that related to matrix-matched and solvent-matched calibration, respectively, at 95% confidence. Accordingly, SSE% values ranging between 80 and 105% were obtained.

The results now described confirmed that the external calibration method could provide reliable results in a good percentage of cases, in spite of the matrix variability existing between different wines. Further checks of the good accuracy achievable with the external calibration were also made. The first check was based on the Reference Material sample, previously adopted for a standard addition-based determination. Even if using the external calibration an accuracy of 97 ± 2% (n = 3), expressed as the ratio between the experimentally determined concentration and the true (assigned) one, was obtained. Finally, the 10 wines already contaminated by OTA were adopted to evaluate the accuracy at those levels. In this case, the increase in OTA response observed when passing from the as such sample to samples resulting from additions of 0.2 and 1.0 μg/L was used to extrapolate the added concentration using the external calibration line; accuracies ranging between 76 and 100%, at 0.2 μg/L, and between 84 and 108%, at 1.0 μg/L, were obtained, resulting

Table 2
Values obtained for the main calibration and performance parameters of the proposed MEPS-HPLC/FLD method when applied to OTA solvent-matched and matrix-matched standard solutions. Note that the matrix-matched calibration was achieved using as matrix a rosé wine virtually free from OTA. Precision values were estimated from replicated analyses at a 0.5 μg/L OTA concentration.

Parameter	Solvent matched calibration	Matrix matched calibration
Linear range	0.02–3.0 μg/L	0.02–3.0 μg/L
Linearity (*R*)	0.9991	0.9988
Regression equation	y = 0.812 x + 0.019	y = 0.784 x − 0.010
slope standard error	0.014	0.015
intercept standard error	0.020	0.022
Limit of detection (*LOD*)	0.08 μg/L	0.09 μg/L
Limit of quantification (*LOQ*)	0.24 μg/L	0.28 μg/L
Precision − $RSD_{intra\text{-}day}$ (%, $n = 8$)	3.8	4.5
Precision − $RSD_{inter\text{-}day}$ (%, $n = 8$)	7.6	8.2

Table 3
OTA concentration levels found in white, rosè and red wines.

Wine sample	OTA concentration (μg/L)	Wine sample	OTA concentration (μg/L)
#1	<LOD	#31	<LOD
#2	0.110 ± 0.008	#32	<LOD
#3	<LOD	#33	<LOD
#4	0.220 ± 0.021	#34	0.110 ± 0.012
#5	0.89 ± 0.05	#35	0.270 ± 0.024
#6	0.120 ± 0.008	#36	0.080 ± 0.006
#7	0.41 ± 0.04	#37	<LOD
#8	0.090 ± 0.007	#38	<LOD
#9	0.160 ± 0.009	#39	0.080 ± 0.005
#10	0.34 ± 0.03	#40	0.62 ± 0.04
#11	0.090 ± 0.006	#41	1.24 ± 0.08
#12	1.07 ± 0.06	#42	<LOD
#13	<LOD	#43	0.090 ± 0.006
#14	<LOD	#44	<LOD
#15	<LOD	#45	0.140 ± 0.010
#16	0.190 ± 0.016	#46	<LOD
#17	0.130 ± 0.009	#47	0.210 ± 0.013
#18	<LOD	#48	<LOD
#19	<LOD	#49	0.110 ± 0.008
#20	<LOD	#50	<LOD
#21	0.210 ± 0.020	#51	<LOD
#22	<LOD	#52	<LOD
#23	0.230 ± 0.022	#53	0.140 ± 0.011
#24	<LOD	#54	0.080 ± 0.006
#25	<LOD	#55	<LOD
#26	0.37 ± 0.03	#56	<LOD
#27	<LOD	#57	<LOD
#28	<LOD	#58	<LOD
#29	<LOD	#59	<LOD
#30	<LOD	#60	<LOD

compliant with the EC Regulation No 401/2006 (EC, 2006b). A final verification of the method accuracy was obtained through participation to a proficiency test (PT) in a inter-laboratory circuit, during which the sample cited as 17143 in the Experimental section, having an assigned OTA concentration of 2.34 μg/kg, was analyzed by the developed MEPS-HPLC/FLD method. As a result, a z-score of −0.8 was obtained by the MEPS-HPLC/FLD method (FAPAS report N. 17143); it is worth noting that a PT can be considered fit-for-purpose if the corresponding z-score lies within the range ±2.

The method repeatability and reproducibility were finally assessed, according to the procedures described in Section 2.6, also on the OTA-free rosé wine spiked with 0.5 μg/L of mycotoxin, chosen as a representative sample for a OTA-contaminated wine. As reported in Table 2, values of 4.5% and 8.2% were found for the two parameters, thus being comparable to those obtained for a 0.5 μg/L OTA solution in solvent (3.8 and 7.6%, respectively). Finally, the solvent-matched calibration, adopted for the determination of OTA concentrations in wines, was replicated four times at time intervals of seven days and the resulting slopes were not statistically different, as assessed through a ***t***-test at 95% confidence level. This result showed the good robustness of the proposed method.

3.5. Evaluation of OTA concentration in several wines

In the last stage of the work sixty different wines were selected for OTA determination, in order to show the method applicability. This sample number could be easily managed using the configured tray of the automatic preparative station described in Section 3.2, since it allowed the preparation of up to 88 samples in one batch. 15 min were required for each preparation; the subsequent chromatographic run had the same duration. The whole procedure could be further automatized by directly connecting the preparative station to the chromatographic system, allowing the use overnight, without the presence of any operator. The values obtained for OTA concentrations in the analyzed wines, each extrapolated using the solvent-matched calibration, are reported in Table 3. As apparent, all concentration values were found to be under the legal limit of 2.0 μg/kg (i.e. ca. 2.0 μg/L) and 55% of them were even below the limit of detection obtained for the solvent-matched calibration (0.08 μg/L).

4. Conclusions

After an appropriate optimization of the operative parameters, MicroExtraction by Packed Sorbent (MEPS) based on a C18 phase proved to be a successful approach to the extraction of Ochratoxin A from wine matrices, preliminary to its determination based on HPLC separation with fluorescence detection. In particular, the remarkable removal of wine interferents achievable using MEPS enabled an accurate determination of the analyte in real samples even using a solvent-matched calibration. This feature, along with the easiness, rapidity and possibility of automation make the proposed MEPS procedure a very promising, reliable alternative to consolidated analytical approaches like SPE or IAC, especially when a significant number of samples has to be analyzed in a relatively short time. The proposed method could then be successfully used for OTA monitoring and for risk-assessment purposes in the context of wine production.

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