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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.

Keywords: Ochratoxin A; Microextraction by packed sorbent; wine; high performance liquid chromatography; fluorescence detection; validation

Chemical compound studied in this article: Ochratoxin A (PubChem CID: 442530)

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 2 B, 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, Fernández-Franzón, Ruiz & Juan-García, 2014; Gil-Serna et al., 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 & Toset, 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 Union for a safe intake according to the Regulation (EC) No 1881/2006 (2006).

The main analytical methods for OTA determination in wine are 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 et al., 2006). The latter method is recommended by the official International Organization of Vine and Wine (OIV) (Resolucìon OENO 16/2001, 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-Beqquali, 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.

90 Therefore, the aim of the present study was to develop and validate a new, simple, fast and
91 accurate method for the determination of OTA in wine using a MEPS extraction combined with
92 HPLC-FLD detection. Besides the parameters generally considered for method validation, such as
93 linearity, LOD, LOQ, precision and accuracy, the method performance was evaluated also in terms
94 of easiness and rapidity, i.e., highly desirable parameters for a successful application in the context
95 of wine production.
96

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.

123 *2.3. Optimization of the MEPS-based method on standard OTA solutions*

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

143

144 *2.4. Comparative experiments on wine samples: sample preparation by Solid-Phase Extraction*
145 *(SPE), Immunoaffinity cleanup (IAC) and MEPS*

146 For the sake of comparison the OTA concentration was determined in a naturally OTA-
147 containing wine sample using a SPE, a IAC or a MEPS procedure for the extraction, all followed by
148 HPLC-FLD analysis under the same conditions. A standard addition approach was adopted for

149 calibration purposes in all cases; in particular, wine aliquots (50 mL) were spiked with OTA at
150 different concentration levels, ranging from 0 to 3.0 μg/L, with two replicates for each level.
151 Standard addition volumes were such to leave the wine sample volume virtually unchanged.
152 *SPE purification.* OTA extraction was performed using Bond Elut C18 (500 mg) cartridges (Varian,
153 Harbor City, USA) and a vacuum manifold (Varian), as reported and validated by Hernàndez et al.
154 (2006), with some modifications. The cartridge was first conditioned with 4 mL of acetonitrile and
155 4 mL of methanol, then it was equilibrated with 4 mL of 2% aqueous acetic acid/ethanol (88:12,
156 v/v). 10 mL of spiked wine, diluted with 10 mL of 2% aqueous acetic acid, were passed through the
157 C18 cartridge. The cartridge was then washed with 2 mL of 2% aqueous acetic acid and 2 mL of
158 methanol/2% aqueous acetic acid (40/60, v/v), before being air-dried. Finally, OTA elution was
159 carried out with 2 mL of acetonitrile. The eluted extract was injected into the HPLC system.
160 *IAC purification.* OTA was extracted according to the method reported by Visconti, Pascale and
161 Centonze (1999), which has become the official method adopted by OIV, as well as by the
162 Association of Official Analytical Chemists (AOAC International). In particular, a 10 mL volume
163 of spiked wine was diluted with 10 mL of a water solution containing PEG (1%) and $NaHCO_3$
164 (5%), mixed and filtered through a cellulose filter Whatman grade-1 (Maidstone, England). A 10
165 mL volume of diluted and filtered wine (equivalent to 5 mL of the original wine) was cleaned up
166 through an OTA CLEAN™ (LCtech GmbH, Dorfen-Germany) immunoaffinity column (3 mL
167 volume, wide bore). The column was washed with 5 mL of a solution containing NaCl (2.5%) and
168 $NaHCO_3$ (0.5%), followed by 5 mL milliQ water. OTA was eluted with 2 mL methanol and
169 collected in a clean glass vial.
170 *MEPS purification*. Each sample of spiked wine was divided into two sample subsets: diluted 1:4
171 and 1:2 (v/v) with 2% aqueous acetic acid; they were then subjected to the optimized MEPS
172 procedure, as described in the Results and discussion section.
173 All the extracts were analyzed by the HPLC-FLD method described in the following section.
174

175

176 *2.5 HPLC-FLD analysis*

177 Chromatographic analysis was performed by an Agilent (Palo Alto, USA) chromatographic
178 system, including a model G1311A pump, a model G1329B autosampler, a Zorbax SB-C18 column
179 (100 mm × 4.6 mm i.d., 1.8 µm packing) and a model G1321A fluorescence detector. The
180 excitation and emission wavelengths adopted for fluorescence detection were 333 and 460 nm,
181 respectively. The elution was carried out at a flow rate of 0.6 mL/min using a binary gradient based
182 on water containing 2% acetic acid (solvent A) and acetonitrile (solvent B). The gradient was run at
183 ambient temperature as follows: (1) from 50% to 75% B in 7 min, followed by washing and re-
184 equilibrating the column. The injection volume was 20 µL. Under these conditions OTA was eluted
185 after 5.3-5.5 min.

186

187 *2.6 Method validation*

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

193 Selectivity was assessed by the analysis of several fortified wines, to ensure the absence of
194 chromatographic interferences. Linearity and linear range were first evaluated in standard solutions,
195 through a calibration curve constructed by plotting OTA peak area vs OTA concentrations, ranging
196 from 0.02 to 3.0 µg/L. The analysis at each concentration was performed in triplicate. Detection and
197 quantification limits (LOD and LOQ respectively) in standard solutions were calculated using the
198 regression line parameters, as follows: LOD = 3.3 σ/b and LOQ = 10 σ/b, where σ is the intercept
199 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 Figure 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 Figure 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 Figure 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 Figure 1b, a recovery higher than 90% was obtained also for V_s = 350 µL and V_e = 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

238 phase in the BIN and a partial elution of OTA extracted in the first stage of sample loading, due to
239 the prolonged withdrawal of sample.

240 After fixing V_s as 350 µL, the influence of the elution volume was checked again, using two
241 further values for V_e, namely 20 and 80 µL (Figure 1c). A V_e = 50 µL was found to be already able
242 to provide a good recovery. Finally, after choosing 350 and 50 µL, respectively, as the best values
243 for V_s and V_e, the evolution of the recovery with OTA concentration was investigated by
244 considering two further values, namely 0.02 and 2.0 µg/L; although the recovery was significantly
245 lower for the lowest concentration, as shown in Figure 1d, the values retrieved for the recovery
246 were generally satisfactory over the investigated concentration range, as required by Regulation
247 (EC) No 401/2006 (2006).

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

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

259

260 *3.2 Application of the MEPS-based method to wine samples: evaluation of matrix interference*

261 Starting from the parameter values optimized on OTA standard solutions the MEPS-based
262 method was applied to OTA-containing wine samples. In this case, after preliminary experiments
263 based on the cited C18 BIN mounted on an eVol® autosampler (SGE), the method was transferred

264 to the MEPS sample preparative workstation HT4000A (HTA Scientific Instruments, Brescia,
265 Italy), in order to achieve automation of the analysis.

266 As described in Figure 2, after washing and conditioning the BIN, wine analysis was started
267 by loading 350 µL (7 × 50 µL) of each sample through the syringe and the C18 sorbent phase at a
268 speed of 3.33 µL/s (level-1 speed). The sorbent bed was then washed first with 20 µL of 2%
269 aqueous acetic acid and then with the same volume of a 2% aqueous acetic acid/methanol mixture
270 (60/40 v/v), to remove eventual interferences, and dried. The adsorbed analyte was subsequently
271 eluted with 50 µL (2 × 25 µL) of acetonitrile/2% aqueous acetic acid (90/10, v/v), which was
272 pulled/pushed through the syringe twice, at the speed of 3.33 µL/s. In view of subsequent analyses,
273 the BIN was washed with 50 µL-acetonitrile/2% aqueous acetic acid (90/10, v/v) for three times
274 after each extraction. To control memory effects blank samples were also randomly extracted on a
275 previously washed BIN and the eluent was analyzed by HPLC-FLD, under the same conditions
276 adopted for real samples. As a result, no significant memory effect was observed. Indeed, the same
277 sorbent could be used reliably for more than 100 subsequent wine extractions during the present
278 work.

279 Before undertaking the systematic application of the MEPS-based method to wine samples an
280 evaluation of eventual interference effects due to the wine matrix was performed. At this aim the
281 only wine found to be virtually free of OTA (a rosé wine, see the Experimental section) was used as
282 a blank matrix and was spiked with 0.5 µg/L OTA, thus obtaining a matrix-matched standard
283 solution of the micotoxyn. An aliquot of the spiked wine was first injected directly, without any
284 dilution, into the HPLC-FLD system. The resulting OTA peak, shown in Figure 3 (trace a), was
285 found to be almost symmetric (symmetry, S, 0.88), with a full width at half height peak (FWHH)
286 equal to 0.094 min. On the other hand, the low peak height (H, 4.6×10^{-3}) suggested the presence of
287 suppression effects due to interfering compounds, although it is not possible to establish if such
288 effects arose from a fluorescence quenching, a chemical interference or both. Another aliquot of the

289 same OTA-spiked blank wine was subjected, undiluted, to MEPS extraction followed by HPLC-
290 FLD analysis, as described before. The resulting OTA peak (see trace c in Figure 3), although
291 significantly higher, as expected, due to the preconcentration associated to the MEPS procedure,
292 was found to be asymmetrical and wide (S 1.43, FWHH 0.23 min, H 7.4 x 10^{-2}). When the extract
293 obtained from the MEPS procedure performed on the same wine previously diluted 1:2 with 2%
294 aqueous acetic acid/ethanol (88/12, v/v) was analyzed by HPLC-FLD the OTA peak (see trace b in
295 Figure 3) appeared symmetrical but still significantly larger than the peak obtained after wine direct
296 analysis (S 1.09, FWHH 0.18 min). It is worth noting that the OTA peak enlargement seems to be
297 related to the MEPS procedure itself, rather than to an effect of wine matrix; indeed, the
298 enlargement occurred also when OTA standard solutions were involved, as clearly inferred by the
299 comparison of traces d and e in Figure 3. The phenomenon could then be due to the higher amount
300 of OTA injected into the HPLC column when the MEPS procedure is performed.

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

368 In particular, the MEPS-HPLC/FLD method was applied, under identical conditions, to eight
369 OTA standard solutions in 2% aqueous acetic acid/ethanol (88:12, v/v), with concentrations ranging
370 between 0.02 and 3.0 μg/L, and to as many samples obtained from the already cited OTA-free rosé
371 wine spiked with OTA at the same concentrations. The solutions were analyzed in triplicate and the
372 corresponding average responses were plotted against OTA concentrations, thus enabling a direct
373 comparison between a solvent-matched and a matrix-matched calibration. The comparison provided
374 excellent results, as emphasized in Table 2, where the main calibration parameters, namely, linear
375 range, linearity (R), regression equation, LOD and LOQ were reported. In particular, the 95%
376 confidence intervals of the respective slopes: 0.81±0.03 and 0.78±0.04 LU min L/μg (where LU
377 represents the luminescence units) were clearly overlapped, indicating no significant signal
378 suppression or enhancement, i.e., a SSE% close to 100%. Moreover, the intercepts of the regression
379 lines were not statistically different from zero (at a 95% confidence level) in both cases, thus
380 indicating the absence of a response due to an interferent eventually present either in the solvent or
381 in the wine matrix. The method showed also promising quantitative performances, as both LOQs
382 were remarkably lower than the maximum level permitted in the European Union (2.0 μg/Kg,
383 which corresponds to as many μg/L, if a wine density closed to unity is assumed) for the OTA
384 concentration in wines.
385 Interestingly, the SSE% was evaluated also after comparing the calibrations lines obtained for
386 the same set of solvent- and matrix-matched standards but without applying the MEPS procedure as
387 a preliminary step. The resulting value, 20%, was dramatically low, thus confirming the precious
388 role of MEPS in removing wine matrix interferents that can lead to a significant suppression of the
389 OTA response.
390 Turning back to the calibrations involving the MEPS step, one could argue that a single
391 successful comparison between solvent- and matrix-matched calibrations does not guarantee that
392 the solvent-matched calibration can be used as a general approach to the quantification of OTA in
393 every possible wine, since wines could be potentially very different in terms of matrix interference.

394 Since further wines virtually free from OTA were difficult to find, the evaluation of matrix effects
395 could be extended only by using standard addition calibrations, which were applied to ten wines,
396 (two for each of the following varieties: Nero di Troia, Cabernet, Merlot, Syrah and Montepulciano)
397 naturally containing OTA levels detectable by the MEPS-based method. As a result, a good method
398 linearity was always found over the explored concentration range, i.e. up to 1.2 μg/L (correlation
399 coefficients of linear regressions ranging in the interval 0.985-0.999). Moreover, t-tests showed nine
400 and seven slopes to be not significantly different from that related to matrix-matched and solvent-
401 matched calibration, respectively, at 95% confidence. Accordingly, SSE% values ranging between
402 80 and 105% were obtained.
403 The results now described confirmed that the external calibration method could provide
404 reliable results in a good percentage of cases, in spite of the matrix variability existing between
405 different wines. Further checks of the good accuracy achievable with the external calibration were
406 also made. The first check was based on the Reference Material sample, previously adopted for a
407 standard addition-based determination. Even if using the external calibration an accuracy of 97 ±
408 2% (n = 3), expressed as the ratio between the experimentally determined concentration and the
409 true (assigned) one, was obtained. Finally, the 10 wines already contaminated by OTA were
410 adopted to evaluate the accuracy at those levels. In this case, the increase in OTA response observed
411 when passing from the as such sample to samples resulting from additions of 0.2 and 1.0 μg/L was
412 used to extrapolate the added concentration using the external calibration line; accuracies ranging
413 between 76 and 100%, at 0.2 μg/L, and between 84 and 108%, at 1.0 μg/L, were obtained, resulting
414 compliant with the Regulation (EC) No 401/2006 (2006). A final verification of the method
415 accuracy was obtained through participation to a proficiency test (PT) in a inter-laboratory circuit,
416 during which the sample cited as 17143 in the Experimental section, having an assigned OTA
417 concentration of 2.34 μg/Kg, was analyzed by the developed MEPS-HPLC/FLD method. As a
418 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 minutes 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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Figure captions

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.

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.

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

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

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

606

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

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Figure 1

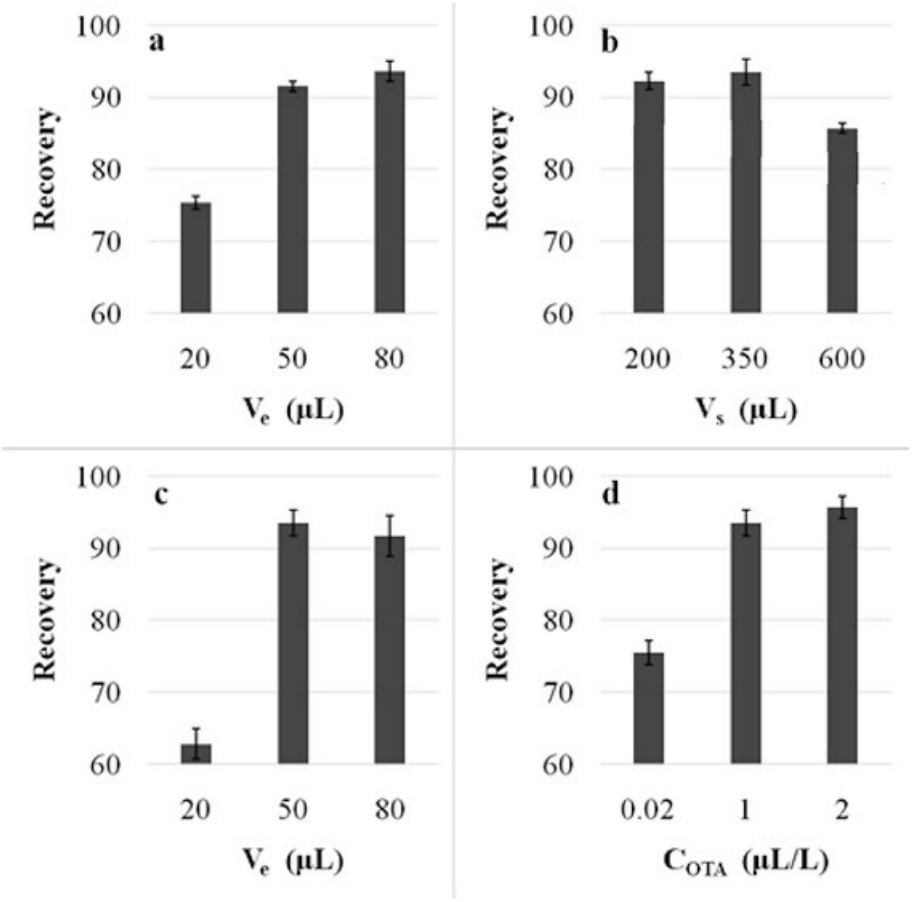
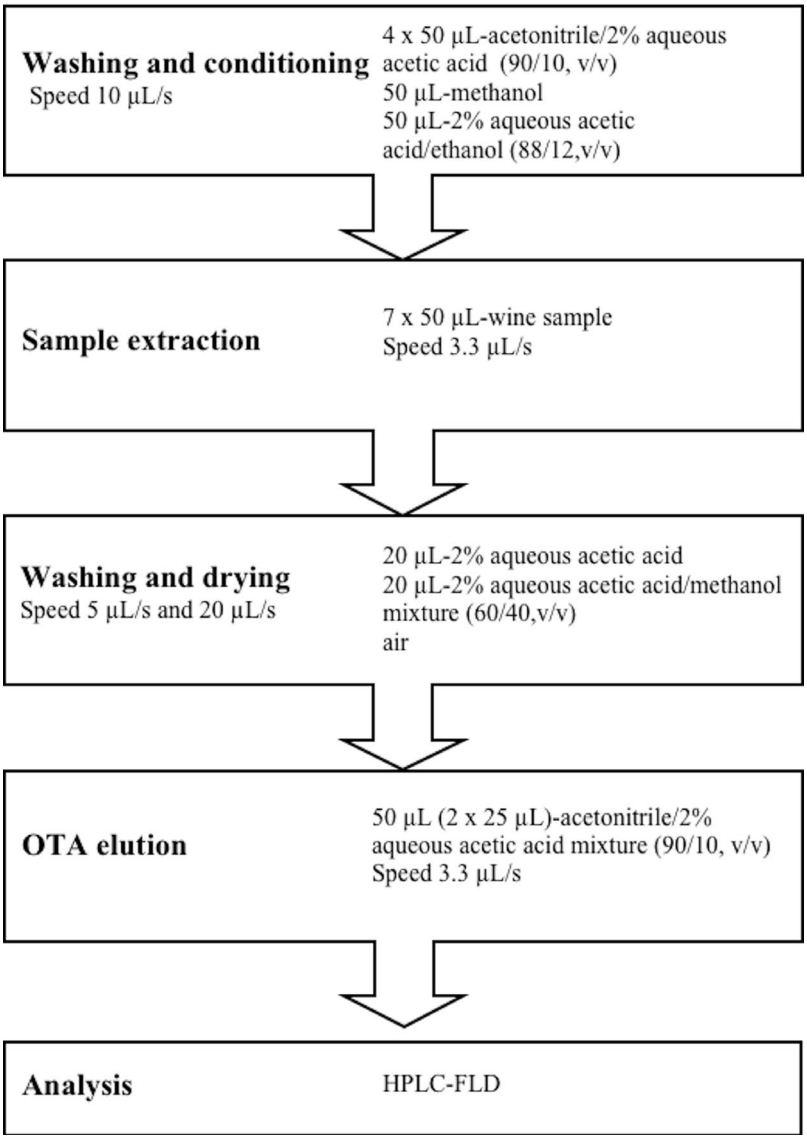

a
b
c
d
Recovery
100
90
80
70
60
20
50
80
200
350
600
0.02
1
2
V_e (µL)
V_s (µL)
C_{OTA} (µL/L)

Figure 2


Washing and conditioning
Speed 10 µL/s
4 x 50 µL-acetonitrile/2% aqueous acetic acid (90/10, v/v)
50 µL-methanol
50 µL-2% aqueous acetic acid/ethanol (88/12,v/v)
Sample extraction
7 x 50 µL-wine sample
Speed 3.3 µL/s
Washing and drying
Speed 5 µL/s and 20 µL/s
20 µL-2% aqueous acetic acid
20 µL-2% aqueous acetic acid/methanol mixture (60/40,v/v)
air
OTA elution
50 µL (2 x 25 µL)-acetonitrile/2% aqueous acetic acid mixture (90/10, v/v)
Speed 3.3 µL/s
Analysis
HPLC-FLD

647 Figure 3

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