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16 **Determination of Ochratoxin A in Italian Red Wines by Molecularly Imprinted Solid-**
17 **Phase Extraction and HPLC Analysis**

18

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21

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24 **Abstract**

25 An extraction method based on molecularly imprinted polymer prepared through a mimic
26 template approach was used for the determination of ochratoxin A in 17 red wines coming
27 from different geographical regions of Italy. Sample loading (wine sample 1:1 diluted with
28 1% v/v aqueous solution of PEG 8000), washing (2 mL water/acetonitrile 4:1 v/v) and
29 elution (2 mL of acetonitrile/acetic acid 98:2 v/v) conditions allowed the optimization of the
30 extraction method, capable of pre-concentrating ochratoxin A below the maximum
31 permitted level of 2 ng/mL. Under optimized conditions, recoveries of ochratoxin A from
32 spiked samples ranged from 88 to 102% with sample volumes up to 20 mL. The HPLC
33 determination by fluorescence detection allowed limit of detection and limit of quantification
34 respectively of 0.075 and 0.225 ng/mL. Sample extractions by an immunoaffinity protocol
35 showed the method to be comparable, demonstrating the potential of the imprinting
36 approach to substitute for the current immunoaffinity method

37

38 **Keywords:** mycotoxin analysis; wine analysis; ochratoxin A; molecularly imprinted solid
39 phase extraction; molecularly imprinted polymer

40

41 INTRODUCTION

42 Ochratoxin A, **1** (Figure 1) is a mycotoxin produced as a secondary metabolite by several
43 toxigenic moulds belonging to *Aspergillus* and *Penicillium* species provided with
44 nephrotoxic, immunosuppressive, teratogenic and carcinogenic (group 2B) properties.^{1, 2}

45 Its widespread occurrence in feed and food chains is due to the contamination of
46 foodstuffs prior to harvest or more commonly during storage.³⁻⁵ Moreover, OTA is
47 chemically stable, thus it survives during storage and food processing and is not destroyed
48 when cooked at high temperatures.^{3, 6} As a consequence, OTA contamination affects
49 many foods and beverages such as different kinds of cereals and derived products, beer,
50 wine, grape juice, coffee beans, dry vine fruits, cocoa, nuts and spices.^{3, 4, 7-9}

51 The internationalization of food trade has imposed a severe regulation to protect
52 consumers from OTA exposure. In particular, for wine and grape juice, the European Food
53 Safety Authorities have enacted the maximum permitted level of OTA at 2 µg/kg.¹⁰ Hence,
54 the development of analytical methods for OTA monitoring characterized by speed,
55 reliability, low cost and sensitivity is considered an important goal in wine quality control.

56 High performance liquid chromatography has become the most important instrumental
57 technique for OTA determination especially when coupled to fluorescence detection which
58 allows the development of a very sensitive method.¹¹ Also the use of tandem mass
59 spectrometric detection has received more of interest in recent years as being an universal
60 approach for multi-residual analysis,^{12, 13} whereas, enzyme-linked immunosorbent assay
61 and lateral-flow immunoassay are used as rapid screening methods.^{14, 15}

62 The complexity of wine matrices requires a proper extraction and clean-up procedure for
63 OTA recovery. The conventional sample treatment to remove matrix components is based
64 on the use of immunoaffinity chromatography which exploits the selective capture of the
65 mycotoxin through the immobilization of anti-OTA antibodies.¹⁶ More recently, the use of
66 molecularly imprinted polymers for the selective extraction of mycotoxins in the so-called

67 “molecularly imprinted solid phase extraction” (MISPE) approach has recently gained
68 importance as a convenient alternative to IAC,¹⁷ and examples of OTA extraction from
69 wine samples have been reported.¹⁸⁻²²

70 The present work concerns the development of a MISPE protocol to extract and
71 preconcentrate OTA from red wines, by using an imprinted polymer whose excellent
72 binding properties have been previously reported by the authors.²³ Several different Italian
73 red wines have been chosen in order to demonstrate the development of an easy,
74 effective and sensitive MISPE protocol for OTA determination in wine matrices.

75

76 **EXPERIMENTAL**

77 **Materials**

78 Ochratoxin A standard solution at a concentration of 10 µg/mL in acetonitrile (standard
79 Oekanal) was supplied by Sigma (Milano, Italy) and was stored at -20 °C. The imprinted
80 polymer was prepared by using as template a mimic of OTA, N-(4-chloro-1-hydroxy-2-
81 naphthoylamido)-L-phenylalanine, **2** (Figure 1) according to published literature.²³⁻²⁵
82 Acetonitrile and methanol (HPLC gradient grade), ethanol (95% v/v), tetrahydrofuran,
83 acetone were supplied by VWR International (Milano, Italy).

84 Citric acid monohydrate, potassium chloride, sodium chloride, sodium hydrogen
85 carbonate, tartaric acid, acetic acid, polyethyleneglycol 8000 were all of analytical grade
86 and were supplied by Sigma.

87 A citrate-tartrate buffer (named “hydroalcoholic citrate-tartrate buffer”) was prepared by
88 mixing 30 mM citric acid, 0.5 mM tartaric acid, 8 mM potassium chloride and titrating at pH
89 3.2 with NaOH 0.1 M. It was mixed with ethanol to obtain a final buffer composition
90 containing 10% v/v of ethanol. It was used to simulate the wine matrix as previously
91 described.²⁶ All the buffered solutions were filtered after preparation on 0.22 µm nylon
92 membrane from Alltech Italia (Milano, Italy) and preserved at 4°C until use. The water for

93 buffer preparation was deionized on mixed ion exchange columns, and it was ultrapurified
94 in Purelab Prima System from Elga (Marlow, UK) for HPLC eluents.

95 The real sample matrices were constituted by eighteen red wines provided with Protected
96 Designation of Origin label, coming from different Italian regions and produced in the years
97 2005-2012. Blank wine samples certified to be free from OTA contamination were kindly
98 provided by Neutron SpA (Modena, Italy).

99 The commercial immunoaffinity columns were OchraTest from VICAM (Watertown, MA,
100 USA). Solid phase extraction was performed with a VacMaster-10 sample processing
101 manifold, equipped with vacuum control valve and PTFE cartridge adapters (VWR
102 International, Milan, Italy).

103

104 **HPLC method**

105 Reversed phase HPLC analysis was used for OTA determination after solid-phase
106 extraction in according with literature.¹¹ LaChrom Elite HPLC system composed of
107 programmable binary pump L-2130, autosampler L-2200, UV detector L-2400,
108 fluorescence detector L-2480 and provided with EZChrom Elite software for the
109 instrumental programming, data acquisition and data processing was from Merck-Hitachi
110 (Milano, Italy).

111 The column used was a 250 mm x 4.6 mm i.d., 5 μ m, Li-Chrospher 100 RP-18 (Merck,
112 Milano, Italy). The mobile phase was composed of acetonitrile / water / acetic acid 40:59:1
113 v/v/v and the elution was performed in isocratic conditions at a flow rate of 0.5 mL/min.

114 The sample volume injected was 10 μ L and OTA was detected by setting the excitation
115 wavelength at 333 nm and the emission wavelength at 460 nm. In these instrumental
116 conditions OTA retention time is 20.3 \pm 0.1 min.

117 Reference standard solutions of OTA of concentration 5, 3, 2, 1, 0.75, 0.5, 0.25 and 0.1
118 ng/mL were prepared both in the eluent solution for the external calibration method and in

119 blank wine sample for the standard addition method, immediately analyzed three times
120 consecutively and peak areas were plotted against concentration. A calibration curve was
121 drawn using a weighted linear regression (weight = 1/conc).

122

123 **Optimization of MISPE protocol**

124 All the solid phase extraction experiments were made in 3-mL polypropylene SPE
125 cartridges, packed with 250 mg of OTA-imprinted polymer. All measurements
126 were performed in triplicate so that the analyte recoveries were evaluated as the averages
127 of the repeated measures in order to estimate the method repeatability. Different
128 experiments of loading, washing and elutions were performed in order to optimize the
129 extraction procedure. Before each experiment the stationary phase was conditioned with
130 five volumes of hydroalcoholic citrate-tartrate buffer.

131 To measure the MISPE cartridge loading capacity 1 mL of 250 ng/mL standard solution of
132 OTA in hydroalcoholic citrate-tartrate buffer was loaded by applying the vacuum. The
133 eluate was analyzed by HPLC. After the sample loading, the cartridge was washed with 5
134 mL of methanol-acetic acid 9:1 v/v to remove OTA.

135 To investigate the effect of different washing solutions on removal of the mycotoxin from
136 the cartridge, 1 mL of OTA solution at the concentration of 25 ng/mL in hydroalcoholic
137 citrate-tartrate buffer was loaded and a vacuum was applied to facilitate the passage of the
138 sample through the cartridge bed. After sample loading, air was passed through the
139 columns for 5 min. Then, the cartridges were sequentially washed with 1 mL of water
140 containing increasing amounts of organic solvent (0, 10, 20, 30, 40, 50, 60, 70, 80, 90, and
141 100% v/v). The organic solvents investigated for elution were: acetonitrile, methanol,
142 tetrahydrofuran and acetone. A final wash with 5 mL of methanol-acetic acid 9:1 v/v was
143 performed to completely remove the mycotoxin from the stationary phase.

144 To investigate the effect of different elution conditions 1 mL of OTA solution at the
145 concentration of 25 ng/mL in hydroalcoholic citrate-tartrate buffer was loaded and the
146 vacuum was applied. Thereafter, air was passed through the columns for 5 min and a
147 washing step with 1 mL of deionized water was performed. The cartridge was then
148 sequentially washed with 1 mL of acetonitrile containing increasing amount of acetic acid
149 (1, 2 and 5% v/v) by drying the stationary phase after each addition. The different aliquots
150 were recovered and analyzed by HPLC to measure OTA concentration. A final washing
151 step with 5 mL of methanol/acetic acid 9:1 v/v was performed to remove all compounds
152 still bound.

153

154 **MISPE of real samples**

155 MISPE protocol was evaluated on wine samples. The analytical performance of the MISPE
156 extraction protocol was compared with those obtained with commercial immunoaffinity
157 columns. All the experiments were performed in triplicate as in the previous protocol
158 optimization with OTA standards.

159 Preliminary wine treatment was performed in accordance with the literature.¹¹ Tannins
160 were precipitated by diluting the red wine samples 1:2 v/v with a 1% v/v aqueous solution
161 of PEG 8000, incubated at 4°C overnight, centrifuged at 8000 rpm for 15 min and filtered
162 on 0.22 µm polypropylene membranes.

163 Extraction of OTA from wine samples was performed by loading 2 mL of pre-treated wine
164 sample on the cartridge and applying vacuum. Afterwards, the cartridge was washed with
165 1 mL of the following solutions: 1) water/acetone 4:1 v/v; 2) water/acetonitrile 4:1 v/v; 3)
166 water/tetrahydrofuran 4:1 v/v. The OTA elution was then performed with 2 mL of
167 acetonitrile/acetic acid 49:1 v/v, as optimized in the MISPE protocol with OTA standards. A
168 final wash of the stationary phase was performed with 5 mL of methanol-acetic acid 9:1
169 v/v. To investigate the minimum volume capable of washing away the retained interferents

170 when loading volumes greater than 1 mL were applied, a set of additional experiments
171 were performed by loading 10 mL of pretreated wine sample were loaded and subsequent
172 washing steps were performed by using increasing amounts of washing solution (2, 3, 4, 5
173 mL).

174 Recovery of OTA from red wine samples was performed on 2 mL of pretreated wine
175 samples spiked with 0.25, 0.5, 1, 2, and 5 ng/mL of OTA. Then, samples were extracted
176 according to the optimized protocol above described. The peak areas measured by the
177 external calibration method allowed the determination of OTA recovery.

178 Pre-concentration of OTA from red wine samples was performed on pretreated wine
179 samples (2, 5, 10, and 20 mL) spiked with amounts of OTA to a final mycotoxin
180 concentration of 2, 0.8, 0.4, and 0.2 ng/mL. In this way, an ochratoxin amount equal to 4
181 ng was added to each volume and loaded into the MISPE cartridge. The wine samples
182 were then extracted and analyzed.

183

184 **IAC procedure**

185 Pretreated wine samples (10 mL) were loaded on the IAC column and passed through it at
186 a flow rate of about 1 drop/s without allowing the column to dry. The column was washed
187 with 5 mL of a 2.5% m/v aqueous solution of sodium chloride, 0.5% m/v sodium hydrogen
188 carbonate, pH 8.1 and then with 5 mL of water, allowing the column to dry by passing air
189 through it. OTA elution was performed with 2 mL of methanol, and the eluate evaporated
190 under a gentle stream of dry nitrogen. OTA was re-dissolved in 0.25 mL of HPLC mobile
191 phase, sonicated for 5 min and immediately analyzed.

192

193 **RESULTS AND DISCUSSION**

194 The high selectivity towards OTA of a molecular imprinted polymer obtained by a thermal
195 polymerization of methacrylic acid as functional monomer and ethyleneglycole

196 dimethacrylate as cross-linker in the presence of *N*-(4-chloro-1-hydroxy-2-
197 naphthoylamido)-L-phenylalanine as mimic template has been previously described.²³⁻²⁵

198 The present work starts from these results to develop a solid phase extraction procedure
199 tailored for red wines as real matrices. To this purpose, we first investigated the behavior
200 of the imprinted polymer in loading and recovery of the analyte and in removing the
201 interfering substances. Once the optimal operating conditions were defined by using a
202 standard solution of OTA, the experimental work concerned the refining of the extraction
203 protocol on Italian red wines.

204

205 **Development of MISPE protocol**

206 A MISPE protocol was developed by using OTA standard solutions diluted in a
207 hydroorganic buffer containing ethanol – the so-called hydroalcoholic citrate-tartrate buffer
208 – that has previously been shown to mimic real wine samples.²⁶

209 The assessment of the loading capacity of the MISPE cartridge allowed us to define how
210 much mycotoxin could be loaded on the stationary phase without detectable amount of
211 OTA in the flow-through. The loading of an amount of 250 ng of ochratoxin A, a very
212 high amount compared to the maximum permitted level in wine samples, allowed us to
213 verify that no target compound was unretained in the loading step.

214 The washing step removed interfering substances from the MISPE cartridge retaining at
215 the same time the target analyte. Thus, these experiments aimed to evaluate the capability
216 of water-organic solvent mixtures to keep loaded OTA bound to the MISPE cartridge. The
217 considered organic solvents have different polarity, although they are all water-soluble
218 allowing their use in aqueous mixture at variable percentages. Figure 2 shows the different
219 trends obtained with different water-organic solvent mixtures. All the profiles show a
220 maximum of OTA elution at a given solvent percentage that is related to the solvent elution
221 strength following the order acetonitrile ~ tetrahydrofuran > acetone > methanol. These

222 results allowed us to make some preliminary considerations about the kind of organic
223 solvent and its relative percentage useful for both washing and elution steps of OTA in the
224 MISPE protocol. However, the final choice was dependent on the basis of the results
225 obtained on real samples by considering that the properties of the organic solvent could
226 also affect the selectivity of the extraction protocol.

227 The recovery of OTA was performed in experimental conditions minimizing the required
228 volume, i.e. the target analyte dilution. The organic solvents that more efficiently eluted the
229 mycotoxin were acetonitrile and tetrahydrofuran, although the best choice for
230 chromatographic analysis reasons was acetonitrile. As a consequence, this solvent was
231 tested in elution experiments, also with the addition of variable amounts of acetic acid as
232 hydrogen bond disruptor. Figure 3 summarizes the results obtained in terms of recovered
233 percentages of OTA: the use of acetic acid allowed the quantitative recovery of OTA
234 (>99%) in 2 mL. As a consequence, OTA elution was performed with 2 mL of
235 acetonitrile/acetic acid 98:2 v/v.

236

237 **Refining of MISPE protocol on real wine samples**

238 The MISPE protocol developed in the previous section was then applied to different wine
239 samples for a further improvement in the presence of real matrices. Therefore, after the
240 preliminary wine treatment, the washing solution composition was refined considering
241 again several washing mixtures by using an OTA-negative certified wine sample.

242 The choice of the tested washing solutions was made on the basis of the experimental
243 results reported in Figure 2, where acetonitrile and tetrahydrofuran seemed to be the
244 strongest eluents, followed by acetone. Hydroorganic mixtures (water / solvent 80:20 v/v)
245 were tested in order to refine the ability to wash away the interfering matrices from the
246 MISPE cartridge while minimally affecting OTA retention. The comparison of
247 chromatographic profiles obtained by the analysis of pretreated red wine samples, loaded

248 and recovered from the MISPE cartridge by using three different washing solutions
249 showed that the difference in the chromatograms mainly concerned the analytical signals
250 in the first five minutes, far away from the peak corresponding to OTA. Thus, a 2 mL
251 washing solution made of water/acetonitrile 4:1 v/v was chosen as appropriate to remove
252 matrix interferents.

253 The minimum volume capable of washing away the retained interferents even in the case
254 of loading volumes greater than 1 mL was furtherly investigated. This step was useful to
255 pre-concentrate the target analyte. No significant differences were observed, confirming
256 that all potentially interfering substances were easily removed from the MISPE cartridge
257 even with large sample volumes. Thus, a minimum volume of 2 mL was then considered
258 as the best choice.

259

260 **Choice of HPLC method calibration**

261 The complexity and the variety of the wine matrix poses the question about what kind of
262 HPLC calibration is more suitable for OTA determination. In fact, if the matrix effect is
263 completely eliminated by the optimized MISPE extraction, then it would be possible to use
264 external calibrators, whereas if a matrix effect is present, the addition standard method
265 should be preferred. However, it should be noted that this last approach is more
266 cumbersome because it requires different calibrations for different matrices and so is less
267 practicable.

268 In order to compare the two approaches, the analytical signals (i.e. peak areas) obtained
269 from wine samples spiked with OTA in the concentration range 0.25 - 5 ng/mL were used
270 to set up a standard addition plot (LoD: 0.080 ng/mL; LoQ: 0.285 ng/mL) and read on the
271 external calibration plot described in section 2.3 (LoD: 0.075 ng/mL; LoQ: 0.225 ng/mL). In
272 this way, added concentrations were plotted *versus* found concentrations. The goodness
273 of fit ($r^2_{adj}=0.9857$, $SEE=0.2167$) highlights that the matrix effect is well compensated so

274 that we chose the calibration plot based on the use of external standards for OTA
275 determination in red wines combined with the optimized MISPE extraction above
276 described.

277 The recovery and pre-concentration tests were performed on a OTA-certified negative red
278 wine according to the optimized MISPE protocol. The experimental results show the
279 overall good performance of the MISPE column. In the concentration range of 0.25 - 5
280 ng/mL of OTA, the recoveries lies between 92 and 110% and a substantial independence
281 of these values from loaded analyte concentration can be observed. When increasing
282 volumes of wine (2 - 20 mL) containing a fixed amount of OTA are loaded, a satisfying
283 target analyte recovery – between 88% and 102% – was obtained, allowing a ten-fold
284 increase of the method sensitivity with respect to the initially optimized MISPE protocol.
285 Hence, this condition leads to a pre-concentration factor of 5 taking into account the initial
286 untreated wine.

287

288 **MISPE of real samples**

289 Finally, in order to evaluate the effect of the matrix variability, the MISPE protocol was
290 applied to 17 red wine samples of different type and coming from several regions of Italy.
291 A HPLC chromatogram of a wine sample eluted by the MISPE cartridge is reported in
292 Figure 4. The amounts of OTA found by MISPE-HPLC and determined on 20 mL of
293 pretreated wine samples were compared with those obtained by the conventional IAC-
294 HPLC procedure. The obtained results (Table 1) show a good agreement between the two
295 extraction methods fit ($r^2_{adj}=0.9817$, $SEE=0.0262$) (Figure 5) without any bias effect due to
296 different geographic origin of wines. Therefore, the MISPE procedure can be considered a
297 reliable alternative to conventional IAC protocol. Although the pre-concentration factor is
298 more reduced for MISPE with respect to IAC, the clean-up procedure is simple and
299 capable of successfully eliminating most of the matrix effects. Moreover, the combination

300 of the MISPE with the sensitive fluorescence detection allows OTA determination well
301 below the legal limit of 2 ng/mL.

302 In conclusion, we have developed a MISPE protocol for extraction and preconcentration
303 for HPLC analysis of OTA in red wine samples. The molecularly imprinted polymer,
304 prepared through a mimic template approach, was used for the preparation of a MISPE
305 stationary phase. The assessment of a solid phase extraction protocol indicates that a
306 careful choice of both the washing and eluting conditions allow the optimization of a
307 reliable MISPE extraction protocol, selective for the mycotoxin, and capable of
308 preconcentrating the analyte well below the maximum permitted levels. Recoveries
309 performed on different Italian red wine samples spiked with OTA showed good
310 reproducibility and agreement with those obtained by the IAC method demonstrating the
311 potentiality of this procedure to substitute for the current IAC-HPLC protocol.

312

313 **ABBREVIATIONS**

314 IAC: immunoaffinity chromatography; LoD: limit of detection; LoQ: limit of quantification;
315 MISPE: molecularly imprinted solid phase extraction; PEG 8000: polyethyleneglycole
316 8000; PTFE: polytetrafluoroethylene; r^2_{adj} : adjusted regression coefficient; SEE: standard
317 estimate of error; SPE: solid phase extraction

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FIGURE CAPTIONS

Figure 1: molecular structures of ochratoxin A and its template mimic

Figure 2: recovery profiles of OTA in the cartridge washing step as a function of different water - organic solvent mixtures. Acetonitrile (open circle); tetrahydrofuran (filled circle); acetone (open square); methanol (filled square).

Figure 3: Recovering of eluted OTA by using acetonitrile with increasing percentages of acetic acid. Acetonitrile (red bar); acetonitrile + 1% v/v acetic acid (orange); acetonitrile + 2% v/v acetic acid (pale green); acetonitrile + 5% v/v acetic acid (green).

Figure 4: HPLC chromatogram of red wine sample (Novello 2012) obtained by MISPE

Figure 5: comparison of OTA determination in wine samples by IAC vs. MISPE

TABLES

Table 1: OTA Determination in Italian Red Wines

Wine denomination	Region of provenience	OTA by MISPE, ng/mL	OTA by IAC, ng/mL
Aglianico (2005)	Campania	0.377±0.049	0.301±0.041
Aglianico (2009)	Campania	0.451±0.054	0.428±0.045
Barbera d'Asti (2010)	Piedmont	0.437±0.055	0.413±0.051
Barbera d'Asti (2012)	Piedmont	0.941±0.095	0.886±0.089
Birbet (2011)	Piedmont	0.271±0.046	0.200±0.019
Bonarda (2011)	Piedmont	0.182±0.022	0.179±0.024
Freisa d'Asti (2011)	Piedmont	0.197±0.029	0.194±0.031
Freisa di Chieri (2011)	Piedmont	< LOD	<LOD
Mastro (2011)	Campania	0.388±0.045	0.312±0.036
Merlot (2011)	Veneto	0.589±0.074	0.544±0.065
Montepulciano (2012)	Tuscany	0.171±0.023	0.165±0.021
Nebbiolo (2009)	Piedmont	0.200±0.019	0.189±0.017
Nero d'Avola (2011)	Sicily	0.294±0.032	0.255±0.026
Novello (2012)	Piedmont	0.548±0.069	0.538±0.062
Primitivo di Manduria (2009)	Apulia	0.492±0.051	0.482±0.047
Rouchè (2011)	Piedmont	0.212±0.027	0.199±0.025
Salento (2011)	Apulia	0.434±0.049	0.423±0.051

Comparison between results by MISPE and by IAC. Each result was the average of three replicates