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Molecularly imprinted polymer-assisted sample clean-up of ochratoxin A from red wine: merits and limitations

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Abstract

A new analytical method for the determination of the carcinogenic mycotoxin ochratoxin A (OTA) in red wines has been developed involving a two-dimensional solid-phase extraction (SPE) clean-up protocol on C18-silica and a target-selective molecularly imprinted polymer (MIP). Prior removal of the interfering acidic matrix compounds by C18 solid-phase extraction was crucial for a successful clean-up as direct sample loading onto the MIP led to poor recoveries. The combined solid-phase extraction protocol afforded extracts suitable for sensitive ochratoxin A quantification by HPLC-fluorescence detection. Preliminary validation of the method performance with spiked (0.033–1.0 ng OTA/ml) and commercial red wines provided recoveries >90% and $R.S.D._r < 10\%$, with limit of detection (LOD) and limit of quantification (LOQ) of 0.01 and 0.033 ng/ml. However, a similarly favorable performance characteristics was observed in control experiments in which the MIP was replaced by the corresponding non-imprinted polymer (NIP). These findings provide evidence that under the employed experimental conditions specific analyte binding to imprinted binding sites plays a minor role in selective OTA retention. In the framework of this study, other problems inherent to MIP-based solid-phase extraction have been addressed. These include the reproducible preparation of MIP materials with consistent molecular recognition characteristics, the potential for repeated use of MIP, unfavorable polymer swelling in application-relevant solvents, potential sample contamination by template bleeding, and slow analyte binding kinetics.

Keywords: Mycotoxin analysis; Ochratoxin A; Molecularly imprinted polymers

1. Introduction

Ochratoxin A (OTA, see Fig. 1A), a secondary metabolite of various genera of Aspergillus and Penicillium [1], is found as a frequent contaminant in food and feed commodities. OTA exhibits multi-facetted toxicity in animals and mankind, including nephrotoxic, hepatotoxic, immunotoxic, teratogenic and carcinogenic effects, and represents therefore a serious health risk to lifestock and population [2,3]. The widespread occurrence of OTA in cereals, maize, rice, beans, nuts, raisins and beverages, such as milk, coffee, grape juice and wine has prompted health regulation authorities to define maximal tolerable daily intake levels, ranging from 1.5 to 15 ng/kg body weight [3].

The availability of sensitive analytical methods for trace level monitoring and quantification of OTA is a crucial requirement to guide efforts focusing on responsible risk assessment, efficient food quality control and development of provisional food processing procedures. Generally, these protocols involve sample clean-up and pre-concentration steps prior to RP-HPLC/fluorescence [4] or MS-detection [5,6] to remove matrix components and enhance sensitivity. Sample treatment for OTA determination involves liquid–liquid and/or solid-phase extraction (SPE), the latter being preferred due to simplicity in operation and reduced solvent requirements.

Particularly efficient SPE can be achieved employing immunoaffinity columns (IACs), comprising anti-OTA antibodies as molecular recognition principle [6–14]. These IACs offer several significant advantages over conventional, less selective SPE materials. Thus, the exquisite OTA specificity and affinity allow to obtain very clean sample extracts, even from highly diluted solution in presence of complex matrices. The IACs operate under biomimetic conditions, thus permitting direct processing of aqueous sample solutions, with benefits in terms of operational simplicity, rapidity, and reduction of the use of organic, potentially toxic solvents.

However, there are also some limitations inherent to IAC-based SPE [10,15]. Due to the high molecular weight,

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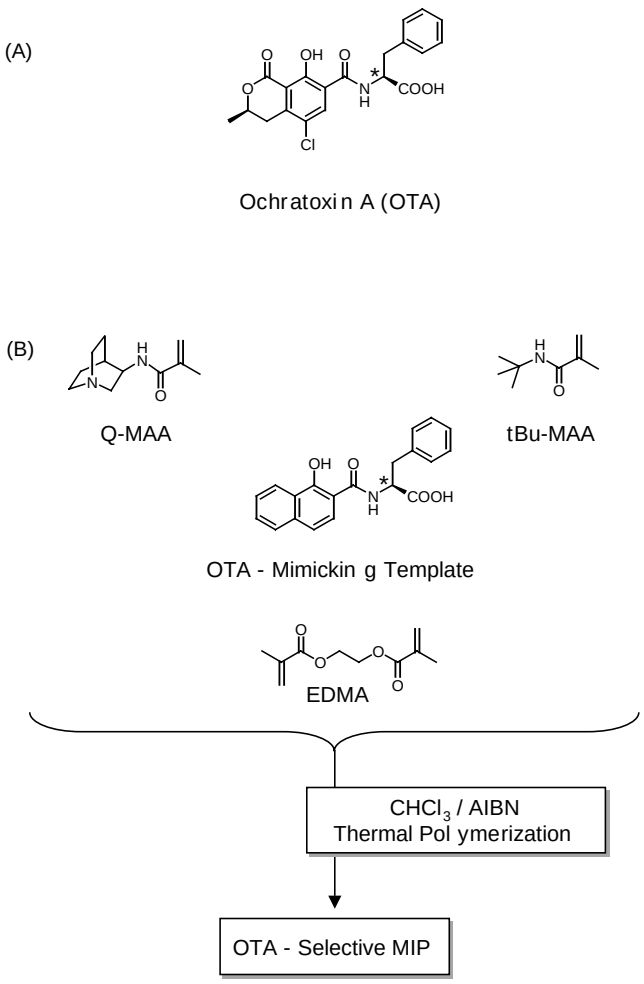

Fig. 1. (A) Chemical structure of ochratoxin A. (B) Preparation of the ochratoxin A-selective molecular imprinted polymer using designed functional and auxiliary monomers and an ochratoxin A-mimicking template. For details see Section 2.

the number of immobilized antibodies is generally low and may lead, especially under sub-optimal binding conditions, to poor binding capacities. Moreover, immunoaffinity materials tolerate only limited amounts of organic solvents, which may preclude direct processing of non-aqueous sample extracts. For optimal performance, immunoaffinity materials often require specific environments (aqueous media, well-controlled pH, ionic strength, and concentration levels of matrix components), which significantly narrow the "optimization space" for analytical method development. A serious concern with immunoaffinity SPE materials is their susceptibility to biodegradation, leading to relatively short shelf life. Analyte release from IACs often involves antibody-denaturating conditions, leading to partial or complete loss of their highly selective binding properties, precluding multiple use scenarios. Finally, the preparation of antibodies is elaborate and requires special skills, which is reflected in the quite high price of IACs.

To address these problems, OTA-selective molecularly imprinted polymers (MIPs) have been considered as potential alternative to immunoaffinity-based SPE [16,17]. MIPs are generated by co-polymerization of functional monomers and cross-linking agents in presence of template molecules, leading to rigid polymeric networks comprising specific binding sites for the template molecules. In some cases, the re-binding affinity and selectivity of these polymer-embedded binding sites for the template molecule (and structurally related molecules) rival those of natural antibodies [18]. As a significant advantage over immunoaffinity materials, MIPs are chemically (and thermally) very stable, allowing for considerable flexibility in terms of solvents and additives in course of SPE method development. The enhanced chemical resistance also may permit MIP cleaning and regeneration under harsh conditions, making them accessible for multiple use. Other arguments in favor of MIPs are ease of preparation from relatively low-cost materials and practically unlimited storage times.

Recently, we reported on the development of an OTA-selective MIP material, specifically designed to recognize and bind OTA under polar protic conditions [17]. In engineering this MIP, we purposefully addressed the electrostatic and hydrophobic molecular recognition increments of OTA, implementing functionally complementary monomers with ion-pairing and hydrophobic adhesion capacity. For this purpose, dedicated functional and auxiliary monomers were designed and synthesized (see Fig. 1B), comprising a sterically demanding tertiary amine (Q-MAA) and the highly hydrophobic *tert*-butyl group (tBu-MMA). As template, we employed a carefully designed mimic, exhibiting a close resemblance to OTA in size, shape, sterochemistry and functionality. This mimic was used to circumvent the necessity of manipulating highly toxic OTA, but primarily to avoid sample contamination in the case of template bleeding [19]. Thermal co-polymerization of these designed components with ethylene glycol dimethacrylate (EDMA), using chloroform as porogenic solvent and bis-azoisobutyronitrile (AIBN) as radical initiator (see Fig. 1B), provided a MIP that was capable of binding OTA with considerable specificity and affinity from polar protic media.

In this contribution, we report our results concerning the application of this engineered MIP material to SPE of OTA from red wines. Red wine was deliberately chosen as a challenging test case to rigorously evaluate the potential of the MIP to master real-world applications. We will describe the development and optimization of the MIP-based SPE protocol and RP-HPLC-fluorescence detection method, and provide information on the performance characteristics of the advanced method. The contributions of "specific analyte binding" to imprinted binding sites will be critically assessed by comparing the performance of the imprinted versus non-imprinted polymers (NIP). In addition, problems inherent with the application of MIP materials in SPE, concerning reproducibility of the molecular recognition properties, polymer swelling, template bleeding, slow mass transfer/binding kinetics, and reusability of MIPs will be addressed.

2. Experimental

2.1. Materials and chemicals

Solvents of HPLC grade quality (methanol, acetonitrile, chloroform, toluene) were purchased from Merck (Darmstadt, Germany). Acetic acid (analytical quality, >99%) was ordered from Fluka (Buchs, Switzerland). Functional monomer Q-MMA, auxiliary monomer tBu-MAA and the OTA-mimicking template (see Fig. 1B) were prepared following the procedures described previously [17]. Ethylene glycol dimethacrylate and bis-azoisobutyronitrile were purchased from Merck (Darmstadt, Germany). Pre-packed SPE extraction cartridges comprising 100 mg C18-adsorbent were from Phenomenex (Torrence, CA). The 1 ml-SPE cartridges and appropriate frits made from polyethylene were purchased from SUPELCO (Bellefonte, PA). Bi-distilled water was produced in-house using a GFL quartz distilling apparatus model 2104, GFL (Burgwedel, Germany). OTA standard material in crystalline form was purchased from Sigma–Aldrich (Vienna, Austria).

2.2. Wine samples

Bottled red wines were purchased at a local supermarket (Vienna, Austria), and the origin and production year reported in Table 1 are taken from the information provided on the labels. Contaminated and non-contaminated red wine samples used for method development were available from prior studies [20].

2.3. Apparatus

The HPLC-apparatus (pump L-7150, programmable autosampler D-7250, fluorescence detector F-1080, interface D-7000) was from Merck–Hitachi (Darmstadt, Germany). Chromatographic data were processed using Chromatography Data Station Software, Version 4.0, from Merck–Hitachi (Darmstadt, Germany). All analyses were performed on a Purospher RP C18 column (125 mm × 4 mm i.d., 5 μm) with an inline-guard column from Merck (Darmstadt, Germany). The column temperature was controlled by a Haake F6 thermostat (Thermo Haake GmbH, Karlsruhe, Germany). SPE was performed with a VacMaster-10 sample processing station, equipped with vacuum control valve and PTFE cartridge adapters (Argonaut Technologies, Foster City, CA).

2.4. Preparation of MIP and NIP materials

The OTA-selective MIP material and the corresponding non-imprinted control polymer used for SPE extraction were prepared following essentially the procedure described in a recent paper [17]. However, to establish unambiguously the reproducibility of the imprinting protocol, the non-commercial components, i.e. functional monomer Q-MAA, auxiliary monomer tBu-MAA and OTA-mimic, were subjected to additional purification steps by flash chromatography and re-crystallization prior to use. With these highly purified components, the MIP material was produced as follows: a 20 ml screw-cap centrifuge vial was charged with a solution of 194 mg (1.0 mmol) functional monomer, 423 mg auxiliary monomer (3.0 mmol), 335 mg OTA-mimic (1.0 mmol), 3.964 g EDMA (20 mmol) and 4.0 mg AIBN (0.025 mmol) in 5.60 ml dry and acid-free chloroform (treated over basic alumina prior to use). The solution was carefully degassed by passing a stream of dry nitrogen through the mixture for 5 min. The vial was sealed and allowed to incubate at 65 °C for 10 h. The corresponding NIP was prepared in an analogous fashion, omitting the template in the polymerization mixture. The resultant polymer rods were broken into small pieces, ground by means of a ball mill and sieved to isolate the fractions with a particle size <65 μm. From these materials, fines were removed by repeated sedimentation in methanol–acetic acid (95:5). For efficient removal of template and non-incorporated components, the polymers were subjected for 24 h to a continuous hot-extraction employing the azeotropic mixture of toluene–acetic acid (comprising 37 mol% acetic acid, boiling point: 100 °C) as extraction medium. Subsequently, the polymers (about 3.0 g) were packed into a flash-chromatography cartridge and rinsed with 1.5 l methanol–acetic acid (90:10) at a flow rate of 3 ml/min. After this treatment, for the MIP the template was no longer detectable in the eluate as judged by HPLC/UV-monitoring.

To establish the reproducibility of the MIP preparation protocol, three batches of polymer following strictly the protocol outlined above were produced. For evaluation of the selectivity and affinity of the obtained MIP materials, the processed and washed polymer beads were packed into 150 mm × 4 mm columns and tested under chromatographic conditions using the racemic OTA-mimic as test analyte. The mobile phase consisted from methanol–acetic acid (95:5) at a flow rate of 1 ml/min. The injected sample amount was 10 μg, and peaks were detected at 254 nm. All chromatographic measurements were performed at 25 °C. The chromatographic runs were performed in triplicate for each column and the data given in Fig. 2 represent the corresponding mean values.

2.5. Packing of SPE cartridges with MIP/NIP materials

The pronounced swelling properties of the OTA-selective MIP (and NIP) material in methanol (swelling factor >1.5) enforced the development of a wet packing procedure. For this purpose, a 100 mg amount of MIP was transferred into a 10 ml screw-cap centrifuge vial and incubated with 1 ml of methanol. The sealed vial was allowed to stand 24 h at ambient temperature with occasional shaking. Then, the slurry was transferred into an 1 ml SPE cartridge equipped with a polyethylene frit. The polymer was allowed to settle for 5 min. The adsorbent bed was stabilized by careful insertion of a second frit, avoiding any compression of the polymer filling. Prior to use, the MIP cartridges were pre-conditioned

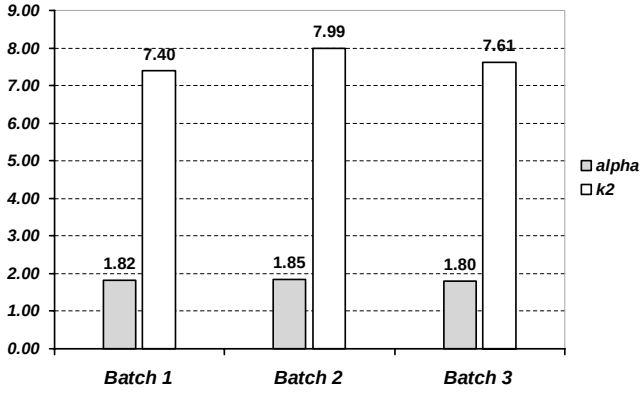

Fig. 2. Reproducibility of molecular recognition properties (retention and selectivity) of the OTA-selective MIP probed with the racemic ochratoxin A-mimic. k_2: retention factor of the more retained template enantiomer; α: chromatographic enantioselectivity factor. Chromatographic conditions: MIP columns (125 mm × 4 mm i.d.); mobile phase: methanol–acetic acid (95:5 (v/v)); flow rate: 1 ml/min; UV-detection: 254 nm; column temperature: 25 °C; injected sample amount: 10 μg.

by washing with methanol–acetic acid (8:2, 10 ml), followed by methanol (10 ml) at 0.1 ml/min. In a few cases, extreme slow flow rates were observed during the pre-conditioning step, indicating that frit-plugging has occurred. These cartridges were excluded from SPE applications. If not used immediately, pre-conditioned MIP cartridges were sealed with appropriate pluggers and stored at 4 °C to prevent drying out by solvent evaporation.

2.6. SPE protocol for clean-up of red wines samples

Cartridges comprising 100 mg C18-adsorbents were equipped with 10 ml reservoirs and mounted onto a 10-position SPE manifold. Prior to use the cartridges were pre-conditioned with 3 ml of methanol followed by 3 ml water. The 10 ml volumes of red wine samples were loaded and allowed to migrate at a flow rate of 0.2 ml/min. After washing the columns with 2 ml of water they were dried employing vacuum for 30 s. The cartridges were then eluted with 3 ml of methanol at a flow rate of 0.2 ml/min to give partially cleaned samples. The C18-cartridges were replaced by pre-conditioned MIP (or NIP) columns, the methanolic extracts loaded and allowed to migrate at a flow rate of 0.1 ml/min. After washing with 2 ml of methanol, the MIP (or NIP) columns were eluted with a total volume of 4 ml methanol–acetic acid (8:2, 2 ml × 2 ml) at a flow rate of 0.1 ml/min. The eluate was immediately dried under a stream of nitrogen at ambient temperature and reconstituted in 500 μl mobile phase for HPLC analysis.

2.7. In-matrix and external OTA calibration curves, determination of recoveries

Aliquots of a methanolic OTA stock solution (40 ng/ml) equivalent to 0.33, 0.66, 1.25, 2.5, 5.0, 8.0 and 10 ng OTA were transferred to 10 ml volumetric flasks. The solvent was removed under a stream of nitrogen and the residues were reconstituted in 10 ml of a non-contaminated red wine, giving samples with spiking levels of 0.033, 0.066, 0.125, 0.25, 0.5, 0.8 and 1.0 ng OTA/ml. These were sonicated for 5 min and then allowed to stand for 3 h at ambient temperature to equilibrate OTA with the red wine matrix. Subsequently, these samples were cleaned-up according to the SPE protocol outlined above and subjected to HPLC analysis to give an "in-matrix" OTA calibration curve. In similar fashion, identical volumes of the OTA stock solution were transferred to HPLC vials, dried under nitrogen and reconstituted in 500 μl of mobile phase. HPLC analysis of these standards gave an "ex-matrix" calibration curve. Comparison of these calibration curves was used to calculate the recoveries of the developed SPE protocol.

2.8. Determination of OTA

For final HPLC analysis of the sample extracts a Purospher RP-C18 column (125 mm × 4 mm i.d., 5 μm) with a Select B inline-guard column was employed. The mobile phase consisted of acetonitrile–water–acetic acid (40:60:2) at a flow rate of 0.8 ml/min. The OTA signal (retention time: 10.20 min) was detected fluorimetrically (λ_{ex} = 333 nm, λ_{em} = 460 nm). The column temperature was kept at 25 °C. Fifty microlitre volumes of reconstituted extracts were injected (equivalent to 1 ml wine). For the quantification of the OTA concentration in contaminated red wines the observed peak areas were correlated to those of the "in-matrix" calibration curve.

2.9. Reusability of MIP cartridges

For investigation of the reuseability of the MIP affinity material, a single MIP cartridge was employed for six consecutive SPE clean-up cycles for a given OTA contaminated wine sample (0.22 ng OTA/ml). In between the cycles, the MIP affinity column was reconditioned by washing with methanol–acetic acid (8:2, 10 ml) and methanol (10 ml).

3. Results and discussions

3.1. Reproducibility of molecular recognition properties of OTA MIPs

The success in developing validated analytical methods for the quantification of trace levels of toxins is intricately linked to the availability of materials and reagents of reproducible quality. For SPE materials, these quality criteria include reproducible affinity, selectivity, and capacity for the analytes of interest. While these characteristics are generally controlled and well-documented for commer-

cially available adsorbents, such as surface-modified silicas and polymers, these aspects have found little attention for MIP-based SPE materials. Neglecting these crucial issues appears problematic, considering the complex nature of molecular recognition principles governing the selective binding of analytes in MIPs. Thus, incremental changes in the imprinting protocols, such as small changes in the functional monomer-template stoichiometry, quality of the porogenic solvents, polymerization temperature, etc. may significantly affect the molecular recognition profiles of the resultant MIPs [21,22]. Especially in cases, where the generation of MIP-based SPE materials involves custom-designed non-commercial components, the capability of producing MIPs with reproducible binding properties needs to be demonstrated.

Addressing these crucial aspects, the batch-to-batch reproducibility of the molecular recognition properties of the OTA-selective MIP used in this study was evaluated. For this purpose, the non-commercial components of the MIP formulation, i.e. the OTA-mimic template, basic functional monomer Q-MAA and the auxiliary monomer tBu-MAA (see Fig. 1B), were prepared following known routes and subjected to strict purification protocols by crystallization and chromatography, respectively. Employing these compounds in combination with cross-linking agent, radical initiator and porogen of well-defined qualities, three batches of MIPs were produced following strictly the reported thermal polymerization protocol. The obtained bulk polymers were separately processed to provide 40–65 μm particles, which were packed into columns and evaluated under identical chromatographic conditions. Using the racemic template as test analytes and a mixture of methanol–acetic acid (95:5) as mobile phase, the enantioselectivity as well as the retention factor of the (more retained) template were measured. The observed enantioselectivities were considered to reflect the operational integrity of the polymer-embedded binding sites, while the retention factors for the template enantiomer may be seen as a measure for the overall affinity, density, and accessibility of these binding domains. As can be seen from Fig. 2, the individual MIP batches exhibit very similar enantioselectivities ($\alpha = 1.82 \pm 0.03$) and retention factors ($k_2 = 7.68 \pm 0.30$). For the individual MIP batches the variation in enantioselectivity was found to be <2%, indicating practically identical binding specificity. Also, the deviations in template retention were similarly low (<4%), demonstrating an excellent reproducibility with respect to binding site affinity and accessibility.

3.2. Packing of MIP SPE cartridges

The MIP material used in this study has been engineered to address specifically the selective molecular recognition requirements for OTA in polar protic environments by implementing in a stoichiometric fashion electrostatic interactions between the functional monomer and template. For this MIP, a favorably high template re-binding capacity (>90% of the employed template) was demonstrated [17], suggesting the preservation of a large fraction of the imprinted binding domains on template removal. A serious disadvantage of these MIP materials, however, consists in pronounced polymer swelling associated with the template release, most probably as a consequence of excessive solvation of "unsaturated" functional groups located within the binding cavities [23]. In fact, the exposure of the dry MIP to polar solvents led to substantial increase in volume, e.g. in methanol by a factor >1.5. These pronounced swelling properties proved prohibitive with respect to the development of a dry-packing procedure for the production of SPE columns. Attempts of wetting dry-packed SPE cartridges with polar solvents resulted in inhomogeneous and compressed adsorbent beds that suffered from severe channeling and/or extremely poor liquid permeability. Therefore, a wet-packing procedure was advanced, comprising column filling of SPE cartridges with MIP pre-swollen in methanol. In this context, the polymer particle size also proved to be a crucial factor. Columns packed with 25 μm particles led to SPE units with extremely poor liquid permeability. MIP particles in the range of 40–65 μm proved an acceptable compromise between bed homogeneity and permeability, allowing with methanol at ambient pressure appropriate flow rates in the range of 0.1–0.2 ml/min.

3.3. Development of the MIP-based SPE sample clean-up protocol for red wines

Our efforts towards the development of a MIP-based SPE sample clean-up protocol for OTA from red wine were primarily guided by application-relevant criteria. Along this line, material costs, operational simplicity and environmental issues were considered as crucial aspects. Specifically, the SPE protocol should allow to process directly untreated red wine samples, avoiding time-consuming dilution, pH adjustment and liquid–liquid extraction steps. Furthermore, SPE should be achievable employing low-cost and low-toxicity solvents for washing and elution steps, without resorting to environmentally harmful aromatic and halogenated solvents. Additionally, the SPE protocol in combination with the employed RP-HPLC-fluorescence detection assay should fulfill at least the minimum method performance requirements defined by CEN committee for determination of OTA [24]. Thus, to be competitive to officially recognized methods, recoveries in the range of 70–100% and $R.S.D._r < 20\%$ in the low contamination level (<1.0 ng OTA/ml) should be achieved.

Based on the requirements stipulated above, first exploratory SPE experiments focused on the most straightforward scenario, consisting in application of untreated red wine samples directly on pre-conditioned MIP cartridges, washing with water, and elution with methanol–acetic acid. The obtained sample extracts showed a good clean-up effect, but suffered from relatively poor recoveries. Thus, with a red wine sample contaminated with 0.22 ng/ml OTA the recov-

ery levels after method optimization were <66%. Clearly, this result was unacceptable considering the above defined method performance criteria. We reasoned that the high concentrations of polar acidic compounds in red wine matrix (pH < 3.7) may severely compete with the analyte for the crucial basic interaction centers at polymer-embedded binding sites. Consequently, the partial disruption of the prime electrostatic interaction between the "basic" MIP and the "acidic" OTA would lead to the observed losses in analyte. For this reason, we decided to perform prior to the MIP treatment an additional SPE step on C18-modified silica, which was expected to remove the major amount of polar acidic compounds while retaining the highly lipophilic target analyte. The feasibility of this concept was first tested using an artificial wine matrix, composed from an aqueous solution of ethanol (12%), tartaric acid (2%) glucose (1%) and OTA (2.5 ng/ml). Loading a 10 ml volume of this mixture on a 100 mg C18 cartridge, washing with water and subsequent elution with methanol resulted in a complete removal of the matrix components and a quantitative recovery of OTA. Loading the OTA containing eluate from this C18-SPE step onto a pre-conditioned MIP column, washing with methanol, and elution with methanol–acetic acid (8:2) resulted in an OTA recovery >96%.

Based on these promising results, the combined C18-MIP SPE protocol was optimized using a naturally contaminated red wine of known OTA content (0.22 ng OTA/ml) as model sample. As optimization parameter, the amounts and nature of the washing and elution solvents as well as the flow rates through the affinity materials were considered. All washing and elution steps could be performed using inexpensive and non-toxic solvents (water, methanol, acetic acid and mixtures thereof). In terms of flow rates, ambient pressure solvent migration through the SPE materials proved appropriate. Under these condition, the flow rates through the C18 SPE cartridges are 0.5 ml/min and about 0.1–0.2 ml/min through the MIP cartridges. Attempts to accelerate the relatively slow MIP-based SPE process by vacuum-enforced increase of the flow rate led to considerable losses of analyte. Thus, at flow rates of 0.5 ml/min the observed OTA recovery was <30%, indicating poor interaction between the MIP-based affinity material and the target analyte.

The optimized C18-MIP protocol did not produce colorless sample extracts, but was capable of removing a major amount of the interfering matrix components. The effectiveness of the sample clean-up procedure could be demonstrated gravimetrically. Thus, evaporation of a 10 ml volume of the naturally contaminated red wine sample used for method optimization provided 287 mg of non-volatile matter. After C18 SPE clean-up this amount was reduced to 7 mg, indicating that most of the matrix compounds are of polar nature. The final MIP clean-up step yielded <1 mg of solid residue containing the analyte of interest, but also still a considerable amount of red colorants.

In the final sample extract, several non-matrix-derived fluorescence-active peaks were observed, providing evidence for template bleeding. Evidently, even after the rigorous washing protocol performed on the MIP material, there are still small amounts of the OTA-mimicking template (and decomposition products thereof) bound to the high affinity binding sites and become slowly released under SPE conditions. This finding is consistent with the observations made with other MIP materials [25] and emphasizes the need to employ analyte mimics as templates for imprinting, if the resultant MIPs are intended to be used for trace analysis applications [19]. Interestingly, the concentrations of MIP-related peaks were significantly higher for pre-conditioned MIP-cartridges stored in methanol for extended time periods. This observation provides evidence that template release from imprinted binding sites is a process of slow overall kinetics and may also be influenced by the swelling state of the polymeric matrix.

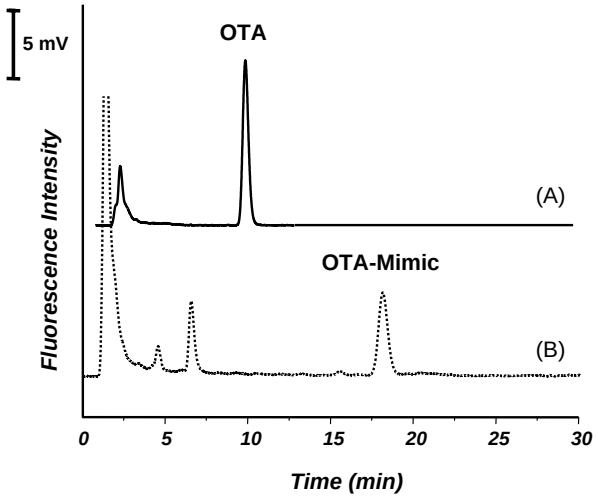

Fig. 3. Demonstration of template bleeding from the ochratoxin A-selective MIP materials. Trace A: chromatogram of an ochratoxin A standard (0.25 ng/ml). Trace B: chromatogram of the extract obtained from an unused MIP cartridge after washing with 4 ml methanol–acetic acid (8:2 (v/v). Chromatographic conditions: Purospher RP-C18 column (125 × 4 mm i.d., 5 μm); mobile phase: acetonitrile–water–acetic acid (40:60:2 (v/v/v)); flow rate: 1 ml/min; fluorescence detection: $\lambda_{ex} = 333$ nm, $\lambda_{em} = 460$ nm; column temperature: 25 °C; injected sample volume: 50 μl of the standard solution and the dried extract, reconstituted in 500 μl mobile phase.

The presence of the template (and template-derived compounds) and the residual matrix components in sample extracts was addressed by a careful optimization of the analytical RP-HPLC procedure used for OTA determination. The mobile phase conditions were optimized to avoid peak overlap between the background signal of the MIP and OTA. Fig. 3 shows the chromatographic traces of an OTA standard (trace A) and of a methanol–acetic acid extract from a MIP cartridge (trace B). Under optimized chromatographic conditions, the OTA peak is well separated from all MIP-derived contaminants, allowing unambiguous analyte identification and quantification.

Fig. 4 shows three chromatographic traces of a OTA spiked red wine (0.22 ng OTA/ml) used for method opti-

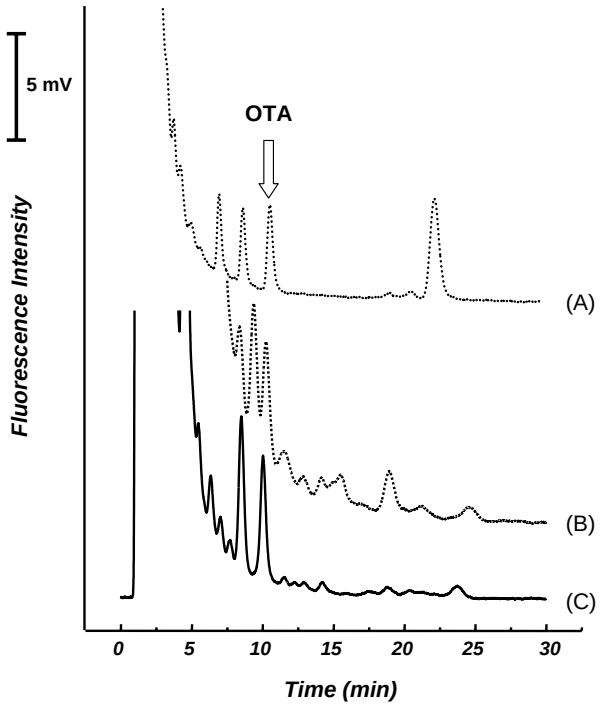

Fig. 4. Chromatographic traces of ochratoxin A-contaminated red wine (0.22 ng/ml) after different SPE steps. Trace A: red wine sample after clean-up with a MIP cartridge only (analyte recovery <70%). Trace B: after C18-based SPE clean-up. C: after combined C18-MIP SPE clean-up (analyte recovery >90%). For chromatographic conditions see Fig. 3.

mization at different levels of sample clean-up. Trace A shows the separation of the sample extract resulting from MIP-based clean-up only. The OTA signal is well separated from interfering matrix components and can be reliably quantified. However, as outlined above, OTA recoveries from MIP material alone were unacceptably low. Trace B represents the chromatogram of the extract resulting after the C18-SPE clean-up step. In this case, the chromatogram exhibits more matrix components than the MIP extract. In addition, the OTA signal is severely obscured by overlapping peaks, precluding any reliable signal integration. Trace C represents the chromatogram of the extract from the combined C18-MIP sample clean-up procedure. Apart from displaying fewer matrix components than the C18 SPE extract, the OTA signal appears to be well isolated from co-eluting matrix peaks, allowing accurate peak integration and quantification, respectively.

For preliminary validation of the performance characteristics of the developed SPE protocol, recovery experiments were conducted with a non-contaminated red wine spiked with 0.033–1.00 ng OTA/ml. These spiking levels were chosen with the intention to cover the most often reported OTA concentration range in contaminated red wines. The results of the recovery experiments are depicted in Fig. 5. The external and the "in-matrix" calibration curves constructed from OTA standards and spiked red wine samples after SPE, respectively, show excellent linearity (linear regression factor $R^2 = 0.999$). OTA recoveries calculated from these data are

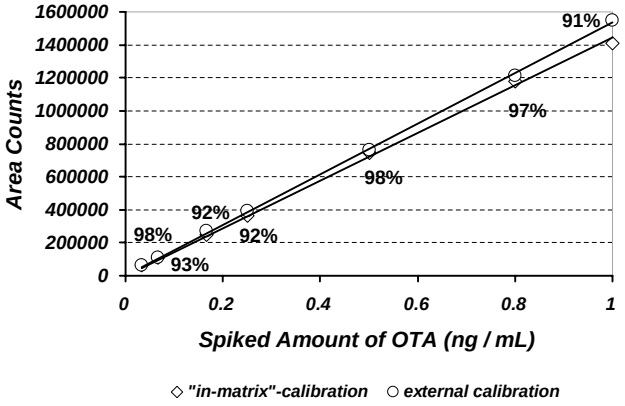

Fig. 5. External and "in-matrix" calibration curves for ochratoxin A. The "in-matrix" calibration curve is derived from spiked red wine (0.033–1.0 ng ochratoxin A/ml) after C18-MIP SPE clean-up. The numbers indicate the ochratoxin A recoveries achieved at different spiking levels.

>90% over the full investigated concentration range. The repeatability of OTA quantification was investigated by performing triplicate determinations at low (0.066 ng/ml) and medium (0.50 ng/ml) spiking levels, giving R.S.D.$_r$-values of 10.1 and 4.3%, respectively. Considering a signal-to-noise ratio of 3, the detection limit (LOD) was judged to be 0.01 ng OTA/ml. Consequently, the limit of quantification (LOQ) was 0.030 OTA ng/ml.

3.4. Determination of OTA in red wines

The developed analytical method was subsequently employed to OTA determination in various red wines. The bottled wines were purchased from a local supermarket and selected to sample products of different geographic origins and production years. The corresponding data are summarized in Table 1. OTA contamination could be detected for

Table 1
OTA contamination levels established with the developed analytical method involving a C18-MIP SPE protocol

Sample	Origin[a]	Production year[a]	OTA contamination[b]
W1	Australia	2001	n.d.[c]
W2	Burgenland, Austria	2001	n.d.
W3	Maule Valley, Chile	2000	n.d.
W4	California, USA	2002	n.d.
W5	Arniston Bay, South Africa	2002	n.d.
W6	Tuscany, Italy	2001	n.d.
W7	Friuli, Italy	2002	0.12 ± 0.001[d]
W8	South Tyrol, Italy	2002	0.20 ± 0.005[d]
W9	Tuscany, Italy	2002	0.22 ± 0.002[d]
W10	Emilia Romagna, Italy	2002	0.22 ± 0.004[d]
W11	Zagreb, Croatia	2002	0.30 ± 0.003[d]

[a] Information on the origin and production year was taken from the labels of the bottled wine samples.
[b] Concentrations are given in μg/l.
[c] n.d.: not detected, OTA level was below the detection limit (LOD < 0.01 μg/l).
[d] Results given are the mean of triplicate analyses.

5 out of 11 investigated red wines in concentrations ranging from 0.11 to 0.30 μg/l. The given OTA concentrations represent the mean values of triplicate determinations performed within a single working day. As basis for quantification the "in-matrix" calibration curve was employed, assuming that it does consider more accurately the impact of the still present matrix components on the analyte recoveries.

The determined OTA contamination levels in red wines correlate remarkably well with those reported by other groups, using primarily immunoaffinity SPE sample clean-up procedures [8–12,14,26]. In addition, our results are consistent with prior observations that red wines grown in warmer, high humidity climates are more likely to be contaminated with OTA [10,26].

3.5. Reusability of MIP SPE cartridges

One of the major advantages MIPs offer compared to immunoaffinity SPE materials is their high chemical robustness, providing the opportunity to clean and reactivate them under relatively harsh conditions for multiple use in SPE applications. Generally, multiple use of immunoaffinity columns is not encouraged by the producers as the exposure to protein-denaturating environments may lead to irreversible changes in the binding domains of the antibodies. Although reusability has been claimed for OTA-selective immunoaffinity SPE materials, complete regeneration of the selective binding properties was found to be challenging and was reported to require extended incubation (>10 h) in special buffers at low temperatures [10].

For the assessment of the reusability of our MIP material, we performed six consecutive sample clean-up cycles for a naturally contaminated red wine (0.22 ng OTA/ml) with a single MIP SPE cartridge. After each cycle, the MIP was subjected to a simple-to-perform regeneration procedure, consisting in washing the polymer consecutively with 10 ml of methanol–acetic acid and methanol. These relatively large amounts of solvents were used to avoid carry-over effects between the individual SPE cycles. Although the polymer showed indications of irreversible adsorption of dye components from the wine matrix, the performance of the MIP with respect to sample clean-up remained uncompromised, as is evident from the data presented in Fig. 6. Even after five reuse cycles, the recoveries were practically identical with those observed with unchallenged polymers, and the corresponding chromatograms neither did show any indication for the appearance of interfering matrix components nor a detectable increase of the baseline signal.

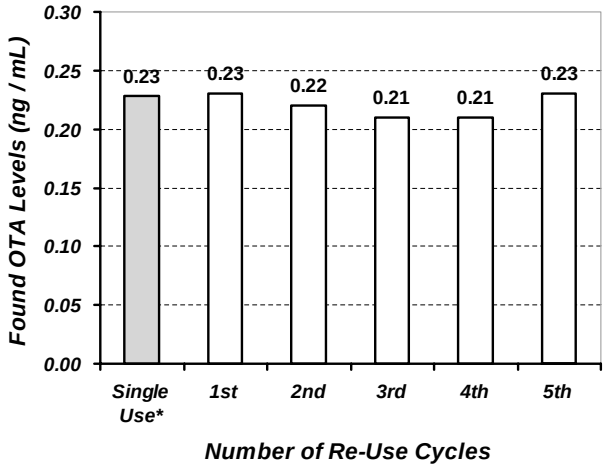

Fig. 6. Ochratoxin A concentrations found for a contaminated red wine sample (0.22 ng ochratoxin A/ml) after multiple SPE cycles on a single MIP cartridge. The "single use" reference represents the mean concentration of a triplicate determination performed with unused MIP SPE cartridges.

3.6. Assessment of the contribution of "specific binding sites" to selective OTA retention

To finalize our investigations, we tried to assess to what extent "analyte-specific imprinted binding sites" of the MIP are involved in the selective extraction of OTA from the red wine matrix. For this purpose, OTA spiked non-contaminated red wine samples (0.20, 0.50 and 1.00 ng OTA/ml) were processed following the developed SPE protocol, but employing the corresponding NIP instead of the MIP material.

Surprisingly, the sample clean-up effects and recoveries observed in these experiments were very similar to those achieved with the MIP material. Very clean chromatograms were observed and recoveries calculated from triplicate determinations were 92, 94 and 84% for samples with a 0.20, 0.50 and 1.00 ng OTA/ml spiking level, respectively. This observation suggests that, at least under the experimental conditions used, selective OTA binding must be effected primarily by the global electrostatic/hydrophobic interaction characteristics of the polymer matrix rather than by analyte-specific binding to imprinted domains.

Given the demonstrated OTA binding capabilities of the employed MIP material [17], this finding appears somewhat unexpected. A possible explanation for the "deactivation" of OTA-selective binding cavities of the MIP may be provided considering the relative concentrations as well as the potential structure similarity of the matrix components to OTA. As outlined above, after C18-SPE the extracts from 10 ml red wine typically contain less than 10 ng of OTA, but in presence of 7 mg (i.e. 10^5 times more) of other non-volatile matrix components. Among these, several compounds may show cross-selectivity to the imprinted binding sites with an affinity similar to or even higher than OTA. If these compounds are present in considerably higher concentration than OTA, the majority of the selective binding sites will become competitively blocked and thus inaccessible for analyte-specific interactions. Consequently, the retention mechanism for OTA will be governed by the most effective residual analyte–polymer interactions, i.e. by electrostatic attraction to the basic functionalities of the incorporated

functional monomer. As this type of interaction is also amply provided by the non-imprinted polymer, successful sample clean-up of OTA from red wine can also be achieved with the NIP material.

In view of this fact, searching sophisticated experimental conditions (e.g. pH, ionic strength, nature and composition of wash and elution media) under which the imprinted cavities maintain their functional integrity may appear pointless. However, one may speculate that "activation" of the yet unused target-specific binding properties of the MIP may enhance binding affinity to a level that allows to perform SPE of OTA from red wine without resorting to pre-purification on C18-materials. Currently ongoing studies along this line show first promising results, which will be communicated in due course.

4. Conclusions

A new analytical method for the quantification of ochratoxin A in red wines has been developed involving a two-dimensional SPE clean-up protocol on C18-silica and a target-selective MIP material. Although appreciable levels of sample clean-up of untreated red wine could be achieved with the MIP alone, the high concentrations of acidic matrix compounds led to unsatisfactory OTA recoveries. Removal of the interfering acidic polar compounds by C18-silica gave extracts that could be further cleaned by MIP-based SPE. This combined SPE protocol afforded samples suitable for sensitive RP-HPLC-fluorescence quantification of ochratoxin A. Method evaluation with spiked (0.033–1.0 ng OTA/ml) and commercial red wines provided recoveries >90% and $R.S.D._r$ <10%, with LOD and LOQ of 0.01 and 0.033 ng/ml.

Control SPE experiments with the corresponding NIP, however, gave similarly favorable sample clean-up effects and analyte recoveries. Thus, the observed clean-up effects are governed by the general adsorption characteristics of the polymers, providing a well-balanced combination of hydrophobic and electrostatic interaction increments, rather than analyte-specific interaction with imprinted binding sites. These findings strongly recommend to consider also the potential of NIPs for selective sample clean-up, as their global adsorption characteristics are similar to those of the corresponding MIPs, while their preparation avoids the use of templates and thus obviates problems associated with bleeding phenomena.

The results of the present study indicate that in terms of SPE applications currently available MIPs are far from being competitive to the excellent target-specific binding properties of immunoaffinity materials. The extreme heterogeneity of matrix components encountered in real-world samples renders selective analyte extraction at the trace level a formidable challenge. However, accepting protocols with added complexity, analytically useful sample clean-up may become feasible by combining MIP (or NIP)-based analyte recognition with additional fractionation steps on conventional SPE materials.

Future research in MIP technology must focus on novel strategies allowing to engineer two or even more dimensional molecular recognition properties into a single polymer, being fully compatible with the given sample environment. Only if this challenge is mastered, MIPs will be capable of addressing the multi-facetted requirements of effective SPE from real-world samples.

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