Determination of Ochratoxin A in italian red wines by molecularly imprinted solid phase extraction and HPLC analysis

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

Cristina Giovannoli*, Cinzia Passini, Fabio Di Nardo, Laura Anfossi, Claudio Baggiani

Department of Chemistry, University of Torino, Via P.Giuria 5 – 10125 Torino, Italy

* corresponding author (tel: +390116705252 ; fax: +390116705242; e-mail: cristina.giovannoli@unito.it)

Abstract

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

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

INTRODUCTION

Ochratoxin A, **1** (Figure 1) is a mycotoxin produced as a secondary metabolite by several toxigenic moulds belonging to *Aspergillus* and *Penicillum* species provided with nephrotoxic, immunosuppressive, teratogenic and carcinogenic (group 2B) properties.[1, 2] Its widespread occurrence in feed and food chains is due to the contamination of foodstuffs prior to harvest or more commonly during storage.[3-5] Moreover, OTA is chemically stable, thus it survives during storage and food processing and is not destroyed when cooked at high temperatures.[3, 6] As a consequence, OTA contamination affects many foods and beverages such as different kinds of cereals and derived products, beer, wine, grape juice, coffee beans, dry vine fruits, cocoa, nuts and spices.[3, 4, 7-9]

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

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

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

"molecularly imprinted solid phase extraction" (MISPE) approach has recently gained importance as a convenient alternative to IAC,[17] and examples of OTA extraction from wine samples have been reported.[18-22]

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

EXPERIMENTAL

Materials

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

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

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

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

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

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

HPLC method

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

The column used was a 250 mm x 4.6 mm i.d., 5 μm, Li-Chrospher 100 RP-18 (Merck, Milano, Italy). The mobile phase was composed of acetonitrile / water / acetic acid 40:59:1 v/v/v and the elution was performed in isocratic conditions at a flow rate of 0.5 mL/min. The sample volume injected was 10 μL and OTA was detected by setting the excitation wavelength at 333 nm and the emission wavelength at 460 nm. In these instrumental conditions OTA retention time is 20.3±0.1 min.

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

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

Optimization of MISPE protocol

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

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

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

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

MISPE of real samples

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

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

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

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

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

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

IAC procedure

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

RESULTS AND DISCUSSION

The high selectivity towards OTA of a molecular imprinted polymer obtained by a thermal 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 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

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

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

Refining of MISPE protocol on real wine samples

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

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

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

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

Choice of HPLC method calibration

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

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

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

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

MISPE of real samples

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

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

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

ABBREVIATIONS

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

REFERENCES

[1] Poland, A.E.; Nesheim, S.; Friedman, L. Ochratoxin A: a review. *Pure Appl. Chem.* **1992**, *64*, 1029-1046.

[2] Pfohl-Leszkowicz, A.; Mandeville, R.A. Ochratoxin A: an overview on toxicity and carcinogenicity in animals and humans. *Mol. Nutr. Food Res.* **2007**, *51*, 61-99.

[3] Duarte, S.C.; Peña A.; Lino, C.M. A review on ochratoxin A occurrence and effects of processing of cereal and cereal derived food products. *Food Microbiol.* **2010**, *27*, 187-198.

[4] Magan, N.; Aldred, D.; Mylona, K.; Lambert, R.J.W. Limiting mycotoxins in stored wheat. *Food Addit. Contam. A* **2012**, *27*, 644-650.

[5] Duarte, S.C.; Lino, C.M.; Peña A. Ochratoxin A in feed of food-producing animals: an undesirable mycotoxin with health and performance effects. *Vet. Microbiol.* **2011**, *154*, 1-13.

[6] Kabak, B. The fate of mycotoxins during thermal food processing. *J. Sci. Food Agric.* **2009**, *89*, 549-554.

[7] Bertuzzi, T.; Rastelli, S.; Mulazzi, A.; Donadini, G.; Pietri, A. Mycotoxin occurrence in beer produced in several European countries. *Food Control* **2011**, *22*, 2059-2064.

[8] Anli, E.; Bayram, M. Ochratoxin A in wines. *Food Rev. Int.* **2009**, *25*, 214-232.

[9] Copetti, M.V.; Pereira, J.L.; Iamanaka, B.T.; Pitt, J.I.; Taniwaki, M.H. Ochratoxigenic fungi and ochratoxin A in cocoa during farm processing. *Int. J. Food Microbiol.* **2010**, *143*, 67-70.

[10] Commission of the European Communities (CEC). Commission Regulation (EC) No. 1881/2006 of 19 December 2006 setting maximum levels for certain contaminants in foodstuffs. *Off J Eur Union L.* **2006**, *364*, 5-24.

342 [11] Visconti, A.; Pascale, M.; Centonze, G. Determination of ochratoxin A in wine and
343 beer by immunoaffinity column cleanup and liquid chromatographic analysis with
344 fluorometric detection: Collaborative study. *J. AOAC Int.* **2001** *84* 1818-1827.
345 [12] Zollner, P.; Mayer-Helm, B. Trace mycotoxin analysis in complex biological and food
346 matrices by liquid chromatography-atmospheric pressure ionisation mass
347 spectrometry. *J. Chromatogr. A* **2006** *1136* 123-169.
348 [13] Sforza, S.; Dall'Asta, C.; Marchelli, R. Recent advances in mycotoxin determination in
349 food and feed by hyphenated chromatographic techniques/mass spectrometry. *Mass*
350 *Spectrom. Rev.* **2006** *25* 54-76.
351 [14] Zheng, M.Z.; Richard, J.L.; Binder, J. A review of rapid methods for the analysis of
352 mycotoxins. *Mycopathologia* **2006** *161* 261-273.
353 [15] Anfossi, L.; Baggiani, C.; Giovannoli, C.; D'Arco, G.; Giraudi, G. Lateral-flow
354 immunoassays for mycotoxins and phycotoxins: a review. *Anal. Bioanal. Chem.* **2013**
355 *405* 467-480.
356 [16] Scott, P.M.; Trucksess, M.W. Application of immunoaffinity columns to mycotoxin
357 analysis. *J. AOAC Int.* **1997** *80* 941-949.
358 [17] Baggiani, C.; Anfossi, L.; Giovannoli, C. Molecularly imprinted polymers as synthetic
359 receptors for the analysis of myco- and phyco-toxins. *Analyst* **2008** *133* 719-730.
360 [18] Maier, N.M.; Buttinger, G.; Welhartizki, S.; Gavioli, E.; Lindner, W. Molecularly
361 imprinted polymer-assisted sample clean-up of ochratoxin A from red wine: merits and
362 limitations. *J. Chromatogr. B* **2004** *804* 103-111.
363 [19] Yu, J.C.C.; Krushkova, S.; Lai, E.P.C.; Dabek-Zlotorzynska, E. Molecularly-imprinted
364 polypyrrole-modified stainless steel frits for selective solid phase preconcentration of
365 ochratoxin A. *Anal. Bioanal. Chem.* **2005** *382* 1534-1540.

366 [20] Yu, J.C.C.; Lai, E.P.C. Molecularly imprinted polypyrrole modified carbon nanotubes
367 on stainless steel frit for selective micro solid phase pre-concentration of ochratoxin A.
368 *React. Funct. Polym.* **2006**, *66*, 702-711.
369 [21] Yu, J.C.C.; Lai, E.P.C. Determination of ochratoxin A in red wines by multiple pulsed
370 elutions from molecularly imprinted polypyrrole. *Food Chem.* **2007**, *105*, 301-310.
371 [22] Cao, J.L.; Kong, W.J.; Zhou, S.J.; Yin, L.H.; Wan, L.; Yang, M.H. Molecularly imprinted
372 polymer-based solid phase clean-up for analysis of ochratoxin A in beer, red wine, and
373 grape juice. *J. Sep. Sci.* **2013**, *36*, 1291-1297.
374 [23] Baggiani, C.; Biagioli, F.; Anfossi, L.; Giovannoli, C.; Passini, C.; Giraudi, G. Effect of
375 the mimic structure on the molecular recognition properties of molecularly imprinted
376 polymers for ochratoxin A prepared by a fragmental approach. *React. Funct. Polym.*
377 **2013**, *73*, 833-837.
378 [24] Baggiani, C.; Giraudi, G.; Vanni, A. A molecular imprinted polymer with recognition
379 properties towards the carcinogenic mycotoxin ochratoxin A. *Bioseparation* **2001**, *10*,
380 389-394.
381 [25] Jodlbauer, J.; Maier, N.M.; Lindner, W. Towards ochratoxin A selective molecularly
382 imprinted polymers for solid-phase extraction. *J. Chromatogr. A* **2002**, *945*, 45-63.
383 [26] Baggiani, C.; Baravalle, P.; Giraudi, G.; Tozzi, C. Molecularly imprinted solid-phase
384 extraction method for the high-performance liquid chromatographic analysis of
385 fungicide pyrimethanil in wine. *J. Chromatogr. A* **2007**, *1141*, 158-164.

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 detemination 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