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Determination of ochratoxin A in red wines by multiple pulsed elutions from molecularly imprinted polypyrrole

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Abstract

Carbon nanotubes (CNTs) were used successfully to enhance the binding capacity of a molecularly imprinted polypyrrole (MIPPy)-modified stainless steel frit for micro-solid phase extraction (μSPE). Low back pressure of this new MIPPy/CNTs-modified stainless steel frit device made it feasible for sample preconcentration by using a syringe pump. For a 3-mL sample of red wine, preconcentration of ochratoxin A (OTA) was achieved within 6 min at a flow rate of 0.5 mL/min. In order to eliminate matrix interferences, two new approaches based on differential or multiple pulsed elution (PE) were developed for the determination of OTA in French (1999), Canadian (2003) and Bulgarian (2004) red wines. Elution of OTA from MIPPy/CNTs, for on-line coupling to high performance liquid chromatography (HPLC) analysis with fluorescence detection (FLD), was achieved by an ion-pairing mechanism. It was found that the % triethylamine (TEA) in the PE solution had a scalable effect on the OTA retention time, as influenced by the formation of three different ion-pairs: $[NH_4^+–OTA^{2-}–NH_4^+]$, $[NH_4^+–OTA^{2-}–TEA^+]$, and $[TEA^+–OTA^{2-}–TEA^+]$. Preliminary method validation showed that the Bulgarian red wine was best analyzed by MIPPy/CNTs–μSPP–PE–HPLC–FLD using 2% TEA. The reproducibility was 8.9% RSD and the limit of detection was 0.08 ppb ($S/N = 3$), which is more than adequate for handling the regulatory level of 2 ppb.

Keywords: Molecularly imprinted polypyrrole; Carbon nanotubes; Stainless steel frit; Micro-solid phase extraction; Ochratoxin A; Multiple pulsed elutions

1. Introduction

The occurrence of ochratoxin A (OTA) as a mycotoxin in red wine was first reported by Zimmerli and Dick (1995). Since then, the development of analytical methods generally involved extraction of OTA by liquid–liquid extraction, clean-up on an immunoaffinity column, and determination by high performance liquid chromatography (HPLC) with fluorescence detection (FLD). An accurate and precise method was developed for the determination of OTA in red, rosé, and white wine by Visconti, Pascale, and Centonze (1999). After simple dilution with water containing polyethylene glycol and $NaHCO_3$, red wine samples were cleaned-up using immunoaffinity columns and analyzed by reversed-phase HPLC with FLD. This method has been adopted by AOAC International and is being considered for adoption by the European Commission of Standardization (CEN) (Visconti, Pascale, & Centonze, 2001). Currently, AflaOchra™ immunoaffinity columns (Vicam Ltd, Watertown, MA, USA) is found suitable for simultaneous detection of aflatoxins and OTA (Visconti & De Girolamo, 2005). Our objective in the present work was focused on the development of a new sorbent for selective SPE that is capable of OTA preconcentration prior to HPLC–FLD analysis.

To date, attempts at designing specific affinity sites for the extraction of biochemical analytes have focused on the use of molecularly imprinted polymers (MIPs). The particles of a MIP material can be either packed into a microcolumn (Feng, Lai, Dabek-Zlotorzynska, & Sadeghi,

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2004; Zhou, Lai, & Miller, 2004; Wu, Lai, & Mayer, 2004) for solid phase extraction (SPE), or glued to the surface of a solid-phase micro-extraction (SPME) fiber (Liu, Zeng, & Fang, 2005). New fabrication techniques continue to be developed for molecularly imprinted solid phase extraction (MISPE) and molecularly imprinted solid phase micro-extraction (MISPME). Pyrrole (Py) can be oxidized to form a polymer that has intrinsic conducting properties (Sadki, Schottland, Brodie, & Sabouraud, 2000). The mechanical, electronic and chemical properties of polypyrrole (PPy) films have been well documented in the literature (Iroh & Su, 2002). PPy has been used in many successful applications ranging from ion-exchange extraction to hydrophobic extraction, based on the selective interaction between the polymer and the target analytes (Saito & Jinno, 2003). In addition, tunable properties of PPy, such as the oxidation/reduction equilibrium, can be exploited to control adsorption and desorption (Kim, Hur, Jung, & Kang, 2004). Because PPy can be prepared on a metal surface by electrodeposition from an aqueous solution of pyrrole, fine stainless steel wires (diameter = 127 μm) were selected as the supporting fiber (Lord et al., 2003). Recently, a MIP was prepared by electropolymerization onto a stainless steel frit, using OTA as the template, in order to make a micro solid phase preconcentration (μSPP) device (Yu, Krushkova, Lai, & Dabek-Zlotorzynska, 2005). The MIPPy-μSPP device afforded selective preconcentration of OTA from red wine samples, and maximum OTA binding was reached after a total loading of 3.2 ng OTA per frit.

Carbon nanotubes (CNTs) represent a new kind of carbon material that has been widely recognized as the quintessential nano-material since their discovery in 1991 (Iijima, 1991). In addition to the potential electrochemical applications of CNTs, their high electrical conductance, tensile strength, chemical stability, ultra-small size and poor solubility make them an attractive structural material for development of novel analytical devices. A uniform coating of PPy onto CNTs was electrochemically synthesized by several research groups (Chen et al., 2000; Chen et al., 2001; Chen et al., 2002). Nano-sized CNTs have been developed as the surface-enhancing component for PPy film to produce an open porous structure with high surface area (Gao, Dai, & Wallace, 2003). Compared to PPy, better thermal stability and higher charge delocalization properties are observed in the resulting PPy/CNTs (Hughes, Chen, Shaffer, Fray, & Windle, 2004). The electrochemical growth of CNTs-conducting polymer composites offers the ability to produce three-dimensional nano-structured films that combine the redox pseudo-capacitive charge storage mechanism of conducting polymers with the high surface area and conductivity of MWNTs (Gooding, 2005). Recently, (Han, Yuan, Shi, & Wei, 2005) electrochemically prepared a PPy/CNTs composite film onto stainless steel sheets by direct oxidation of pyrrole in 0.1 M aqueous solution of dodecylbenzene sulfonic acid (DBSA) containing a certain amount of CNTs.

Combining CNTs and the molecularly imprinted polypyrrole (MIPPy) technology dawned on us as a unique idea to overcome the limits of conventional MIP particles that could not be packed with sub-micron interstitial spaces. Recently, a MIP was prepared in our lab by electrochemical polymerization of pyrrole onto a stainless steel frit, using OTA as the template and CNTs as nano-structured fibers, to make a μSPP device (Yu et al., 2005). The ultra-high surface-to-volume ratio and nano-structured surface morphology were utilized to enhance the binding capacity of these new MIPPy/CNTs-modified stainless steel frits. After removing the OTA template with 1% triethylamine (TEA) in methanol, the μSPP device was coupled on-line to HPLC with FLD, using 20:80 v/v acetonitrile–ammonia buffer (20 mM NH_4Cl/NH_3, pH = 9.2) as the mobile phase. The use of MIPPy/CNTs-modified stainless steel frits for rapid extraction of trace OTA amounts was compared with traditional μSPE cartridges and MIPPy-modified stainless steel frits (without CNTs). Our MIPPy/CNTs–μSPP–HPLC–FLD results clearly demonstrated a significant enhancement of selective binding capacity for OTA at levels below 1 ppb (= 1 μg/L or 1 ng/mL) even in the presence of abundant red wine matrix components. Using a sample volume of 3 mL red wine for pre-concentration, it was possible to determine OTA down to a detection limit of 0.012 ppb, or a quantification limit of 0.041 ppb. (Aresta, Vatinno, Palmisano, & Zambonin, 2006) recently reported another cost-effective solid phase microextraction prior to LC–FLD for the determination of OTA in wine samples. A good detection limit has been demonstrated at the level of 0.07 ppb, which is well below the European regulatory level of 2 ppb. However, a long extract time of 1 h was needed for the SPME–LC–FLD method.

In the present work, the MIPPy/CNTs–μSPP–HPLC–FLD method was further developed for more accurate determination of OTA in red wines. Due to the high complexity of red wine matrices, multiple pulsed elutions (MuPEs) was adopted to eliminate some matrix interferences for accurate OTA determination. On-line coupling of μSPE (for sample purification and analyte pre-concentration during direct loading onto the injection valve) with MuPEs was successful in the HPLC–FLD determination of OTA in three red wines from France, Canada and Bulgaria.

2. Experimental

2.1. Reagents and materials

OTA, pyrrole (Py), ethylene glycol dimethacrylate (EGDMA), and triethylamine (TEA) were purchased from Sigma–Aldrich (Mississauga, ON, Canada). Tetrabutylammonium perchlorate (TBAP) was obtained from Fluka (Buchs, Switzerland). Acetonitrile and methanol were HPLC-grade solvents obtained from Caledon (Georgetown, ON, Canada). Distilled deionized water (DDW)

(18-MΩ cm) was supplied from a Millipore Milli-Q water system (Bedford, MD, USA). MWNTs with ~95% purity were obtained from Shenzhen Nanotech Port (Shenzhen, China).

OTA standard solutions were prepared according to the procedure established by González-Peñas et al. (2004). Three bottles of French (1999), Canadian (2003) and Bulgarian (2004) red wine were purchased from a store in Ottawa.

2.2. Preparation of MIPPy/CNTs-modified stainless steel frits

MIPPy/CNTs composite films were grown using an electrochemical method in which MIPPy and CNTs were simultaneously deposited on a stainless steel frit with a pore size of 0.5 μm and an active bed volume of 1.5 μL (Upchurch Scientific, Oak Harbor, WA, USA). The concentrations of Py, TBAP and EGDMA were 0.1 M, 0.1 M and 0.2 M, respectively, in the pre-polymerization mixture with acetonitrile as the solvent. OTA (75 μM) and 0.02–0.4% by weight of suspended CNTs were present when Py was electropolymerized onto the stainless steel frit serving as the working electrode. A platinum counter electrode and an Ag/AgCl reference electrode were used to complete a three-electrode setup for the electropolymerization in a potentiostatic mode. A Stoneheart BC 1200 high-performance potentiostat (Madison, CT, USA) was used to apply a constant potential of +0.85 V on the frit. The applied potential was switched off when MIPPy/CNTs with a dark brown color was observed. Similarly, PPy/CNTs-modified stainless steel frits were prepared, in the absence of 75 μM OTA, to serve as a control for comparison. More details of the instrumentation for electrodeposition of MIPPy have been described in a separate report (Yu et al., 2005).

2.3. MIPPy/CNTs–μSPP–HPLC determination of OTA

Our method development of MIPPy/CNTs–μSPP–HPLC for the extraction and determination of OTA from red wine samples was described in a separate report (Yu & Lai, 2006). The major instrumentation is briefly as follows. The MIPPy/CNTs–μSPP–HPLC consisted of a solvent pump (Shimadzu LC-6A, Kyoto, Japan), injector valve (Valco Cheminert VIGI C2XL, Houston, TX, USA) equipped with a 100-μL sample loop, column (Phenomenex polymerX RP-1, pH stability 0–14, 5 μm, 250 mm × 4.6 mm, Torrance, CA, USA), fluorescence detector (Perkin–Elmer LC 240, Boston, MA, USA), an integrator (Spectra Physics 42900, San Jose, CA, USA) and data acquisition system (PeakSimple, Torrance, CA, USA). A sample (0.1–3.0 mL) of OTA standard solution (0.1–1.0 ppb), red wine, or spiked red wine was pumped through the MIPPy/CNTs-modified stainless steel frit device from a syringe. All samples were acidified with 1 M HCl (1% by volume) to provide nearly quantitative recovery of OTA for μSPP. After washing the device with 100 μL of DDW to eliminate HCl and polar organics, the pre-concentrated OTA was pulse-eluted with 100 μL of 1% TEA in MeOH. The mobile phase transported the extracted analyte from the MIPPy/CNTs-modified frit to the HPLC column. For HPLC analysis of the eluate, 20:80 (v/v) acetonitrile–ammonia buffer (20 mM NH_4Cl/NH_3, pH = 9.2) was used as the mobile phase at a flow rate of 1 mL/min. Fluorescence detection of OTA was performed at optimal excitation and emission wavelengths of 380 nm and 440 nm. The % recovery of OTA by the MIPPy/CNTs device and PPy/CNTs control were calculated from OTA peak areas measured in PPy/CNTs–μSPP–HPLC analysis versus direct HPLC analysis of the same 1.0-ppb OTA standard solution. After each μSPP–HPLC analysis, 1% TEA in methanol was used in a 200-μL aliquot for the regeneration of MIPPy/CNTs. The MIPPy/CNTs was finally washed with 100 μL of 1 M HCl (1% by volume) for surface reconditioning.

After MIPPy/CNTs–μSPP–HPLC analysis of the red wine, a sample was spiked with 1.0 ppb OTA for re-analysis to confirm the adequate chromatographic separation between OTA and all wine matrix components. This new OTA peak area was used in a standard addition calculation to determine the original concentration of OTA in the red wine.

2.4. Measurement of binding capacity by frontal chromatography

The binding capacity of the modified frits to retain OTA was measured by frontal chromatography similar to Baggiani, Giovannoli, Anfossi, and Tozzi (2001). Briefly, one pump was used to elute an acidic mobile phase (water–acetonitrile–acetic acid, 98:2:1, v/v/v) over the non-modified frit or modified frit. When the fluorescence signal reach the steady baseline, same mobile phase containing 50 ppb OTA was switched over by another pump at a flow rate of 0.5 mL/min. The breakthrough volume was measured by multiplying the flow rate by the frontal time (when fluorescence signal reach half height of the 50 ppb OTA signal). Correction for dead volumes (from pump to frit) was made. Any difference in breakthrough volume was determined between the non-modified frit and modified frit. Note that the fluorescence detection condition was adjusted to 330 nm for excitation and 460 for emission to attain a better sensitivity for OTA in the acidic medium. After each frontal chromatography run the modified frit was washed extensively with 1% TEA/methanol.

3. Results and discussion

3.1. Preparation of MIPPy/CNTs-modified stainless steel frits

When growing composite films of CNTs and MIPPy in the present work, a polymerization electrolyte (0.1 M

TBAP) was used in conjunction with 0.1 M pyrrole monomer and 0.5 mg/mL of suspended CNTs in acetonitrile. There was no need to oxidize the suspended CNTs (using a 3:1 v/v of sulfuric acid and nitric acid refluxed at 130 °C for 1 h) for surface functionalization. Electrodeposition of MIPPy with CNTs enabled the nanotubes to be immobilized easily in the pores of the stainless steel frit (acting as the working electrode). The immobilized MIPPy/CNTs showed good mechanical stability for μSPP applications (even under a high pressure flow of HPLC mobile phase). SEM analysis revealed that the CNTs, with a diameter ranging from 30 to 80 nm, are randomly distributed in the frit pores. As shown in Fig. 1, most of the CNTs are in the form of small bundles or individual tubes. They were coated with MIPPy, providing surfaces in close proximity that were rapidly accessible by OTA molecules. This large total surface area (all contained within a very compact size of the frit with an active bed volume of 1.5 μL only) was believed to be very beneficial for μSPP performance. A standard solution of OTA (50 ppb) was used in frontal chromatography to determine the binding capacity (2270 ± 5 ng OTA). This frit had an internal volume that facilitated efficient desorption of the OTA analyte into a small eluate plug (100 μL) for HPLC analysis. Note that the molecular recognition and binding properties of OTA–MIPPy were superior to those of the non-imprinted control polymer, as detailed in our previous report (Yu et al., 2005).

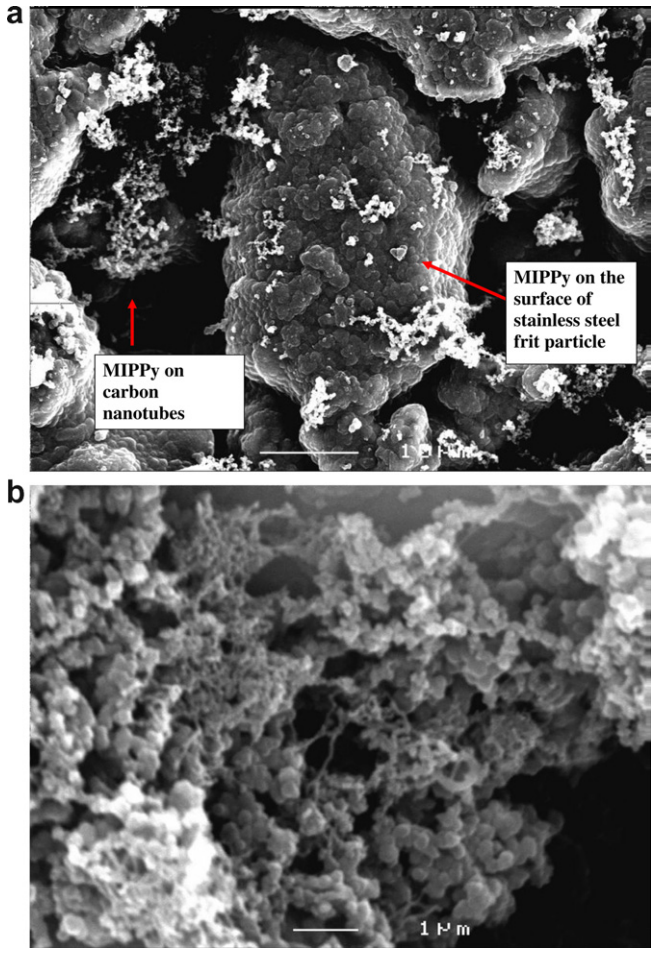

Fig. 1. (a) SEM image of MIPPy on the surface of stainless steel frit particles and carbon nanotubes and (b) SEM image of MIPPy on CNTs.

3.2. MIPPy/CNTs–μSPP–PE–HPLC–FLD analysis of red wine

One common problem in the HPLC–FLD determination of OTA would be the interferences from fluorescent matrix components (such as flavonoids) that are usually present in red wines. If these components can be selectively removed from a red wine sample before HPLC analysis, the accuracy of OTA determination would be greatly improved. Unfortunately, sample cleanup by conventional SPE using a C18 cartridge tended to extract both OTA and the interferences indiscriminately. To enhance the binding differences in μSPP between OTA and red wine matrix components, a MIPPy/CNTs-modified stainless steel frit was fabricated based on the molecular imprinting technique. High selectivity was achieved by using this frit in the sample loop of the injection valve for on-line cleanup of red wine samples prior to HPLC analysis. In detail, each 100 μL of sample was manually loaded from a syringe to go through the frit over approximately 20 s. Pulsed elution (PE) with 100 μL of 2% TEA was then performed in accordance with a previous report. HPLC analysis of the eluate followed, using 20:80 v/v acetonitrile–ammonia buffer (20 mM NH_4Cl/NH_3) as the mobile phase at an optimum pH of 9.2 and a flow rate of 1 mL/min (Yu et al., 2005). From the chromatogram shown in Fig. 2, it can be seen that the interference of major wine matrix components with 1 ppb of spiked OTA ($(t_R = 8.8 \pm 0.1$ min)) became practically negligible. The Bulgarian wine showed a different profile from the French and Canadian wines, presumably due to the fact that these wines were made form different grapes, under different

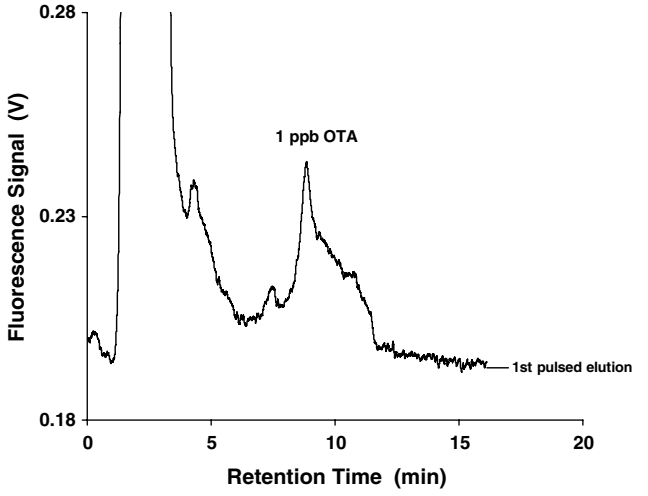

Fig. 2. Analysis of 100 μL Ontario red wine (spiked at 1 ppb OTA) using MIPPy/CNT-modified stainless steel frit for μSPE on-line prior to PE with 1% TEA in methanol for HPLC–FLD.

growing conditions, and perhaps in different wine making styles. When the frit was calibrated using standard OTA solutions, a linear response and constant recovery (nearly 100%) were obtained. However, it was only possible to determine OTA down to a detection limit ($S/N = 3$) of 0.08 ppb, or a quantification limit ($S/N = 10$) of 0.27 ppb. One obvious limitation was OTA peak broadening due to the PE chemistry.

3.3. Investigation of pulsed elution chemistry

Standard solutions of 10 ppb OTA were prepared in different solvents (containing 0–3% TEA) for a systematic investigation of the PE chemistry. These solutions were first analyzed by direct HPLC, and the observed OTA retention behaviors would be similar to those obtained from actual PE (of OTA from the MIPPy/CNTs-modified frit) with the same solvents. As shown in Fig. 3, the OTA retention times turned out to be very different. More importantly, the best OTA peak height and shape were obtained for OTA prepared with 1% TEA in the mobile phase (retention time = 7.5 min). Inferior peak height and shape were observed for OTA prepared with either 0% TEA in the mobile phase (retention time = 6.5 min) or with 3% TEA in the mobile phase (retention time = 9.5 min). Apparently, three different ion-pairs were formed in these solutions, respectively: $[NH_4^+–OTA^{2-}–TEA^+]$, $[NH_4^+–OTA^{2-}–NH_4^+]$, and $[TEA^+–OTA^{2-}–TEA^+]$. These results indicated that it would be best to use 1% TEA in the mobile phase for PE in all subsequent red wine analyses by MIPPy/CNTs–μSPP–PE–HPLC–FLD.

3.4. Differential pulsed elution and doubled pulsed elutions

In our study, one French red wine and one Canadian red wine did not pose any problems with the OTA determination by MIPPy/CNTs–μSPP–PE–HPLC–FLD (using 1% TEA in the mobile phase for PE). However, difficulty was encountered when we tried to analyze a Bulgarian red wine for OTA, as illustrated in Fig. 4. Obviously, analytical selectivity must be enhanced for the elimination of all interference due to matrix components in the Bulgarian red wine. One plausible approach was differential pulsed elution (DPE), which had been described in our previous reports (Mullett & Lai, 1999a, 1999b; Mullett, Lai, & Sellergren, 1999; Mullett, Dirie, Lai, Guo, & He, 2000; Lai & Wu, 2003; Feng et al., 2004). DPE involved essentially an intermediate wash step that would remove much of the wine matrix components from the MIPPy/CNTs-modified stainless steel frit. Several organic solvents were evaluated for their DPE effectiveness, by using 100 μL of each solvent to wash the frit before PE–HPLC–FLD analysis. Ideally, DPE must eliminate all the wine matrix components quantitatively while keeping a good amount of OTA in the frit for subsequent HPLC–FLD detection. As summarized in Fig. 5, acetonitrile was bad for eluting the bound OTA. Acetonitrile/mobile phase (1:1 v/v) was good for eluting all the peaks, including OTA. THF was bad for eluting matrix component 1 and OTA, and not capable for eliminating matrix component 3 (retention time = 2.4 min) in the frit. Dichloromethane and methanol were good for eluting OTA, but bad for matrix components 1 and 3.

In an alternate approach, 1% TEA in the mobile phase was used to perform DPE. The top chromatogram in Fig. 6a resulted from the MIPPy/CNTs–μSPP–PE–HPLC–FLD analysis of a Bulgarian red wine that had been spiked with 10 ppb OTA. The second chromatogram was obtained from the MIPPy/CNTs–μSPP–PE–HPLC–FLD analysis of the original Bulgarian red wine. These results clearly demonstrated a significant overlap between a wine matrix component peak (retention time = 6.7 min) and the OTA peak (retention time = 7.0 min). When a 2nd PE was performed with 1% TEA in the mobile phase,

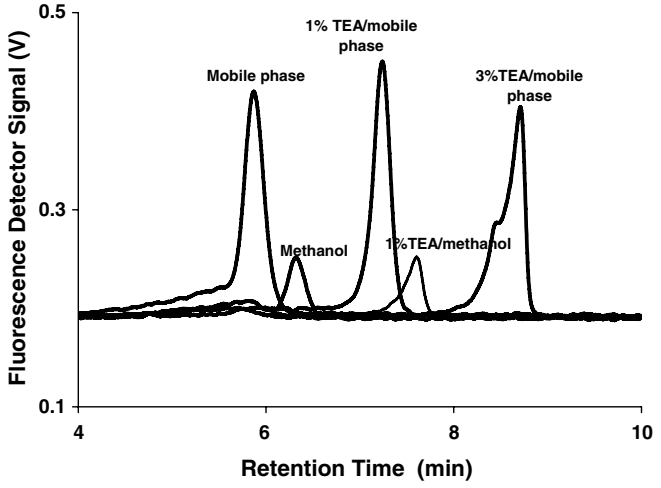

Fig. 3. Direct HPLC analysis of 10 ppb OTA standard solutions prepared in different solvents for investigation of the PE chemistry. TEA = triethylamine, and mobile phase = 20:80 v/v acetonitrile/ammonia buffer (20 mM NH_4Cl/NH_3, pH = 9.2).

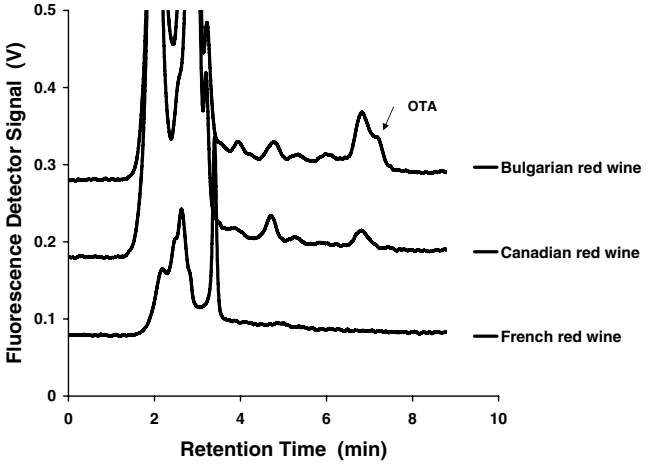

Fig. 4. MIPPy/CNT–μSPE–PE–HPLC–FLD analyses of three red wines from France, Ontario (Canada) and Bulgaria, using 1% TEA in mobile phase for PE.

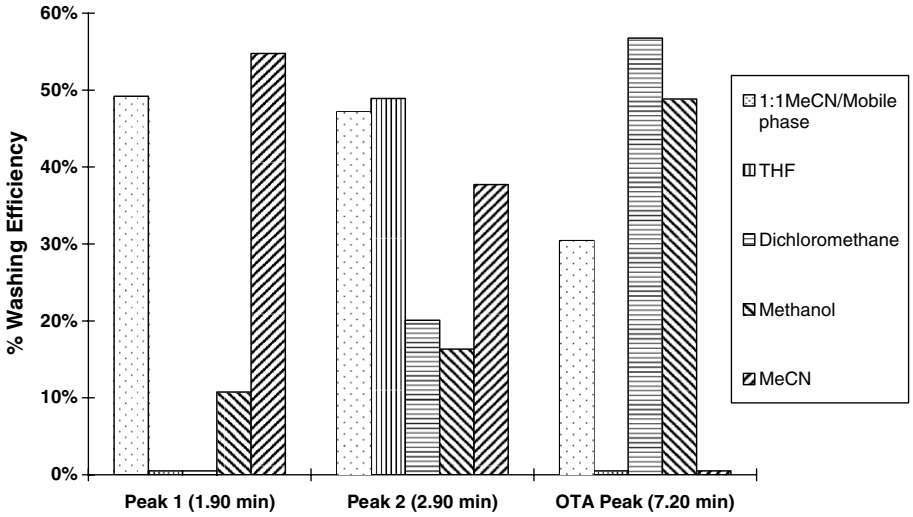

Fig. 5. Evaluation of organic solvents for their DPE effectiveness on OTA, by using 100 μL of each solvent for washing the MIPPy/CNT-modified stainless steel frit prior to PE–HPLC–FLD analysis.

the third chromatogram was obtained. This new result looked promising because the wine matrix component and OTA peaks were totally resolved down to the baseline. It was readily explained by the fact that the weakly bound wine matrix component had been largely eluted during the 1st PE, while nearly half of the strongly bound OTA was kept on the MIPPy/CNTs-modified stainless steel frit. Further data analysis was carried out to subtract the second chromatogram from the first one, yielding a blank-subtracted chromatogram that revealed essentially the OTA peak. As can be seen in Fig. 6b, its signal-to-noise ratio is slightly better than the chromatogram obtained from the 2nd PE. This comparison clearly suggests that the 2nd PE provided a good opportunity to determine OTA, after using the 1st PE (essentially as a DPE) for elimination of the wine matrix components. This new approach, now called double pulsed elutions (DoPEs), is uniquely based on both the strong molecular recognition property of MIPPy and a high OTA binding capacity of the MIPPy/CNTs-modified stainless steel frit.

One limitation of the above DoPEs approach (using 1% TEA in the mobile phase) was a small blank signal due to OTA bleeding from the MIPPy/CNTs-modified stainless steel frit. In search of a better method for red wine analysis by PE–HPLC–FLD, the PE chemistry was further optimized. A range of 0–3%TEA in the mobile phase was evaluated for use in the 1st PE, and HPLC chromatograms thus obtained from the analysis of a 10-ppb OTA standard solution are shown in Fig. 7. These new results looked very interesting in that the % TEA contained in the PE solution had a scalable effect on the OTA retention time. Indeed, there was a linear dependency. Judging from the largest peak area and an optimal retention time for OTA, all subsequent experiments in our Bulgarian red wine analysis used 2% TEA in the mobile phase for PE.

3.5. Multiple pulsed elutions (MuPEs) with 2% TEA

In Bulgarian red wine analysis using 2% TEA in the mobile phase for pulsed elution, the results showed that the OTA peak (at 5 ppb and 10 ppb in two spiked wine samples) was successfully separated from the earlier-observed interference peak (retention time = 6.9 min). However, when OTA was spiked up to the 20-ppb level, the analyte peak shifted to an earlier retention time to cause partial overlap with the matrix component peak in front. This suggested that the retention time for OTA was influenced by the relative concentration of TEA in the PE solution (which dictates the shift of equilibrium among the three ion-pairs: $[NH_4^+–OTA^{2-}–NH_4^+] \leftrightarrow [NH_4^+–OTA^{2-}–TEA^+] \leftrightarrow [TEA^+–OTA^{2-}–TEA^+]$). Note that this observation is not really bad because 20 ppb OTA is a very high level that is rarely found in red wines. The Bulgarian red wine was analyzed, before and after spiking at 10 ppb OTA, by MIPPy/CNTs–μSPP–PE–HPLC–FLD using 2% TEA in the mobile phase. Fig. 8 shows the chromatograms obtained from both the 1st and 2nd PEs. By the standard additions method, an original OTA concentration of 0.9 ± 0.1 ppb OTA was calculated from the 1st PE peak area (after subtraction of the small OTA bleeding background). Moreover, the OTA peak areas obtained from the 2nd PE, the 3rd PE, and all the three PEs were used for repeating the standard additions calculation. These calculation results (for the original concentration of OTA in the Bulgarian red wine) are summarized in Table 1. An OTA concentration of 0.89 ppb was determined to be a conservative level in this difficult-to-analyze Bulgarian red wine. Standard addition curves for OTA determination in Bulgarian red wine by MIPPy/CNT–μSPE–PE–HPLC–FLD using peak areas obtained from multiple PEs was shown in Fig. 9. Compared to the existing non-PE methods, multiple PEs showed not only good linearity, but also high sensitivity when the summation of three PE peaks was used

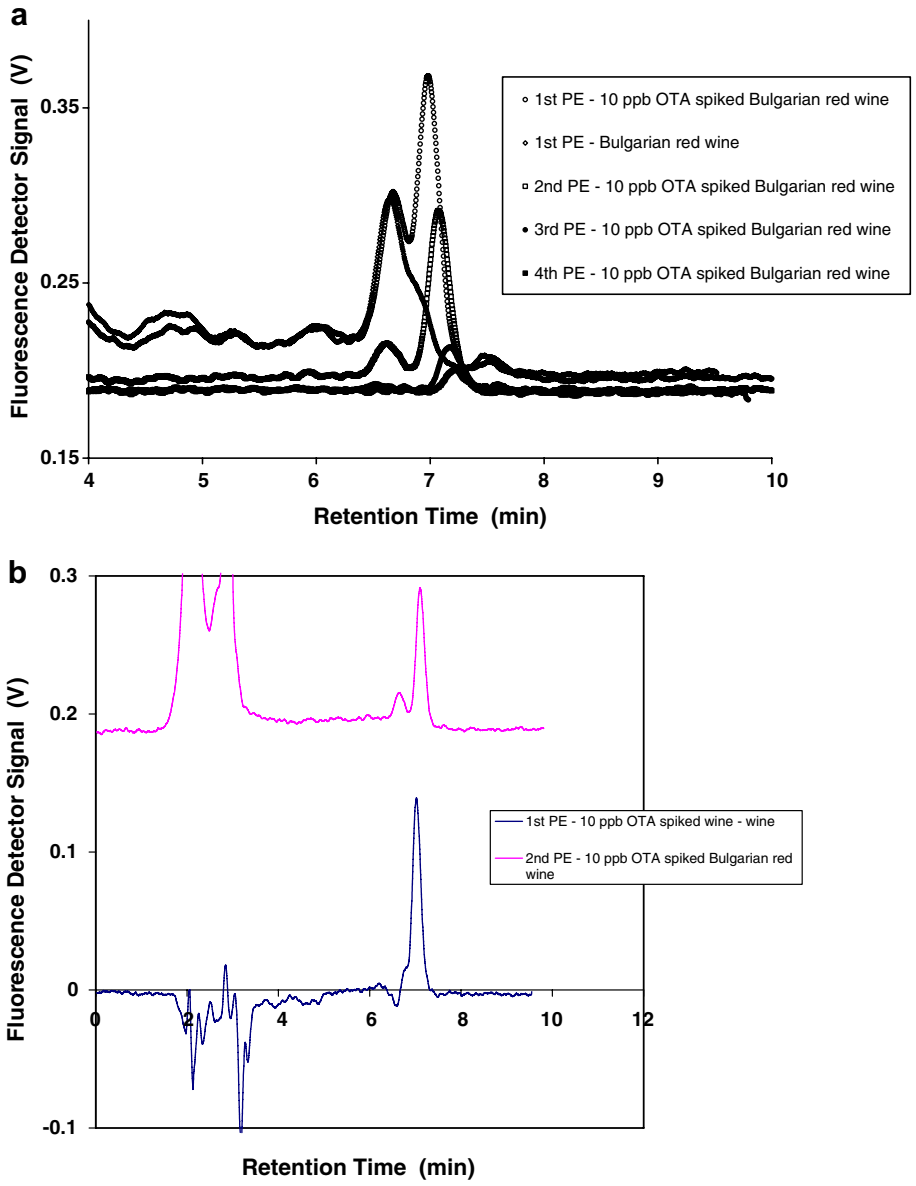

Fig. 6. (a) Analysis of Bulgarian red wine, before and after spiking at 10 ppb OTA, by MIPPy/CNT–μSPE–PE–HPLC–FLD using 1% TEA in mobile phase for multiple PE. Mobile phase = 20:80 v/v acetonitrile/ammonia buffer (20 mM NH_4Cl/NH_3, pH = 9.2) and (b) comparison of a blank-subtracted chromatogram (bottom) with the chromatogram obtained from 2nd PE (top).

for calibration. The reproducibility is 8.9 %RSD and the limit of detection was 0.08 ppb ($S/N = 3$), which is more than adequate for handling the regulatory level of 2 ppb.

3.6. Elimination of template bleeding and carry over problems

It is commonly recognized that direct imprinting with the target analyte as template molecule poses a template bleeding problem. Nonetheless, this issue was recently addressed by Turner et al. (2004). It was found that a continuous leaching of OTA template at the scale of ~50–100 ppb only occurred when vinylimidazole and diethylamino ethyl methacrylate were used as functional monomers. For MIPs prepared by using methacrylic acid/acrylamide as functional monomer, OTA leaching was negligible after extensive washing. In our development of pyrrole-based MIPs, OTA template bleeding from the frit was monitored over storage time periods ranging from hours to days. Usually, a couple of pulsed elutions readily cleaned out any OTA leakage prior to using the frit for MIPPy/CNTs–μSPP–PE–HPLC analysis of a new red wine sample over the next 30 min. Hence, it was unnecessary to adapt the concept of analyte mimicking (with OTB or OTC) as the direct imprinting procedure guaranteed the best possible recognition sites.

To investigate the problem of OTA carry over after each MIPPy/CNTs–μSPP–PE–HPLC analysis, MuPEs were

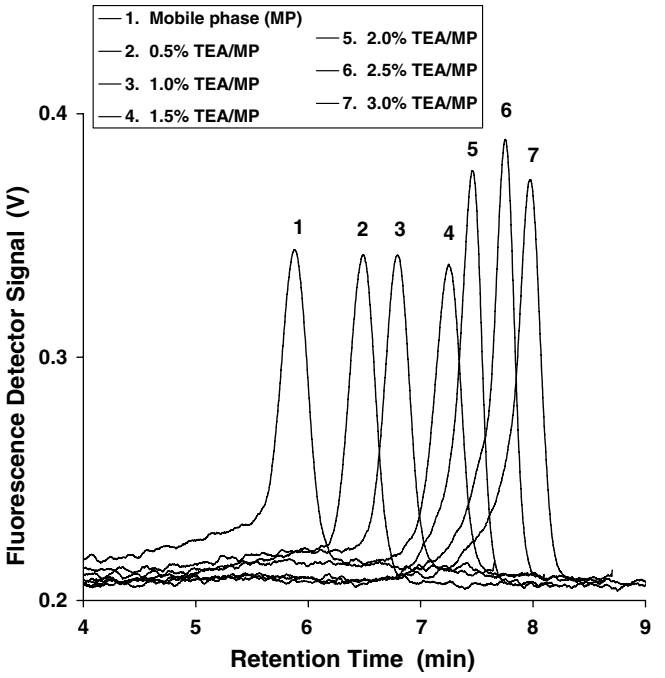

Fig. 7. HPLC chromatograms from analysis of 10-ppb OTA standard solution by MIPPy/CNT–μSPE–PE–HPLC–FLD using 0%-3%TEA in mobile phase for 1st PE.

conducted after loading a standard solution of 1 ppb OTA onto the frit. It was found that the 2nd PE (with 100 μL of 1% TEA in methanol) yielded an OTA peak area that was as much as 25% of that obtained in the first pulsed elution. Fortunately, the 3rd PE yielded an OTA peak area only as little as 1% of the 1st PE. These observations signified that a 2nd PE must always be made to clean out any residual OTA from the MIPPy/CNTs-modified stainless steel frit. After that, even without making a 3rd PE, the extent of carryover would be equivalent to 1% of 1 ppb (= 0.01 ppb) OTA. Such a low analyte concentration was already at the detection limit of MIPPy/CNTs–μSPP–PE–HPLC for red wine analysis. Alternatively, a single PE with 300 μL (of 1% TEA) would be sufficient to clean out the frit. In any case, the mycotoxin can be completely removed with no significant carryover from one SPP cycle to the next.

Finally, the new MIPPy/CNTs-modified stainless steel frit seemed to be stable and robust under ordinary lab operation conditions. Our observations showed that its

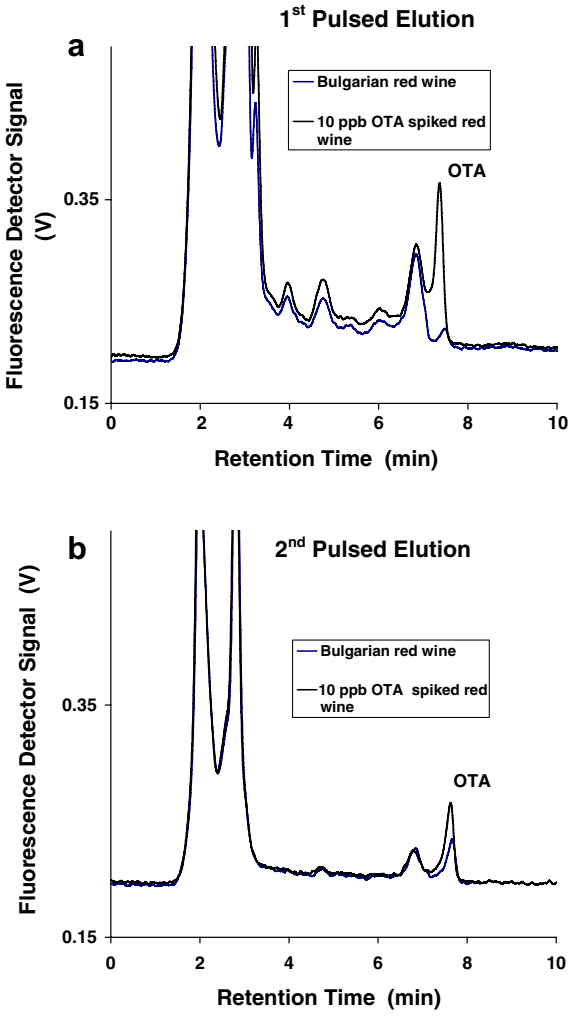

Fig. 8. Analysis of Bulgarian red wine, before and after spiking at 10 ppb OTA, by MIPPy/CNT–μSPE–PE–HPLC–FLD using 2% TEA in mobile phase for multiple PEs: (a) 1st PE chromatogram, and (b) 2nd PE chromatogram.

binding capacity remained relatively constant over the first 15 days, but decreased to about 80% of the original value after being used for more than 80 times. Such good long-term stability can be attributed to the stability of cross-linking PPy with EGDMA. Should physical pore blocking eventually happen, the frit can easily be replaced by a new one (which costs $3 each to purchase from a scientific company, followed by modification with MIPPy/CNTs in our lab).

Table 1
Determination of OTA in Bulgarian red wine by measuring the OTA peak areas obtained from the 1st PE, 2nd PE, 3rd PE, as well as the summation of all three PEs

OTA peak area (after blank subtraction)	Bulgarian red wine	5 ppb Spiked	10 ppb Spiked	20 ppb Spiked	OTA concentration in original Bulgarian red wine (ppb)
1st PE	13,100	259,649	396,748	786,995	0.9 ± 0.3
2nd PE	3875	92,115	105,878	222,854	1.5 ± 0.3
3rd PE	7195	55,552	39,161	107,860	1.8 ± 0.4
1st + 2nd + 3rd PEs	24,170	40,7316	541,787	1,117,709	1.2 ± 0.3

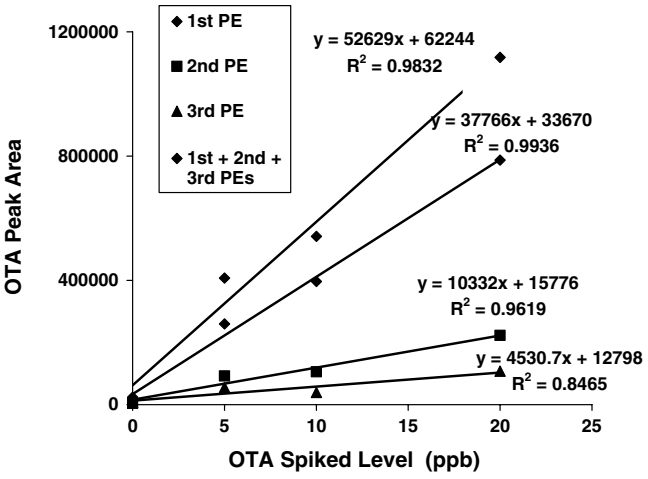

Fig. 9. Standard addition curves for OTA determination in Bulgarian red wine by MIPPy/CNT–μSPE–PE–HPLC–FLD, using peak areas obtained from multiple PEs.

4. Conclusions

The MIPPy/CNTs-modified stainless steel frit was utilized as a μSPP device for on-line PE–HPLC–FLD determination of OTA in three red wines of different origins. The frit possessed a high binding capacity that better handled the rich components in complex wine matrices. Reasonably low back pressure was observed for this new MIPPy/CNTs-modified stainless steel frit device when a syringe pump was used for sample preconcentration. For a 3-mL sample of red wine, preconcentration was achieved within 6 min at a flow rate of 0.5 mL/min. With LOD of 0.08 ppb and a total analysis time of 30 min for one sample, our method was very competitive as compared to the SPME method developed by Aresta et al. (Aresta et al., 2006). Two new techniques based on DoPEs and MuPEs were developed successfully for the elimination of wine matrix interferences. The Bulgarian red wine was best analyzed by MIPPy/CNTs–μSPP–PE–HPLC–FLD using 2% TEA in the PE solution. A conservative OTA concentration of 0.9 ppb was finally determined in this difficult-to-analyze wine.

Current efforts in our lab are focused on how to control the three-dimensional network of CNTs in various stainless steel devices (such as a syringe needle for biomedical analysis using an autosampler). As PPy is an inert extraction phase that is biocompatible, MIPPy/CNTs-modified stainless steel frits can potentially be used for pharmacokinetic, clinical, forensic, food safety, and environmental health analyses. Future applications would see better quality and cost-effectiveness in these industrial applications.

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