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**Development of Polypyrrole Films by Electrochemical Imprinting for
Molecular Recognition of Ochratoxin A
-Applications in Surface Plasmon Resonance Sensor and Micro Solid
Phase Extraction**

by
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A dissertation submitted to the Faculty of Graduate Studies in partial fulfillment of the
requirements for the degree of Doctor of Philosophy

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Abstract

This thesis describes research on a new electrochemical imprinting technique, in which pyrrole was used as a functional monomer for the preparation of molecularly imprinted polymers (MIPs). The molecularly imprinted polypyrrole (MIPPy) was electrochemically deposited onto the substrate from a pre-polymerization mixture containing ochratoxin A (OTA) as the template molecule. After removal of OTA templates, the binding properties of MIPPy were characterized by surface plasmon resonance (SPR), and high performance liquid chromatography (HPLC). Applications of the MIPPy were to serve as the sensing element for SPR and as the selective sorbent coating the pores of a stainless steel frit to make a novel micro solid phase extraction (μ SPE) device.

For sensor development, a MIPPy film was synthesized *in situ* onto a miniaturized SPR sensor. Film growth of MIPPy and subsequent binding event were monitored by the SPR angle (θ_r). Sensor performance was investigated by running OTA standard solutions into the integrated 20 μ L flow cell. After 300 seconds, nonlinear regression was used to determine the maximum binding signal ($\Delta\theta_{300}$). SPReeta results showed that $\Delta\theta_{300}$ was measurable for OTA concentrations down to 0.05 ppm. No significant binding of the wheat extract with MIPPy was observed when

acetonitrile/water (1:1 v/v) was used as the sample loading mobile phase.

To utilize the selectivity, the MIPPy was prepared onto a stainless steel frit to make a novel μ SPE device. Compared to non-imprinted polypyrrole (Ppy), MIPPy enhanced the selective binding of OTA. MIPPy- μ SPE-HPLC results clearly demonstrated that the MIPPy- μ SPE device afforded selective preconcentration of OTA from red wine samples, at OTA concentration levels as low as 0.05 ppb, prior to HPLC analysis. Enhancement of the total number of specific binding sites of MIPPy-on-a-frit was achieved by incorporating carbon nanotubes (CNTs) as nano fibers. The results clearly demonstrated a significant enhancement of selective binding capacity for OTA at sub-ppb levels 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 12 ppt, or a quantification limit of 41 ppt.

Compared with conventional preparation of MIP bulks, the electrochemical preparation of MIPPy for sensor or SPE applications is greatly simplified and attains satisfactory performance.

Publications and Presentations

Refereed Journals

1. Jorn C.C. Yu and Edward P.C. Lai, "Ion-Pairing HPLC Analysis of Red Wines by Multiple Pulsed Elutions of Ochratoxin A from Molecularly Imprinted Polypyrrole Modified Stainless Steel Frit with Carbon Nanotubes", *Journal of Chromatography A* (Submitted, 21 November 2005).
2. Jorn C.C. Yu and Edward P.C. Lai, "Molecularly Imprinted Polypyrrole Modified Carbon Nanotubes on Stainless Steel Frit for Selective Micro Solid Phase Pre-concentration of Ochratoxin A", *Reactive & Functional Polymers*, accepted (October 15, 2005).
3. Jorn C.C. Yu, Svetla Krushkova, Edward P.C. Lai and Ewa Dabek-Zlotorzynska, "Molecularly Imprinted Polypyrrole Modified Stainless Steel Frits for Selective Solid Phase Pre-concentration of Ochratoxin A", *Analytical and Bioanalytical Chemistry* 382 (2005) 1534-1540.
4. Jorn C.C. Yu and Edward P.C. Lai, "Interaction of Ochratoxin A with Molecularly Imprinted Polypyrrole Film on Surface Plasmon Resonance Sensor", *Reactive & Functional Polymers* 63 (2005) 171-176.
5. Jorn C.C. Yu and Edward P.C. Lai, "Polypyrrole Modified Stainless Steel Frits for On-line Micro Solid Phase Extraction of Ochratoxin A", *Analytical and Bioanalytical Chemistry* 381 (2005) 948-952.
6. Jorn C.C. Yu, Edward P.C. Lai, Susan Sadeghi, Surface Plasmon Resonance Sensor for Hg(II) Detection by Binding Interactions with Polypyrrole and 2-Mercaptobenzothiazole, *Sensors & Actuators B: Chemical*, 101 (2004) 236-241.
7. Jorn C.C. Yu, Edward P.C. Lai, Polypyrrole Film on Miniaturized Surface Plasmon Resonance Sensor for Ochratoxin A Detection, *Synthetic Metals*, 143 3 (2004) 253-258.

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3. J.C.C. Yu, S. Krushkova, E.P.C. Lai and E. Dabek-Zlotorzynska, "Molecularly Imprinted Polypyrrole Modified Stainless Steel Frits for Selective Micro Solid Phase Preconcentration", poster # 37, 28th International Symposium on Capillary Chromatography and Electrophoresis, Las Vegas, Nevada, May 22-25, 2005.
4. T. Lu, E.P.C. Lai, J.C.C. Yu, E. Dabek-Zlotorzynska, "Rapid Analysis for Flavonoids in Bee Pollens by Capillary Electrophoresis", poster # 110, 14th International Symposium on Capillary Electroseparation Techniques, Rome, Italy, September 12-15, 2004.
5. Edward P.C. Lai, Jorn C.C. Yu, Simon N.S. Zhou, Molecularly Imprinted Polymers for Mycotoxin Analysis by Surface Plasmon Resonance Sensor, Laser-Ablation Time-of-Flight Mass Spectrometry, and Ion-Pair High Performance Liquid Chromatography, the 87th Canadian Chemistry Conference and Exhibition, 2004.
6. Jorn C.C. Yu, Edward P.C. Lai, Susan Sadeghi, A Surface Plasmon Resonance Sensor Based on Self-Assembled Monolayer and Electrochemical Deposition for Mercury Ion in Water Analysis, the 87th Canadian Chemistry Conference and Exhibition, 2004.
7. Jorn C.C. Yu, Simon N.S. Zhou, Edward P.C. Lai, Molecularly Imprinted Polymers on Surface Plasmon Resonance Sensor for Ochratoxin A (OTA) detection, the 87th Canadian Chemistry Conference and Exhibition, 2004.
8. Jorn C.C. Yu, Simon N.S. Zhou, Edward P.C. Lai, Development of Molecularly Imprinted Polymers on the Surface Plasmon Resonance Biosensor, Varian Lecture, Carleton University, 2004.
9. Jorn C.C. Yu, Edward P.C. Lai, Electro-polymerized Polypyrrole Films for

Determination of Ochratoxin A by Surface Plasmon Resonance, The 6th Annual Chemistry and Biochemistry Graduate Research Conference, Concordia University, 2003.

10. Jorn C.C. Yu and Edward P.C. Lai, Electro-functionalized Polypyrrole Films for Determination of Ochratoxin A by Surface Plasmon Resonance, The 6th Annual Chemistry and Biochemistry Graduate Research Conference, Concordia University, Montreal, 14th-15th Nov, 2003.
11. Jorn C.C. Yu, Edward P.C. Lai, Susan Sadeghi, Surface Plasmon Resonance Sensor for Mercury Determination by Electrochemical Deposition in Water Analysis, poster presentation, the 39th IUPAC Congress and the 86th Conference of The Canada Society for Chemistry, 2003.
12. Edward P.C. Lai, Sherry Y. Feng, Simon N. Zhou, Jorn C.C. Yu, Functional Polymers and Molecularly Imprinted Polymers for Selective Bioanalytical Separations, oral presentation, the 39th IUPAC Congress and the 86th Conference of The Canada Society for Chemistry, 2003.
13. Jorn C.C. Yu, Edward P.C. Lai, Determination of Mycotoxin Binding Interactions of Functionalized Polypyrrole Film by Surface Plasmon Resonance and Laser Ablation Time-of-Flight Mass Spectrometry, poster presentation, the 49th International Conference on Analytical Sciences & Spectroscopy, June 1-4, 2003, Ottawa, Canada.
14. Susan Sadeghi, E.P.C. Lai, J.C.C. Yu, A sensor based on self-assembled monolayer for detection of mercury ion by surface plasmon resonance, the 49th International Conference on Analytical Sciences & Spectroscopy, June 1-4, 2003, Ottawa, Canada.

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List of Abbreviations

BSA	bovin serum albumin
CNT	carbon nanotube
DoPE	double pulsed elution
DDW	distilled deionized water
HPLC	high performance liquid chromatography
IAC	immunoaffinity column
LOD	limit of detection
LOQ	limit of quantification
MPE	multiple pulsed elution
MIPPy	molecularly imprinted polypyrrole
MISPE	molecularly imprinted solid phase extraction
MWCNT	multi-walled carbon nanotubes
NIP	non-imprinted polymer
μ SPE	micro solid phase extraction
OTA	ochratoxin A
OTB	ochratoxin B
PE	pulsed Elution
Py	pyrrole
PPy	polypyrrole
ppm	parts per million (10^{-6})
ppb	parts per billion (10^{-9})
ppt	part per trillion (10^{-12})
RIU	refractive index unit
SPR	surface plasmon resonance
θ_r	SPR angle
SPReeta	a miniaturized surface plasmon resonance sensor registered by instrument
SPE	solid phase extraction
SPP	solid phase preconcentration
SWCNT	single-walled carbon nanotubes
TBAP	tetrabutylammonium perchlorate

Chapter 1 Introduction

1.1 Molecularly Imprinted Polymers

Over the last decade of development, molecularly imprinted polymers (MIPs) have been proven regarding their potential as synthetic receptors for applications in several analytical techniques, such as liquid chromatography, capillary electrophoresis, capillary electrochromatography, solid phase extraction, immunoassay, and chemical sensors [1]. MIPs are polymers synthesized with the presence of a target compound (an imprint or template molecule) through polymerization. After removal of template from the MIPs, specific binding cavities with a shape and functional groups complementary to the target compound will be created within the polymer network. Figure 1-1 shows a schematic drawing of MIP with a recognition binding cavity [2]. MIPs have been considered as synthetic antibodies that mimic the function of naturally raised antibodies which exhibit molecular recognition properties. With the advantages of molecular recognition, many studies combining with MIPs have been reported, such as a quartz-crystal microbalance (QCM) for amino acid sensing [3], cholesterol sensor [4], colorimetric tests for sensing morphine [5], molecularly imprinted quantum dot photoluminescence for caffeine detection [6], MIP modified voltammetric sensor for vanillylmandelic acid [7], capacitive chemical sensor for amino acid [8], just to name a

few.

Figure 1-1 Schematic concept of MIP with a recognition binding cavity (adopted from Reference 2)

1.2 Current State of Knowledge

1.2.1 Theory of Molecularly Imprinted Polymer

An original theory, the lock-and-key mechanism, was proposed by Nobel laureate Emil Fischer in his attempt to explain enzyme–substrate interactions [9]. Unfortunately, this theory was later disproved by X-ray diffraction studies, in which enzymes showed a more flexible substructure that refuted the lock-and-key principle. However, the idea of a matrix designed to recognize a particular substrate remained as the cornerstone of molecular imprinting and has been the primary goal of research since the 1970s [10].

The theory behind molecular imprinting is that the MIP has built-in prearranged functional groups that provide spatial interactions against the template molecule. Formation of a complex between the template molecule and functional monomers is the first step in the preparation of MIP. Monomers that can interact with the template most intensively will give the complex stability. The key step is the polymerization of functional and cross-linking monomers in the presence of the template molecule. The functional monomers are spatially fixed in the polymer via their interaction with the imprint molecules during the polymerization reaction. Subsequent removal of the imprint molecules leaves behind imprints in the polymer network. These imprints enable the polymer selectively to rebind the template molecule from closely related compounds in a sample mixture. This is how the molecular imprinting works. Binding affinity and selectivity comparable to those of antigen–antibody systems have been achieved.

From the practical understanding of MIP development, a number of rules of thumb have emerged in the literatures that are helpful when developing particular MIPs [9]. It has been believed that the quantity and quality of the MIP recognition sites are related to the mechanisms and strength of the monomer-template interactions present in the pre-polymerization mixture. As general guidelines, “The more stable or stronger the

monomer-template complex is, the more selective the MIP will be” has been presumably used as a rule in the selection of functional monomers [11]. As for the selection of porogen solvents, it is generally accepted that the MIPs synthesized with aprotic porogens usually have higher affinity and selectivity than those prepared using porogens with moderate or strong hydrogen bonding capacity [12]. These general guides were drawn from the conventional preparation of MIPs, which were prepared by using methacrylic acid (MAA) as a functional monomer, ethylene glycol dimethacrylate (EGDMA) as a cross-linker and an aprotic solvent as the porogen. Thus, hydrogen bonding and electrostatic interactions could operate strongly in the non-aqueous environments. In aqueous environments, hydrogen bonding and electrostatic interactions could be disrupted. Hydrophobic interactions, which are non-specific, could govern analyte retention. An ideal MIP is generally the result of good spatial orientation and intermolecular interactions (such as electrostatic, hydrogen bonding and hydrophobic interactions) in both non-aqueous and aqueous environments. In SPE procedures, the MIP could be washed with an appropriate solvent that is capable of disrupting the non-specific interactions of analytes with the polymer matrix. Pulsed elution has been developed for the elimination of non-specific bindings by Mullett *et al* [13].

1.2.2 Conventional Preparation of MIPs

To make MIPs, selection of suitable functional monomers, cross-linkers, porogen solvents, initiators and general polymerization procedures are factors pertaining to the interactions with the template molecule before polymerization. Due to the complexity of the factors, such as functional monomer-template complexation, solvent effect, cross-linking, that drive the imprinting process, the performance of any new MIP toward the target molecule is difficult to predict. The development of a MIP for a specific separation still relies on experiments and practical observations. The specificity of a MIP is governed by the factors mentioned above at the preparation stage and an optimal condition at the binding and elution stage.

Many vinyl monomers are available commercially at a low cost, and free radical polymerization is usually the method of choice for preparing MIPs [14]. Commonly used monomers are listed in Fig. 1-2.

Acidic (a)

Basic (b)

Neutral (n)

Figure 1-2 Monomers commonly used in the non-covalent approach of molecularly imprinting. Acidic; aI: methacrylic acid (MAA); aII: p-vinylbenzoic acid; aIII: acrylic acid (AA); aIV: itaconic acid; aV: 2-(trifluoromethyl)-acrylic acid (TFMAA); aVI: acrylamido-(2-methyl)-propane sulfonic acid (AMPSA). Basic; bI: 4-vinylpyridine (4-VP); bII: 2-vinylpyridine (2-VP); bIII: 4-(5)-vinylimidazole; bIV: 1-vinylimidazole; bV: allylamine; bVI: N,N'-diethyl aminoethyl methacrylamide (DEAEM), bVII: N,N'-diethyl aminoethyl methacrylamide (DEAEM), bVIII: N,N'-diethyl aminoethyl methacrylamide (DEAEM), bIX: N,N'-diethyl aminoethyl methacrylamide (DEAEM), bX: N,N'-diethyl aminoethyl methacrylamide (DEAEM), bXI: N,N'-diethyl aminoethyl methacrylamide (DEAEM). Neutral (n); nI: acrylamide; nII: N-methylacrylamide; nIII: N-ethylacrylamide; nIV: N-vinylcarbazole; nV: acrylonitrile; nVI: methyl methacrylate; nVII: styrene; nVIII: ethyl vinyl ether.

N-(2-aminethyl)-methacrylamide; bVIII: N,N'-diethyl-4-styrylamidine; bIX: N,N,N,-trimethyl aminoethylmethacrylate; bX: N-vinylpyrrolidone (NVP); bXI: urocanic ethyl ester. Neutral; nI: acrylamide; nII: methacrylamide; nIII: 2-hydroxyethyl methacrylate (2-HEMA); nIV: trans-3-(3-pyridyl)-acrylic acid; nV: acrylonitrile (AN); nVI: methyl methacrylate (MMA); nVII: styrene; nVIII: ethylstyrene. (adopted from Reference 14)

Macroscopic polymer networks have been most widely synthesised and studied in the preparation of MIPs, as these tend to be insoluble materials that provide rigidity and mechanical stability to an imprinted binding site. To induce radical polymerization, an appropriate initiator is required. Commonly used cross-linkers and initiators are listed in Fig. 1-3 and Fig. 1-4.

Figure 1-3 Commonly used cross-linkers for molecular imprinting. xI: p-divinylbenzene (DVB); xII: 1,3-diisopropenyl benzene (DIP); xIII: ethylene glycol dimethacrylate (EGDMA); xIV: tetramethylene dimethacrylate (TDMA); xV: N,O-bisacryloyl-L-phenylalaninol; xVI: 2,6-bisacryloylamidopyridine; xVII: 1,4-phenylene diacrylamide; xVIII: N,N'-1,3-phenylenebis(2-methyl-2-propenamide) (PDBMP); xIX: 3,5-bisacrylamido benzoic acid; xX: 1,4-diacryloyl piperazine (DAP); xXI: N,N'-methylene bisacrylamide (MDAA); xXII: N,N'-ethylene bismethacrylamide; xXIII: N,N'-tetramethylene bismethacrylamide; xXIV:

N,N'-hexamethylene bismethacrylamide; xXV: anhydroerythritol dimethacrylate; xXVI: 1,4;3,6-dianhydro-d-sorbitol-2,5-dimethacrylate; xXVII: isopropylenebis(1,4-phenylene) dimethacrylate; xXVIII: trimethylpropane trimethacrylate (TRIM); xXIX: pentaerythritol triacrylate (PETRA); xXX: pentaerythritol tetraacrylate (PETEA). (adopted from Reference 14)

Figure 1-4 Chemical structures of commonly used initiators: iI: azobisisobutyronitrile (AIBN); iII: azobisdimethylvaleronitrile (ABDV); iIII: dimethylacetal of benzil; iIV: benzoylperoxide (BPO); iV: 4,4'-azo(4-cyanovaleric acid).

1.2.3 Thermodynamic Principles of MIP Formation

Thermodynamic studies by Whitcombe and Vulfson on the prediction of MIP selectivity offered another facile approach to investigate the factors determining selectivity [15]. A thermodynamic analysis of monomer–template interactions predicting ligand-receptor binding constants was proposed by Nicholls *et al* [16]:

$$\Delta G_{\text{BIND}} = \Delta G_{\text{t-r}} + \Delta G_{\text{r}} + \Delta G_{\text{h}} + \Delta G_{\text{vib}} + \sum \Delta G_{\text{p}} + \Delta G_{\text{conf}} + \Delta G_{\text{vdw}}$$

where

ΔG_{BIND} = complex formation energy;

$\Delta G_{\text{t+r}}$ = translational and rotational energy;

ΔG_r = restriction of rotors upon complexation;

ΔG_h = hydrophobic interactions;

ΔG_{vib} = residual soft vibrational modes;

$\sum \Delta G_p$ = sum of interacting polar group contributions;

ΔG_{conf} = adverse conformational changes;

ΔG_{vdw} = favorable van der Waals interactions.

This equation describes a relation of molecular level events prior to polymerization. While the general concept of molecular imprinting is straightforward, the involved molecular interactions are influenced by many different parameters. Thermodynamic control has already been applied to MIP designs to test the validity of the postulated hypotheses on computer simulations [17]. Although stable monomer–template complexes may be assembled prior to polymerization, the actual conditions during the polymerization, including elevated temperature and UV radiation, may significantly alter the nature of the complex.

1.2.4 Computational Modeling

With the development of computer technology and quantum chemistry, the

computational study of MIPs has emerged in order to make a good selection of functional monomers that maximize the molecular recognition property of MIPs. Wulff *et al* used electrostatic potential surface obtained by MolCad to indicate the shapes of occupied and unoccupied molecular cavities of MIP [18]. There was a report on using molecular modeling software to study the functional monomer–template conformation before polymerization [19]. Chianella *et al* employed a virtual library of functional monomers to screen against the target template molecule, and the selectivity of MIP was greatly improved [20,21]. These results suggested that computer modeling of MIP synthesis, analysis and evaluation would be a promising method for fast, accurate, safe and economical study of MIPs. Recently, a theoretical and experimental study of nicotinamide MIPs with different porogens was conducted by Wu *et al* [12]. Good correlations have been found between the interaction energy and the selectivity. When the porogen had poor hydrogen bonding, the interaction energy was mainly influenced by the dielectric constant of the solvent. When the porogen had strong capacity in forming hydrogen bond, both the dielectric constant of the solvent and the hydrogen bonding interference would affect the formation of the template–monomer complex. This computational study strongly suggested that the interaction energy between the template and monomer varied with the pre-polymerization solution composition. Using aprotic

solvents (such as chloroform, toluene and acetonitrile), the interaction energies are mainly influenced by their dielectric constants. The smaller the dielectric constant value is, the stronger complexation between template and monomer will be. Using solvents (such as methanol) with a high capacity in forming hydrogen bond, the hydrogen bonding would affect the formation of the template–monomer complex, thus influencing the interaction energy. This successful prediction model may provide a fast way for the selection of a good solvent for a given template and functional monomer system.

Pavel and co-workers used computational tool not only to investigate the monomer-template interaction but also to simulate the template-MIP interaction [22,23]. They used molecular dynamics simulations for different molecular systems in order to predict the interaction energies, the closest approach distances and the active site groups between the simulated molecular systems and different bio-ligands. It was found that electrostatic interactions play the most significant role in the formation of molecular imprinting materials. Acrylic acid, methacrylic acid, acrylamide, acrolein, acrylonitrile, styrene, etc. (a total number of 25 monomers) were simulated. The minimized structures of five ligands (theophylline, theobromine, theophylline-8-butanoic acid, caffeine and theophylline-7-acetic acid) were obtained with the use of molecular mechanics approach. The simulated results indicated that ligands interact with either

–COOH or CH₂-CH– functional group at the MIP surface. It was also found that molecular substrate without functional side groups can also be used for molecular imprinting. With several successes in MIP computational modeling, some experimental parameters seem not fully comparable regarding to the reality. It does not account for effects such as disruption of monomer-template complexes by the cross-linker, modulation of the reactivity of monomers as they increasingly incorporate into the growing polymer network, and the influence of the porogen solvent.

Even though the preparation of MIP is relatively easy, the complexity of multiple interactions among the pre-polymerization components makes the computer modelling of MIP still a big challenge. A simplified computational approach is often employed to develop a promising prediction of the resulting MIP. Predictive modeling of functional monomer interactions and the resulting binding site performance will be a promising approach toward more rational design of synthetic strategies for improved MIP technology. It can be expected that upon establishment of more complex models that provide better resemblance of the actual MIP preparation conditions, computational modeling will develop into a useful complementary tool efficiently reducing the large number of polymers currently screened to achieve optimal recognition properties. However, the accuracy of predictive simulations will strongly depend on the complexity

of the implemented model and on experimental conditions. Therefore, refined simulations along with supporting spectroscopic data should lead to more accurate modeling of pre-polymerization complexes and binding sites. Spectroscopic studies gathered evidence for self-assembly based on ionic and π - π stacking interactions rather than previously assumed hydrogen bridge bonding have led to a refined model of the pre-polymerization complex [24].

1.2.5 MIP for Solid Phase Extraction

Isolation, pre-concentration and clean-up is common sample pre-treatment procedure used to eliminate some complex matrix components before instrumental analysis in order to obtain better selectivity. In pharmaceutical, clinical, environmental and food chemistry applications, solid-phase extraction has been widely employed for the isolation and pre-concentration of target analytes as well as the clean-up of sample matrices [25]. However, unknown matrix interference components can be co-extracted with the target analytes when conventional SPE sorbents, such as C18, ion-exchange and size-exclusion materials, are used. The co-extraction and co-elution problem produce bad influences on detection specificity. For example, detector signals could be suppressed or enhanced when matrix components are co-eluted with the analyte. These effects are particularly problematic in the case of trace analysis. To improve the

selectivity of SPE, many affinity-based sorbent materials have been developed to selectively extract the target analytes. Materials, such as boronate, lectin, protein A or G, synthetic dyes, immobilized metal ions, aptamers, peptides, antibodies and molecularly imprinted polymers (MIPs), have been developed for seeking better selectivity in SPE [26, 27]. These affinity materials are usually bound to solid supports and have been used for analysis or isolation of target analytes in either offline or online modes. Among these affinity techniques, both antibodies and MIPs can be used as single analyte- or group-selective sorbents. Although an antibody can selectively recognize an analyte, it can also recognize one or more structurally related analogues with different affinities, depending on spatial interactions.

To date, the particles of an MIP material can be either packed into a micro-column [28,29,30] for selective solid phase extraction (SPE), or glued to the surface of a solid-phase micro-extraction (SPME) fiber [31]. New fabrication techniques continue to be developed for molecularly imprinted solid phase extraction (MISPE) and molecularly imprinted solid phase micro-extraction (MISPME).

1.2.6 New Development of MIPs

The interfacing of a suitable transducer to MIPs is still growing and expected to have more significant impact in the field. A recent evaluation of the performance of

MIP films for coulometry was conducted by using an applied positive potential to induce adsorption of the target molecules [32]. One possible recognition mechanism for the interaction of the MIPs toward the template under the influence of an applied potential has been proposed [33]. The resultant sensor showed a high degree of sensitivity, selectivity and a broad linear measuring range, which has been demonstrated by a comparison of sensor response to structurally related analogues of morphine. Imprinting a polymer matrix with binding sites located at the surface has been shown to be advantageous as the sensor interface. The binding sites are more accessible, the mass transfer is faster, and the binding kinetics is faster. More interestingly, such surface imprinted MIPs are potentially suited for the recognition of large-molecular-weight peptides and proteins. However, these materials are not widely used as yet because their preparation is less straightforward and requires specially adapted protocols [34].

Molecular imprinting at the nano-scale to make nano-MIPs has gained more and more attention in this field [35]. Nano-MIPs can be synthesized by precipitation or emulsion polymerization, and be suspended in aqueous media. Their applications are expected to be more compatible with biological analysis. Applying MIP to modify gold nanoparticles for the construction of a sensing system has been shown to be a promising strategy [36]. Surface imprinted nano wire has also been developed for the application

of MIP on biological analysis [35].

Chen *et al* observed the enthalpic changes attributed to rebinding of template molecules to the MIP by microcalorimetric studies [37]. Enthalpic changes of binding was measured by the adjustment of pH at which rebinding was performed to elucidate the nature of the binding mechanism. This investigation confirmed that interactions such as π - π stacking and electrostatic interaction can be observed. Although the contribution of π - π stacking to stabilization of the binding pocket (5–10 kJ/mol) is less than that of electrostatic interactions (tens of kJ/mol), it may yet have an important role in determining the shape of the cavity and molecule orientation within the specific binding site. It suggests that a simple one-point interaction is insufficient to induce selectivity regardless of the strength of this interaction. Selectivity of non-covalent MIP materials requires those conditions mimicking biological receptor entities where molecular recognition relies on electrostatic interactions and secondary interactions such as hydrophobicity. In fact, hydrophobicity and macroscopic phase separation have been observed in molecularly imprinted systems and are considered significant factors in determining the extent of imprinting effects as well as providing insight into the distribution of binding sites within the polymer structure. Evidence is emerging that physical characteristics of MIPs have significant bearing on their inherent recognition

properties. Spivak *et al* have determined that shape selectivity is an important contributing factor to overall MIP selectivity [38]. Twelve secondary amines with different side chains were imprinted, and the enantioselectivity was evaluated by HPLC for each amine on each imprinted polymer. It was derived that branched-structure templates produce higher selectivity MIP than their straight-chain counterparts. Shape selectivity is determined by steric exclusion or an optimal spatial fit which maximizes binding interactions. Moreover, the importance of shape selectivity to molecular recognition by MIPs highlights the importance of the building polymer matrix and complicates any prediction of selective binding from the structure of monomer-template complexes.

1.3 Statement of the Problem

Since MIPs are prepared in the form of a macroporous monolith, grinding and sieving process is required to yield proper particle sizes for analytical applications. This preparation of MIPs inherited some drawbacks, such as intensive labor, inefficient yield, and potential exposure to hazardous airborne particles when toxic molecules are imprinted. The intensive labor involves time-consuming grinding and sieving of a bulk MIP to obtain fine particles for the subsequent SPE or LC applications. Techniques for bead or monolithic formation have been investigated to obtain more homogeneous MIP

particles within a particular size range [39,40]. Precipitation polymerization or emulsion polymerization has gained attention due to their better control of polymer sizes and morphologies [41]. However, polymerization of this type usually requires either the use of special dispersing phases or complicated swelling processes [42]. A more general imprinting method applicable to a wide range of imprint molecules and giving uniform MIP spheres is still needed.

1.4 Methodology

1.4.1 Pyrrole as a Functional Monomer

The possibility of inducing selectivity by the presence of the template during polymerization has been tested with several non-crosslinked electrogenerated polymers, such as polypyrrole, polyphenol, and poly(o-phenylenediamine) (o-PPD) [43]. These polymers can be prepared by electropolymerization from a solution of their monomers. Compared with the well-established acrylate or vinyl derivatives, there has been little work in MIPs utilizing electropolymerizable polymers. Molecularly imprinted polypyrrole has previously been shown to be a promising material for molecular recognition as demonstrated by Bachas *et al* [44] and Nagaoka *et al* [45]. Enantioselectivity towards amino acids by molecularly imprinted polypyrrole has been studied [51]. Recently, polypyrrole-based MIP was developed on platinum-black

electrode via electropolymerization for the detection of bovine leukemia virus glycoproteins [46]. A caffeine-imprinted polypyrrole has also been synthesized using galvanostatic electropolymerization of pyrrole monomer directly onto gold electrode of quartz crystal microbalance [47]. The creation of imprinted sites with this system is based on irreversible over-oxidation that polypyrrole undergoes at potential values higher than those for its reversible doping and undoping, thus inducing charge and complementary structure effects. These studies are based on an extension of the properties of permselectivity against anions (and selectivity towards cations) of thin, overoxidized polypyrrole films, which have led to important analytical applications involving the determination of cations in presence of anionic interferents. During the overoxidation process, the template species is expelled, so this new approach to molecular imprinting avoids the template-extraction procedure and undesired bleeding effects.

Pyrrole ring can also contribute π - π and electrostatic interactions with the template molecule [48]. In a study of pyrrole interaction with alcohols [49], it has been shown that the pyrrole ring can act as a π -base against the OH group of an alcohol. The driving force for the stabilization of the complexes is the strong hydrogen bonding interactions localized on the C-3 atom of the pyrrole ring (being the electrostatic

interaction between this negatively charged center and the positively polarized H atom of the alcoholic OH group). The interactions between pyrrole and a wide range of potential target analytes make pyrrole an attractive functional monomer. With such superior properties, the choice of pyrrole as a functional monomer is gaining more and more attention for the preparation of polypyrrole-based MIPs, as summarized in Table 1-1. In the case of using pyrrole as a functional monomer and OTA as a template, the hydrogen atom in the N-H group of the pyrrole unit can be a hydrogen bond donor to interact with the template molecule (Fig. 1-5) [50].

Table 1-1 Development and Applications of Pyrrole-based MIPs

Target Analyte	Application	reference
<p>L-glutamate</p>	Sensor (quartz crystal microbalance)	[51]
<p>L-lactic acid</p>	Enantioseparation	[52]
<p>1-naphthalenesulfonate</p>	Sensor (quartz crystal microbalance)	[45]
<p>Taurocholate</p>	Sensor (quartz crystal microbalance)	[53]
<p>Bovine leukemia virus (BLV) glycoprotein gp51 (gp51) (No structure available from the article)</p>	Pulsed amperometric detection (PAD)	[46]

L-,D- Tyrosine

Sensor (Electrochemical)

[32]

Caffeine

Sensor design (piezoelectric quartz crystal)

[47]

Glutamic acid

MIPPy nanowires for bioanalytical applications

[35]

Ochratoxin A

SPR sensor and micro solid phase extraction

[54,55]

Figure 1-5 The proposed molecular imprinting of OTA molecule within the conducting polypyrrole polymer.

1.4.2 Electrochemical Imprinting

Polypyrrole (PPy) has been used for many successful applications ranging from ion-exchange extraction to hydrophobic extraction, based on the selective interaction between the polymer and the target analytes [56]. In addition, tuneable properties of the polymer, such as the oxidation/reduction equilibrium in conductive PPy, can be exploited to control adsorption and desorption [57]. Because PPy can be prepared on a metal

surface by electrodeposition from an aqueous solution, fine stainless steel wires (diameter = 127 μm) were selected as the supporting fiber [58].

Non-covalent approach to form MIPs is one accepted mechanism for the formation of specific binding sites. Electrooxidation of the electropolymerizable monomer occurs at the anode and the resulting polymer, usually conducting polymer, deposits onto the electrode surface. The electropolymerization of conducting polymers can be achieved through galvanostatic, potentiostatic and cyclic voltammetric methods. During the electrodeposition of the conductive polymer, the template molecules will be trapped in the polymer matrix as a result of the ability of these molecules to interact with the electropolymerizable monomer. In general, the great attraction of using electrochemical imprinting to prepare recognition elements are ease of preparation (by cycling potential sweeps from solutions of their monomers and templates) and the possibility of obtaining very thin films with good reproducibility on many conductive substrates. These are especially attractive in developing applications for analytes of large molecular size, which would otherwise be significantly impeded in diffusing through the polymeric layer.

1.5 Objectives of Thesis

Ochratoxin A (OTA) is a secondary metabolite of genera of *Aspergillus* and

Penicillium. It has been found as a contaminant in food and feed commodities. OTA exhibits multiple toxicities in animals and mankind, including nephrotoxic, hepatotoxic, immunotoxic, teratogenic and carcinogenic effects, which represent a serious health risk to livestock and general population. 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 (5.0 ng/kg body weight) [59].

The development of validated methods for OTA identification generally involved extraction by liquid–liquid extraction, clean-up by an immunoaffinity column (IAC), and identification by HPLC with fluorescence detection [60]. Unfortunately, the IACs are relatively costly and short shelf life. The development of a new sorbent for selective SPE that is capable of OTA preconcentration prior to instrumental analysis is an important issue. An affordable analytical method, compared to the use of IAC columns, will make the screening of foodstuff more frequent in general laboratories in both developed countries and poor countries, thus protecting human health. Conventional preparation of MIP bulks for OTA recognition is challenging as little reports can be found from the literature, all listed in Table 1-2.

It was the objective of my thesis research to develop molecularly imprinted

polypyrrole for OTA recognition via electrochemical imprinting. Easy preparation and transformation was expected with the new approach. Furthermore, it has been revealed by N1s X-ray photoelectron spectroscopy (XPS) studies that the hydrogen of N-H on pyrrole ring within PPy undergoes protonation under acidic conditions. Under alkaline conditions, the N-H will be deprotonated [61]. The removal of OTA template molecules from MIPPy could be achieved by perturbing the hydrogen bonding between the OTA analyte and the MIPPy binding cavity. Uptake of target analyte would be enhanced when the N-H on pyrrole ring is protonated, as illustrated in Fig. 1-6. The binding cavity within MIPPy will revert back when the target molecule is eluted; it remembers the configuration of the target molecule. A model of polypyrrole cross-linked by ethylene glycol dimethacrylate was shown in Fig. 1-7.

Figure 1-6 Association mechanism of OTA with PPy

Figure 1-7 Model of polypyrrole cross-linked by ethylene glycol dimethacrylate

Table 1-2 Functional Monomers Used for OTA MIP Preparation from the Literature

Functional Monomer	Structure	Reference
N-Phenylacrylamide		29
methacrylic acid acrylamide (dual functional monomer)		62
methacrylic acid		63

In this research work, molecularly imprinting via the electrochemical imprinting route has been investigated to prepare polypyrrole-based MIPs. It has been

demonstrated that molecular imprinting by electropolymerization of pyrrole was an effective way of growing a thin MIP coating directly onto a variety of sensors and conductive surfaces. This new spin-off technology of MIP modified stainless steel frits for selectivity and sensitivity enhancement in biochemical and food analyses has expanded the use of MIPPy. The molecular recognition properties of the MIPPy-modified frits were competitive to those reported conventional MIPs, monoliths specific for OTA and IAC columns. Good combination of new nano-material and MIP technology was demonstrated a unique idea that can pack sub-micron interstitial frit spaces to overcome the limits on conventional preparation of MIP particles. No time-consuming steps of grinding the bulk polymer and removing the template molecules were found to be a great advantage of this work.

Chapter 2 Experimental Section

2.1 Surface Plasmon Resonance Sensor

The phenomenon of anomalous diffraction on diffraction gratings due to the excitation of surface plasma waves was first described by Wood [64]. This surface plasma wave (SPW) is a TM-polarized wave (magnetic vector is perpendicular to the direction of propagation of the SPW and parallel to the plane of dielectric interface). The physical description of the phenomenon has been reported by Raether et al [65]. Optical excitation of surface plasmons by the method of attenuated total reflection was demonstrated by Kretschmann and Otto [66]. Surface plasmon resonance (SPR) can be excited in thin metal films under certain, precisely defined geometric, optical and electronic conditions. The physical quantity obtained from a SPR measurement is a refractive index value, either derived from a spectrally or angularly resolved position of the resonance.

Plasmon resonance is observed when the component of the evanescent field wave vector parallel to the metal/ dielectric interface (K_x) is equal to the surface plasmon wave vector (K_{sp}), as given by the following equation:

$$K_x = \frac{\omega}{C} \sqrt{\epsilon_1} \sin \theta = K_{sp} = \frac{\omega}{C} \sqrt{\frac{1}{\epsilon_2} + \frac{1}{\epsilon_m}}$$

where ω is the optical frequency, C the free space velocity of light, ϵ_m is the real

part of the dielectric constant of the metal, ϵ_1 is the dielectric constant of the prism, ϵ_2 is the dielectric constant of a dielectric applied to the metal, and θ is the angle of incidence of the optical beam at the metal/dielectric interface.

The Texas Instruments (TI) surface plasmon resonance sensor, known as SPReeta, uses a small self-contained device to perform all light incidence activities and measure reflective intensities with the established Kretschman geometry. In the SPReeta device, light is produced from a light-emitting diode (LED) at the near infrared wavelength of 840 nm. The light is immediately plane polarized and incident on the dielectric/gold metal interface. The externally facing gold surface (400 to 500 angstroms thick with a length of 4.5 mm and a width of 0.1 mm) is contained within an airtight channel of a flow cell into which sample solutions can be introduced. To account for non-uniformity in the gold surface, the sensor is first initialized in air, and the SPR curve is determined by normalizing the signal in liquid with the signal in air. Receptor or sorbent materials can be covalently linked or become adsorbed to the gold surface. Sample solutions containing the analyte of interest can be detected by direct binding of analyte molecules to the receptor or sorbent.

SPReeta evaluation module (SPR-EVM-BT) was purchased from Nomadics (Stillwater, OK, USA). The major components of the module were a SPReeta sensor,

integrated flow cell, interface controller, and application software (Version 6.54). Specific features of the module are described in detail by the manufacturer [67] and some research groups [68,69]. A 12-bit analog-to-digital converter was utilized to provide a high resolution of 8×10^{-7} refractive index units (RIU). The module was combined with a Compaq Presario 1700 notebook computer to afford portability. For SPR measurements, the sample solution was introduced by a KD Scientific 100 syringe pump (New Hope, PA, USA) at a flow rate of 3-12 mL/hr.

By the design of SPReeta sensor, the SPR signal, or SPR curve, was obtained from a comparison of the air signal and sample signal:

$$SPRcurve = \frac{signal_{Sample}}{signal_{Air}} = \frac{reflectivity_{Sample} - reflectivity_{Sample_background}}{reflectivity_{Air} - reflectivity_{Air_background}}$$

The optical path inside a SPReeta device is shown in Fig. 2-1.

Figure 2-1 SPReeta, miniaturized SPR sensor.

2.2 High Performance Liquid Chromatography (HPLC)

The HPLC system consisted of a solvent pump (Shimadzu LC-6A, Kyoto, Japan), injector valve (Valco Cheminert VIGI C2XL, Houston, TX, U.S.) equipped with a 100- μ L sample loop, Phenomenex polymerX RP-1 (5 μ m, 250 mm x 4.6 mm, Torrance, CA, U.S.) or Phenomenex Synergi MAX-RP column (4 μ m, 150 mm x 4.6 mm) column was used, a fluorescence detector (Perkin-Elmer LC 240, Boston, MA, U.S.), an integrator (Spectra Physics 42900, San Jose, CA, U.S.) and data acquisition system (PeakSimple, Torrance, CA, U.S.).

For HPLC, 20:80 v/v acetonitrile-ammonia buffer ($\text{NH}_4\text{Cl}/\text{NH}_3$, 20 mM, pH = 9.5)

was used as the mobile phase at a flow rate of 1 mL/min. Note that good stability of the HPLC columns Phenomenex polymerX RP-1 was specified for pH = 1.0-14 and Synergi MAX-RP was specified for pH = 1.5-10. The excitation and emission wavelengths for fluorescence detection were set at 380 and 440 nm. Coupling of the μ SPE device for on-line extraction and pulsed elution to HPLC system was described in each chapter.

2.3 Reagents and Materials

Pyrrole, Ochratoxin A (OTA), Triethylamine (TEA) purchased from Sigma-Aldrich (Mississauga, ON). Sodium chloride (NaCl) was obtained from BDH (B30123). Tetrabutylammonium perchlorate (TBAP) was obtained from Fluka (Buchs, Switzerland). Acetonitrile, methanol, and ethanol were all HPLC-grade obtained from Caledon (Georgetown, ON). Acetic acid was purchased from Fisher Scientific (Fair Lawn, NJ). 18-M Ω -cm distilled deionized water (DDW) was supplied from a Millipore Milli-Q water system (Bedford, MD).

RIDA[®] ochratoxin A columns (Art. No. R 1303) were purchased from R-Biopham AG (Darmstadt, Germany).

MWCNTs with ~95% purity were obtained from Shenzhen Nanotech Port (Shenzhen, China). These MWCNTs were prepared by the catalytic decomposition of CH₄, with diameters ranging from 10 to 30 nanometers and lengths ranging from five

hundred nanometers to five hundred micrometers [70].

2.4 Electropolymerization

Good conditions for the electropolymerization of pyrrole onto a conductive substrate using a potentiostat was described by Wu *et al* [71]. In this study, the SPReeta gold surface or the stainless steel frit was connected as the working electrode to a Stoneheart BC 1200 high-performance potentiostat (Madison, CT). A platinum counter electrode and a Ag/AgCl reference electrode were used to complete a three-electrode setup for the electropolymerization. For pyrrole electropolymerization, a constant potential of +0.76 V to +0.85 V was applied on the working electrode.

Chapter 3 Polypyrrole Films on Miniaturized Surface Plasmon Resonance Sensors for Ochratoxin A Detection

3.1 Introduction

Pyrrole (Py) can be oxidized to form a polymer that has intrinsic conducting properties [72]. Polypyrrole (PPy) is one of the widely studied conductive polymers. The mechanical, electronic and chemical properties of polypyrrole have been well documented in the literature [73,74]. A large amount of research was to address how polymerization conditions would affect the properties. Sree *et al* synthesized thin PPy films chemically by interfacial polymerization [75]. Using a variety of dopants and FeCl₃, de Souza *et al* prepared PPy films directly on glass substrates as volatile organic solvent sensors [76]. PPy is relatively easy to make by electro-oxidation and the thin film is chemically stable. Based on electrochemically deposited PPy with dopants, an array of sensors was developed for the detection of volatile aromatic hydrocarbons [77].

Surface plasmon resonance (SPR) concerns an electromagnetic charge-density wave that propagates along the interface of a metal (gold) and a dielectric. Any changes in dielectric properties of the interfacial region influence the excitation of surface plasmons, thus forming the basis for SPR sensing. This optical technique can measure the thickness and dielectric constant of thin organic films deposited on the gold surface [78]. Thickness resolution is typically at the angstrom level, and films up to several

hundred nanometers thick can be studied. SPR monitoring of the synthesis of a conducting polymer film (polyaniline) has been described by Kang *et al* [79]. SPR is also a facile method to detect molecular interactions with thin organic films [80]. The stability of SPR measurements is good, and sensitivity to environmental factors (e.g., temperature fluctuations, pressure changes, or mechanical disturbances) is minimal [81]. A novel electrochemical SPR biosensor has been fabricated for the measurement of horse radish peroxidase, based on a change in the dielectric constant of a polyaniline film [82]. A real-time and label-free parallel DNA sensor has been characterized by SPR imaging [83]. Garland *et al* have demonstrated a time-resolved SPR technique for studying the correlation between the optical and electrochemical parameters of a thin (~ 590 Å) film gold electrode in aqueous 0.05 M NaNO₃ [84].

The interaction of OTA with Py is a prerequisite in order to evaluate the possibility of using Py for OTA imprinting. In this Chapter, the binding property of PPy towards OTA was first investigated by using a miniaturized SPR sensor. The SPReeta gold surface was assembled with a Pt counter electrode and a Ag/AgCl reference electrode for electrochemical polymerization. SPR was employed not only to monitor the electrochemical polymerization of Py on the gold surface, but also to investigate the binding properties of PPy in real time. Application of the PPy functionalized SPReeta

for OTA sensing was evaluated. Binding of OTA was demonstrated for the 1-10 $\mu\text{g/mL}$ levels.

3.2 Experimental

3.2.1 Modification of SPReeta Sensing Surface

For *in situ* pyrrole electropolymerization, the flow cell (volume = 20 μL) was modified to accommodate three electrodes as shown in Fig. 3-1.

Figure 3-1 Schematic diagram of SPReeta with *in situ* electropolymerization setup.

The SPReeta gold surface acted as the working electrode, which was coupled to a Pt counter electrode and a Ag/AgCl reference electrode for the electropolymerization. Solutions of 1 mM sodium chloride and 1 mM pyrrole were mixed in Milli-Q water for use as the polymerization solution. Depending on the selected data analysis method and

smoothing mode, the SPReeta data rate could be varied from 1-30 Hz. In this work, a polynomial fit about the SPR minimum was chosen to determine the SPR angle. A data rate of 2 Hz was adequate for monitoring the kinetics of PPy electrodeposition.

3.2.2 Binding Interaction of OTA and PPy Film

The PPy film was tested for binding with 0.1-10 $\mu\text{g/mL}$ OTA in water-methanol (9:1) at a flow rate of 3 mL/hr. Sufficient interaction time was allowed for observing an increase of 0.010° in SPR angle. A binding rate (= SPR angle increase / time) was calculated for each of the several OTA concentrations.

3.3 Results and Discussion

3.3.1 Electropolymerization of Pyrrole

The SPReeta evaluation module demonstrated to be an effective device for SPR measurements. This optical phenomenon can detect changes in refractive index of the medium adjacent to the gold surface [85]. As illustrated in Fig. 3-2, a plot of reflectance versus incident angle gives a minimum at the SPR angle, which corresponds to the excitation of surface plasmons at the gold surface. The SPR angle increases with increasing RI of the interfacial region. When 1 mM Py solution in 1 mM NaCl was added in the SPReeta flow cell, the SPR angle and refractive index were $69.24100 \pm 0.00048^\circ$ and 1.333663 ± 0.000004 RIU, respectively. Either signal could

serve as a reference baseline for monitoring the electropolymerization of Py.

Previous works had reported that acetonitrile would lead to better quality PPy films than aqueous solutions [86]. With the SPReeta sensor, however, acetonitrile could not be employed due to solvent attack on some of the opaque materials. The effect of electropolymerization potential on the rate of PPy formation on stainless steel had previously been documented [74]. A potential of +0.76 V was determined by cyclic voltammetry to be optimal for the present work. The resultant PPy formation on the gold surface was investigated by the SPReeta in a plot of reflectance versus incident angle, as shown in Fig. 3-3. After starting electropolymerization, the SPR profile shifted progressively to higher incident angles. The SPR angle monitoring mode was next selected to detect Py electropolymerization in all subsequent experiments.

Figure 3-2 Reflectance versus incident angle plots for monitoring PPy film formation on the SPReeta gold surface.

Figure 3-3 Effect of applied potential (+0.76 V) on SPR angle increase for different NaCl electrolyte concentrations and 1 mM Py. The y-axis on the right shows the corresponding adlayer thickness. Note that film thickness reached a maximum after only 1.3 s.

A control SPReeta experiment was conducted under +0.76 V using different NaCl electrolyte concentrations. As shown in the lower curves of Fig. 3-3, the SPR angle was generally affected by the applied potential. A greater increase in SPR angle was observed with decreasing electrolyte concentration. These results might be partly explained by an influx of electrons charging up the electrical double layer at the gold

surface, until saturation. It is interesting to see that only the Py solution (containing 0.001 M NaCl) was able to sustain a continuous increase in SPR angle, largely due to PPy formation on the gold surface, until the applied potential was switched off. By comparing the results obtained for the Py solution and the 0.001 M NaCl solution, a net increase in SPR angle would indicate the PPy formation.

Figure 3-4 Typical SPR sensogram for *in situ* formation of PPy onto sensor surface

An adlayer thickness could be calculated from the increase in SPR angle, following the propagation depth formula provided by the software of Nomadic Inc. The refractive index of the aqueous pyrrole solution was 1.334. As shown in Fig. 3-4, the

film thickness was estimated to be $25.6 \pm 0.3 \text{ \AA}$ (based on a refractive index of 1.423 [87] and density of 1.5 g/mL [88] for bulk PPy) after applying +0.76 V for 1.3 s. A typical PPy modified SPReeta gold sensor surface is shown in Fig. 3-5.

Figure 3-5 A typical PPy modified SPReeta gold sensing surface

An exact measurement of the PPy film thickness was deemed not critical because OTA binding would mainly occur on the film surface. It had previously been reported that the polymerization time would be a fundamental parameter for determining the final

level of doping, the kinetics of adsorption and the homogeneity of the PPy film [76]. For SPR measurements, the evanescent field decays exponentially from the gold surface. A film thickness of 25 Å was deemed optimal for subsequent OTA binding in terms of good sensitivity and adequate capacity.

3.3.2 Binding Interactions of OTA and PPy film

Binding interaction of OTA and PPy film was reported by the increase of SPR angle when an OTA solution was introduced onto the PPy modified SPR surface. Any increase in SPR angle would be related to the amount of OTA molecules bound onto the PPy film. The PPy films studied were found to exhibit good binding with OTA in water-methanol (9:1). Binding with 10-µg/mL OTA, as shown in Fig. 3-6, produced an increase of 0.010° in SPR angle after 256 s (< 5 min) of interaction time.

Figure 3-6 Binding of PPy film with OTA in water-methanol (9:1) at a flow rate of 3 mL/hr.

At a flow rate of 3 mL/hr, approximately 0.2 mL of sample solution (containing 2 µg of OTA) would be required. A binding rate (= SPR angle increase / time) was calculated for each of several OTA concentrations, in order to construct a standard calibration curve. As shown in Fig. 3-7, fair linearity was obtained in the concentration range from 0.1 to 10 µg/mL (with $R^2 = 0.9689$). It is well known that PPy film can adsorb a lot of different biomolecules (DNA, proteins, etc.). Although it is not surprising that OTA bound the PPy film, these results present the first experimental evidence for such binding directly by SPR, particularly in the water-methanol (9:1)

studied. It also suggested that PPy as a thin film was promising for OTA imprinting, based on its binding capability toward OTA molecules.

Figure 3-7 Standard calibration curve of binding rate (=SPR angle increase/time) versus OTA concentration.

3.4 Conclusion

The SPReeta device is a miniaturized SPR sensor that affords portability to monitor binding interactions in real time. Selectivity of the gold surface can be modified by adding a functional film. Successful modification has been demonstrated in this work by rapid electropolymerization of Py *in situ*. For subsequent OTA detection based on an increase in SPR angle, the linear range extended from 0.1-10 μg/mL. This result is exciting, in that the OTA binding functionality of

electropolymerized PPy films has been demonstrated via SPR measurement for the first time. The rapid binding time (< 5 min) and small sample volume (0.2 mL) promise development into analytical sensor applications.

In order to construct a sensor for analytical applications, the specificity of the binding must be augmented. One plausible scheme would be to continue the experiment with BSA, followed by addition of antibodies against OTA. This would demonstrate whether the present method can be used to detect OTA in complex medium (such as foodstuff) without any significant cross-reactivity of the OTA antibodies with other matrix components. Guedon *et al* has reported on the characterization and optimization of a real-time, parallel, label-free, polypyrrole-based DNA sensor by SPR imaging [83]. They immobilized 15-mer oligonucleotide probes at multiple spots on the PPy-functionalized gold surface for quantifying DNA hybridizations. SPR imaging allowed real-time measurements on several DNA spots at a time. They further demonstrated that a surface density of PPy/DNA probes of 590 pg/mm² optimized the hybridization signal. It would be interesting to follow their imaging technique, showing how more or less competitive the SPReeta is for OTA detection. In order to improve the selectivity of the PPy SPR sensor, molecularly imprinted PPy would next be developed on the SPReeta sensor for OTA detection.

Chapter 4 Interaction of Ochratoxin A with Molecularly Imprinted Polypyrrole Film on Miniaturized Surface Plasmon Resonance Sensor

4.1 Introduction

Molecularly imprinted polymers (MIPs) have been considered to be the man-made material which can provide high affinity to specific target molecules. The concept underlying molecular imprinting is self-assembly of functional monomers around the template molecule, followed by polymerization in the presence of cross-linkers to form a solid material. Upon removal of the template, recognition cavities are created that are complementary in both shape and functionality to the original template. The binding of functional monomers to the template can be achieved by non-covalent interactions, thereby allowing considerable flexibility in the choice of functional monomers for imprinting various types of templates [89]. MIPs have been developed for a variety of applications including chromatography, enzymatic catalysis, solid-phase extraction, and sensor technology [90]. The molecular imprinting technique has been applied in quartz crystal microbalance (QCM) [91] and capillary electrochromatography column [92], to serve as the sensing element and the stationary phase respectively. In the field of modern analytical chemistry, sensors with a high capacity for transducing chemical signals are highly desirable [1]. However, there are challenges associated with

the development of MIP-based sensors, such as the paucity of a general procedure for preparing MIP films, difficulty in integrating them with a transducer, and the lack of sensitivity in transforming the binding event into measurable signals [93].

Taniwaki and co-workers demonstrated that surface plasmon resonance (SPR) sensors are useful for measuring thin film thicknesses (with a resolution at the angstrom-to-nanometer level) and detecting molecular interactions [94]. Previously, Lai *et al* prepared a MIP overlayer (from methacrylic acid and ethylene glycol dimethacrylate) for use as the sensing element in SPR detection of theophylline, caffeine, and xanthine [95]. Kugimiya and co-workers synthesized a MIP on a SPR device by co-polymerizing N,N,N-trimethylaminoethyl methacrylate, 2-hydroxyethyl methacrylate and ethylene glycol dimethacrylate in the presence of *p*-vinylbenzeneboronic acid ester for the detection of sialic acid [96]. Li *et al* fabricated a MIP with L-phenylalanine ethyl ester and used SPR for monitoring the adsorption and elution processes *in situ* [97].

Polypyrrole (PPy) is one of the most promising materials for multi-functionalized applications. The preparation, characterization, properties and applications of PPy has been reviewed by Wang *et al* [98]. According to the synthetic methods, various parameters (such as doping anions, additives and oxidants) have different effects on the PPy properties. For example, PPy doped with small-sized anions act as anion exchange

materials. [99]. Minehan and co-workers reported the use of PPy for binding DNA [100]. They found that the positively charged surface largely enhanced the adsorption of negatively charged colloidal particles through electrostatic interaction to form surface complexes. Electrochemical polymerization results in thin PPy films on the working electrode surface [101], which would be suitable for SPR sensor applications.

Recently, the possibility of inducing selectivity by the presence of a template during polymerization was tested by Blanco-Lopez and co-workers with several non-crosslinked electrogenerated polymers such as PPy [43]. The binding capability of PPy with OTA in a water/methanol mixture has been demonstrated by monitoring the SPR signal in Chapter 2. In this chapter, the use of electrochemical imprinting technique to prepare a molecularly imprinted polypyrrole (MIPPy) film onto the SPReeta surface is described. The objective of this chapter is to investigate the interaction of MIPPy films with OTA in wheat and wine extracts. Each MIPPy film was prepared electrochemically on the SPReeta gold surface from a pre-polymerization mixture of pyrrole and OTA in ethanol/water (1:9 v/v). This preparation provided a simple and rapid way to synthesize a sensing element with targeted selectivity. The OTA binding property of each MIPPy film was directly monitored by SPR, and pulsed elution was developed for regeneration of the MIPPy film surface. Interference by the matrix of

wheat and wine extracts was evaluated for minimization.

4.2 Experimental

4.2.1 Preparation of MIPPy Film

In order to reduce the consumption of toxic and costly OTA template, the mole ratio of OTA to Py for MIPPy preparation was 1:20 (rather than 1:4 or 1:5). To prevent the damage of SPReeta by organic solvents, ethanol/water (1:9 v/v) was selected as the pre-polymerization solvent. The electrochemical setup for polymerization was described in Chapter 2. Briefly, one drop ($\sim 100\ \mu\text{L}$) of pre-polymerization solution was placed on the gold surface to cover also the counting and reference electrodes. A schematic diagram is shown in Fig. 4-1. A Stoneheart BC 1200 high-performance potentiostat (Madison, CT) was used to apply a constant potential of +0.76 V on the gold surface.

Figure 4-1 Electrochemical preparation of MIPPy on SPReeta gold surface

4.2.2 Evaluation of Binding Properties

The MIPPy film was tested for binding with 0.05 to 1 ppm OTA in ethanol/water (1:9 v/v). Each sample solution was switched on-line by using a 6-way selection valve (V241, Upchurch Scientific, Oak Harbour, WA). The sample solution was introduced by a syringe pump (KD Scientific 100, New Hope, PA) at a flow rate of 3 mL/hr. The binding events were monitored by an increasing SPR angle. For pulsed elution (PE), 20 μ L of 1% acetic acid in methanol/water (1:9 v/v) was introduced through a 6-port low-pressure injection valve (V-451, Upchurch Scientific). The flow injection system

was shown in Fig. 4-2.

Figure 4-2 Flow injection system for evaluation of MIPPy binding properties.

4.3 Results and Discussion

4.3.1 MIPPy Film Growth and SPR Angle

MIPPy films were synthesized on the SPReeta gold surface at an applied potential of +0.76 V. The film growth was monitored by an increasing SPR angle. A typical sensogram for monitoring the growth of MIPPy film is shown in Fig. 4-3.

Figure 4-3 Typical sensogram for monitoring the growth of MIPPy film.

After the first 5 s of rapid growth, a moderate increase of SPR angle was observed indicating a slower film growth due to the poor conductivity of the film. The SPR angle stopped increasing as soon as the potential was switched off. Using the SPR angle for DDW on gold as a reference, the formation of MIPPy resulted in a shift from $69.1548 \pm 0.0005^\circ$ to $69.8627 \pm 0.0005^\circ$. A picture of typical MIPPy film electrodeposited onto the SPReeta gold sensing area is shown in Fig. 4-4.

Figure 4-4 A typical MIPPy film electrodeposited onto the SPReeta gold sensing area

4.3.2 MIPPy Binding Properties

After the formation of a MIPPy film on the SPReeta surface, DDW was used to rinse the flow cell. Note that the MIPPy film exhibited hydrophilic properties in comparison with the bare gold surface. The OTA template molecules were removed from the MIPPy film by pulsed elution (PE) with 1% acetic acid in methanol/water (1:9 v/v) at a flow rate of 3 mL/hr. As illustrated in Fig. 4-5, the acetic acid solution was able to elute OTA efficiently. After the PE, the baseline for DDW decreased by 0.008°

in SPR angle.

Figure 4-5 Multiple pulsed elutions of OTA template with 1% acetic acid in ethanol/water (1:9 v/v).

All data from the binding tests were analyzed by non-linear regression, as illustrated in Fig. 4-6, to determine the increase of SPR angle after a binding time of 300 s (Δ_{300}). In the lower concentration range from 0.05 to 0.5 ppm, Δ_{300} showed a good linear correlation ($R^2 = 0.9983$) with OTA concentration. Based on a signal-to-noise ratio of 3, the detection limit was estimated to be 0.01 ppm. As illustrated in Fig. 4-7, negative deviation from linearity became significant above 0.5 ppm.

Figure 4-6 Non-linear regression of binding data for 0.05 ppm OTA

Figure 4-7 Standard calibration curve for MIPPy-SPReeta determination of OTA.

For optimization of the binding conditions in OTA determination, a flow injection analysis (FIA) system was assembled with MIPPy-SPReeta as the detector. A syringe pump delivered the mobile phase flow over the MIPPy. OTA binding with MIPPy was determined by an increase of SPR angle after each sample injection (5 ppm OTA standard solution, 20- μ L aliquots). Multiple injections of 1% acetic acid in 1:9 methanol/water could effectively remove the bound OTA. This regenerated the MIPPy film surface, as shown in Fig. 4-8. Subsequently, multiple injections of the OTA sample caused step-by-step increases of SPR angle. This confirmed the regeneration of the MIPPy film.

Figure 4-8 Regeneration of MIPPy film surface by multiple injections of 1% acetic acid in 1:9 methanol/water (v/v).

Figure 4-9 Matrix interference effects of wheat and red wine extracts, in comparison with 10 ppm OTA.

4.3.3 Interferences from Matrix Components

When wheat and wine extracts were tested using DDW as the mobile phase for flow injection analysis (FIA), there were different increases in SPR angle. These results suggested that some matrix components could bind with the MIPPy through nonspecific interactions. One strategy for the elimination of nonspecific bindings was to change the eluent strength of the mobile phase, as illustrated in Fig. 4-9. It was found that nonspecific binding of matrix components from a wheat extract were eliminated when 1:1 acetonitrile/water (v/v) served as the mobile phase for flow injection analysis. The results demonstrated the selectivity of the MIPPy-SPReeta toward OTA was influenced

by the binding condition. Compared to Turner and co-workers [62], it was interesting to see the better selectivity of MIPPy was found in an aqueous/organic mixture. Note that the selectivity of MIPPy was demonstrated by the approach of real sample testing. In future investigations, it would be interesting to determine the cross-reactivity of OTA-MIPPy-SPReeta sensor against other mycotoxins, such as citrinin, that occur in different foods.

However, this new mobile phase was unable to eliminate interferences from wine matrix components. It suggested that the high concentration of acidic matrix components (such as tartaric acid, malic acid, and lactic acid) in wine extracts might bind with the MIPPy to cause interferences. These acidic interferent compounds would need sample pretreatment before MIPPy-SPReeta analysis. In solid phase extraction (SPE) applications, it is generally known that MIPs do not show their selectivity in the pre-concentration step because of the existence of both specific and non-specific interactions. A washing step is usually necessary for disrupting the non-specific interaction between the interferents and the MIP. Hence, optimization of the washing solvent composition is critical for the success of any MIP-SPE method development.

4.4 Conclusions

In this study, the MIPPy film was synthesized within 3 minutes by taking advantage of electrochemical polymerization *in situ* on the SPReeta sensor surface. The cost of OTA template was greatly reduced by using one drop (100 μ L) of pre-polymerization mixture to cover the effective sensing surface. The SPR angle increased when binding of OTA molecules occurred, within a linear correlation range from 0.05-0.50 ppm. These results demonstrated a novel combination of SPR device and MIP technology in terms of short preparation time, low consumption of print molecules, and real-time sensing. Unfortunately, the sensitivity of SPReeta was unable to detect real-world sample for OTA, which occurs at the ppb level. Sample preconcentration will be needed before the use of MIPPy-SPR for sensing. This conclusion redirected my research to the development of MIPPy on stainless steel frits for solid phase extraction, as described in the next several chapters.

Chapter 5 A Novel Micro Solid Phase Extraction Device Developed by Electropolymerization of Pyrrole onto Stainless Steel Frit Pore Surfaces

5.1 Introduction

In order to expand the application of molecularly imprinted polypyrrole (MIPPy) for solid phase extraction (SPE), a novel idea in combining electropolymerizable pyrrole (Py) and porous stainless steel frits was carried out to make a new micro solid phase extraction (μ SPE) device. In this work, the preparation of PPy onto a frit was first developed. The binding property was investigated by coupling the PPy-on-a-frit to a high performance liquid chromatography (HPLC) system.

PPy is one of the most functional materials for modern scientific applications in biochemical sensor, catalysis, corrosion resistance, chromatographic separation, energy storage, and ion exchange [71]. A thin PPy film can be electrochemical polymerized on the working electrode surface which can be utilized for solid phase extraction (SPE) [102,103]. PPy with high extraction efficiency toward polar compounds and aromatic compounds have been demonstrated [104]. High efficiency can also be achieved for anionic compounds due to the ion exchange property of PPy. Bagheri *et al* applied PPy as a sorbent for SPE of environmental pollutants from water [105]. Wu *et al* applied PPy for in-tube solid phase microextraction for the determination of amphetamine,

methamphetamine and their methylenedioxy derivatives in urine and hair [106]. Yu *et al* formed PPy films via electropolymerization on the gold surface of a miniaturized surface plasmon resonance device for detecting ochratoxin A (OTA) [107]. Recently, Qu *et al* reported on a well-ordered microstructure of polypyrrole standing upright on a stainless steel working electrode [108]. This introduced a new possibility of modifying stainless steel surfaces by electropolymerization of pyrrole for SPE applications. The ion exchange property of PPy has been recently investigated by Wu *et al* [106]. PPy was formed in its oxidized form carrying positive charges. The counter anions were incorporated into the film from the electrolyte. They determined the ion exchange property of the resultant polymer, e.g. ClO₄-doped PPy film exhibited cation exchange properties. The multifunction interactive properties (non-polar interactions, acid-base interactions, dipole-dipole interactions and hydrogen bonding) make polypyrrole a unique polymer as a solid phase extraction sorbent.

SPE cartridges and discs, designed for concentrating analytes or desalting buffer, have been miniaturized down to the μ L range [109]. The micro-disc mixed-mode (non-polar and strong cation exchange (SCX)) SPE cartridges for rapid sample extraction and enrichment was demonstrated by Li *et al* for the determination of fluoxetine and metabolites at low levels in rat serum [110]. Viberg *et al* packed C18 particles into a

capillary for the pre-concentration of heterocyclic aromatic amines at low levels in urine and blood [111]. They developed on-line coupling of in-capillary μ SPE with capillary electrophoresis (CE) and nano-electrospray ionization - mass spectrometry (nESI-MS). Micro sample volumes (0.25–1 μ L) and solvent consumption (5 μ L) were demonstrated as advantages.

High performance liquid chromatography (HPLC) with fluorescence detection is the most commonly used method for OTA determination in wines and foods [112]. Sample preparation and clean-up using SPE and immunoaffinity columns (IAC) afford good detection limits [113]. HPLC methods can also be used for the determination of OTA in animal tissues and human fluids. They are needed for monitoring studies of OTA occurrence in the food chain and for studies dealing with the OTA carry-over [114]. Testing on human urine samples, Domijan *et al* confirmed that humans were frequently exposed to OTA [115]. In this chapter, the use of PPy-on-a-frit for μ SPE of OTA is developed and evaluated. This μ SPE technique was readily coupled on-line by pulsed elution (PE) to HPLC. The μ SPE-PE-HPLC system was used to determine the binding performance of PPy-on-a-frit. This new method was successfully demonstrated for the analysis of OTA-spiked red wine, beer, and orange juice.

5.2 Experimental

5.2.1 Preparation of PPy Modified Stainless Steel Frits

Pyrrole was electrochemically polymerized onto a stainless steel frit with a pore size of 2 μm (Upchurch Scientific, Oak Harbour, WA). The concentrations of Py, TBAP were both 0.1 M in the pre-polymerization mixture using acetonitrile as solvent. For electrochemical preparation of PPy, the frit was connected to serve as the working electrode. A platinum counter electrode and a Ag/AgCl reference electrode were used to complete a three-electrode setup for the electropolymerization at a potentiostatic mode, as shown in Fig. 5-1. A constant potential of +0.85 V was applied on the frit for PPy electrodeposition onto the frit surface. The applied potential was switched off after 30 minutes of polymerization. PPy was observed as the frit turned into a dark brown color from the original shiny grey.

Figure 5-1 Preparation of PPy-on-a-frit by electrochemical polymerization

5.2.2 Evaluation of PPy for μ SPE

OTA standard solutions were prepared according to the procedure established by González-Peñas *et al* [116]. Different concentrations of OTA in the range from 0.1 to

10 pg/ μ L (or ppb) were tested to evaluate the PPy-on-a-frit for μ SPE. Percentage binding of OTA to the PPy was calculated from the breakthrough peak areas measured with the PPy-on-a-frit versus a blank frit.

5.2.3 On-line μ SPE-PE-HPLC Instrumentation

A schematic diagram of the on-line μ SPE-PE-HPLC system is shown in Fig. 5-2.

Water was used as the mobile phase for μ SPE and PE, at a flow rate of 0.5 mL/min.

Figure 5-2 Schematic diagram of on-line μ SPE-PE-HPLC system.

After an OTA standard solution or spiked sample was loaded onto the PPy-on-a-frit, 20 μ L of 1% TEA in acetonitrile was injected to pulsed elute any and all

extracted compounds from the frit. When the extract reached the sample loop of the second injector valve, the valve was switched. The HPLC mobile phase carried the extract over to the HPLC column for separation, with fluorescence detection. A calibration curve was constructed from the peak areas obtained in μ SPE-PE-HPLC analysis of the OTA standard solutions mentioned above.

5.2.4 μ SPE-PE-HPLC Analysis

Red wine, beer and orange juice were purchased from the local market in Ottawa. Before μ SPE-PE-HPLC analysis, each sample was spiked with 10 ppb OTA. Such spiking facilitated the confirmation of chromatographic separation between OTA and all extracted matrix components. Recovery was calculated by comparing the OTA peak area from spiked samples with the peak area for the OTA standard solution.

5.3 Results and Discussion

5.3.1 PPy-on-a-frit for μ SPE of OTA

Orchratoxin A (OTA) is an acidic molecule. The carboxylic group has a $pK_a = 4.4$ and the phenolic group has a $pK_a = 7.1$ [117]. For the μ SPE of OTA, 1% acetic acid was added to the sample solution to prevent acid dissociation. 20 μ L of standard solutions were injected to test for binding of OTA. The % binding of OTA was calculated by dividing the breakthrough peak area obtained with PPy-on-a-frit by the breakthrough peak area obtained with a blank frit. It was found that the μ SPE with PPy-on-a-frit afforded 80% recovery of OTA at the concentration level of 10 ppb. This % recovery was deemed adequate for quantitative analysis of real-world samples. More importantly, it was achieved within a short residence time of 1.2 s under a flow rate of 0.5 mL/min. A comparison of OTA breakthrough peak areas was shown in Fig. 5-3 for different concentrations.

Figure 5-3 Comparison of % recoveries in μ SPE for different OTA concentrations

It is worth to note that OTA in anionic form was expected to be more efficiently incorporated than the neutral form based on the anion versus cation exchange properties of PPy. However, a higher % recovery was found at low pH (= 2.5). No significant correlation was found between OTA % recovery and the pH of loading mobile phase. This result suggests that extraction of OTA by the PPy might not be controlled by the ion exchange properties of PPy. Rather, the extraction was dominated by hydrophobic interactions and hydrogen bonding with the PPy.

In OTA HPLC analysis, most authors use a mobile phase containing a mixture of either acetonitrile or methanol with water. We used acetonitrile because it was equally effective as methanol in OTA resolution. The HPLC separation of OTA with 20:80 v/v acetonitrile-ammonia buffer ($\text{NH}_4\text{Cl}/\text{NH}_3$, 20 mM, pH = 9.5) as the mobile phase gave good elution of OTA as a sharp peak with a retention time of 4.7 ± 0.1 min.

5.3.2 On-line μSPE -PE-HPLC

1% TEA in ethanol, methanol or acetonitrile was found to be effective for the pulsed elution (PE) of OTA from the PPy-on-a-frit. 1% TEA in acetonitrile provided the best PE, probably due to its hydrophobic property. When the 20- μL extract was carried over by the mobile phase to the HPLC column for separation at a flow rate of 1.0 mL/min, a retention time of 4.7 ± 0.1 min ($n=3$) was again obtained for OTA. The calibration curve for μSPE -PE-HPLC showed a good linearity from 0.1-10 pg/ μL . Since $y = 1772913(\pm 143280) x + 11756(\pm 14400)$ ($R = 0.9807$). The LOD and LOQ were 0.1 ppb and 0.3 ppb OTA, respectively. A typical μSPE -PE-HPLC chromatogram is shown in Fig. 5-4 (a) for 10 ppb OTA. The PE solution not only successfully eluted OTA from the PPy, but also regenerated its functionality for μSPE . No significant memory effect was observed after PE was conducted three times before the next sample loading. Thus, the PPy-on-a-frit was highly reusable.

Figure 5-4 μ SPE-PE-HPLC chromatograms for (a) 10 pg/ μ L OTA and (b) red wine spiked with 10 pg/ μ L OTA.

5.3.3 μ SPE-PE-HPLC Analysis of Red Wine, Beer, Orange Juice

The specificity of the on-line μ SPE-PE-HPLC method was checked by adding (or spiking) OTA working standard solution to real-world samples, which should result in an increase in peak height. A red wine spiked with 10 ppb OTA was analyzed by μ SPE-PE-HPLC. As shown in Fig. 5-4(b), OTA appeared at 4.7 ± 0.1 min with good resolution from the matrix components. This result clearly demonstrated the capability of PPy-on-a-frit to bind OTA in the presence of red wine matrix components during the μ SPE.

Analysis by μ SPE-PE-HPLC was next performed on an orange juice and a beer (each spiked with 10 ppb OTA). OTA was identified with good resolution for the

orange juice and the beer, as shown in Fig. 5-5(b) and 5-6(b). These results verified the capability of PPy-on-a-frit to bind OTA in the presence of orange juice and beer matrix components during the μ SPE. The recoveries of $70\pm3\%$ and $65\pm5\%$ were obtained from triplicate analyses. They are adequate for the quantification of 1-10 ppb OTA. Note that these two types of samples were chosen to cover the range from acidic to neutral matrices, with pH=3.97 for orange juice and pH=5.00 for beer (as compared with pH=2.00 for red wine) [118]. When the beer was acidified with 1 M HCl, the % recovery of μ SPE was enhanced to $80\pm5\%$. This enhancement was verified to not have arisen merely from the pH effect on OTA fluorescence (quantum yield) as direct analysis of the acidified beer (spiked) produced the same peak area as the original beer (spiked).

A well established method of OTA determination uses liquid-liquid extraction followed by immunoaffinity column (IAC) for extracts clean-up [119]. Due to the high cost of immunoaffinity columns (IAC), alternative solid phase extraction techniques are worth developing for the advantages of rapid analysis, low cost, and long shelf-life. Several SPE techniques have been investigated for OTA extraction [120,121], including molecularly imprinted solid phase extraction with mimic template imprinting [122] or direct template imprinting. The method of μ SPE-PE-HPLC has demonstrated a shorter analysis time, easier sample preparation, and smaller sample volume.

Figure 5-5 μ SPE-PE-HPLC chromatograms for orange juice sample (a) and sample spiked with 10 pg/ μ L OTA (b).

Figure 5-6 μ SPE-PE-HPLC chromatograms for beer sample (a) and sample spiked with 10 pg/ μ L OTA (b).

5.4 Conclusions

Electropolymerization of PPy on a stainless steel frit has successfully made a new μ SPE device. It was the frit that provided a porous structure and high surface area for 80% binding of OTA at the 10 ppb concentration level. This good recovery was achieved within a short residence time of 1.2 s under a flow rate of 0.5 mL/min. The binding capacity of two frits was estimated to be 2 ng of OTA, which was sufficient for the analysis of 20- μ L samples. On-line μ SPE-PE-HPLC results clearly demonstrated the capability of PPy-on-a-frit to bind OTA in the presence of red wine, beer and orange juice matrix components. The technique of PPy on a stainless steel frit for μ SPE is simple. As validated on-line with PE-HPLC in this work, the new method can be indifferently used in case of unexpected impurities from any sample matrix in food inspection and bio-monitoring programs. Future development should be focused on the improvement of μ SPE selectivity by altering the N-substitution of Py, changing the doping ions, modifying the mobile phase. The technique of molecular imprinting will be adapted in the next chapter.

Chapter 6 Molecularly Imprinted Polypyrrole for Micro Solid Phase Extraction and Preconcentraion of Ochratoxin A

6.1 Introduction

The modification of stainless steel frit surface by polypyrrole (PPy) was described and demonstrated a success for micro solid phase extraction in Chapter 5. Simple, rapid, and reusable PPy-on-a-frit coupled to HPLC was the main advantage of using PPy for the micro solid phase extraction of OTA. In this chapter, molecular imprinting technique is employed to improve the selectivity of PPy-on-a-frit. The new molecularly imprinted polypyrrole on a frit (MIPPy-on-a-frit) has been served as a preconcentration device for OTA extraction from red wine samples.

Recent trends in sample preparation include miniaturization, rapid processing, automation, high-throughput performance, on-line coupling with analytical instruments, and reduction in solvent volume [123]. PPy-modified stainless steel frits have been demonstrated for on-line μ SPE of OTA [124]. Functional polypyrrole with recognition toward alkaline cations was successfully synthesized by chemical modification of PPy [125]. The use of molecularly imprinted polymers (MIPs) as a selective solid phase for OTA sample preparation is potentially attractive, mainly because they afford selectivity, robustness, and long shelf lives. Maier *et al* adopted a dummy imprinting strategy using an OTA mimic as template [126]. Their control SPE experiments with the

corresponding NIP, however, gave similar sample clean-up effects and analyte recoveries. They have pointed out major challenges in the development of OTA MIPs, i.e. reusability, consistency in binding properties, OTA template bleeding, slow analyte binding kinetics, and cross-reactivity due to polymer swelling.

Electrochemical imprinting is a relatively new technique that has been reported by some research groups. One group used electropolymerization of 2-mercaptobenzimidazole (2-MBI) to prepare a MIP as the sensing layer on a capacitive chemical sensor for fenvalerate [127]. Another group prepared molecularly imprinted electropolymerized carbon electrodes for the binding of resorcinol [128]. Both reports adopted the molecular imprinting by the presence of template during the electropolymerization. These authors were careful in comparing the properties of a polymeric thin film with a bulk imprinted polymer. In this Chapter, a new molecularly imprinted polypyrrole (MIPPy) was prepared on stainless steel frits by electrochemical imprinting using Py as functional monomer, tetrabutylammonium perchlorate (TBAP) as electrolyte with the present of OTA in the prepolymerization mixture. These MIPPy-modified frits were easy to prepare, robust, and consistent in binding properties. They were used as a micro solid phase preconcentration (μ SPP) device for OTA prior to HPLC determination. A MIPPy- μ SPP-HPLC method was developed for the selective

pre-concentration of OTA in red wine analysis.

6.2 Experimental

6.2.1 Preparation of MIPPy-modified Stainless Steel Frits

75 μM OTA was present when Py was electropolymerized onto each stainless steel frit with a pore size of 2 μm (Upchurch Scientific, Oak Harbour, WA). The concentrations of Py, TBAP and EDGMA were 0.1 M, 0.1 M and 0.2 M, respectively, in the pre-polymerization mixture with acetonitrile as the solvent. Electrochemical preparation of MIPPy was achieved using a potentiostat, as described in Chapters 2 and 5. The applied potential was switched off when MIPPy with a dark brown color was observed. Similarly, PPy-EDGMA- and PPy-modified stainless steel frits were prepared, in the absence of OTA, to serve as two controls for comparison.

6.2.2 MIPPy- μSPP -HPLC Analysis of OTA

The HPLC setup is illustrated in Fig. 6-1. A 3-mL sample of 0.1-5.0 ppb (or ng/mL) OTA standard solution, red wine, or spiked red wine was pumped (KD Scientific 100, New Hope, PA, U.S.) through the MIPPy-modified stainless steel frit device from a syringe at a flow rate of 0.2-1.5 mL/min. 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 polar organics and HCl, the pre-concentrated OTA was eluted with 100-500 μL of 1% TEA/MeOH.

Figure 6-1 MIPPy-μSPP-HPLC instrumentation

The eluate was dried off-line under nitrogen gas, if necessary, and reconstituted by 100 μL 1%TEA/MeOH. For HPLC analysis of the eluate, 20:80 v/v acetonitrile-ammonia buffer ($\text{NH}_4\text{Cl}/\text{NH}_3$, 20 mM, pH = 9.2) was used as the mobile phase at a flow rate of 1 mL/min. Fluorescence detection of OTA and sample matrix components was performed at optimal excitation and emission wavelengths of 380 and 440 nm. The % recovery of OTA by the MIPPy and PPy control were calculated from OTA peak areas measured in MIPPy-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- μ L aliquot for the on-line regeneration of MIPPy. The MIPPy was finally washed with 200 μ L of DDW for surface re-conditioning.

After MIPPy- μ SPP-HPLC analysis of each red wine, the sample was spiked with 0.1 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.

6.2.3 IAC-HPLC Analysis of OTA

The use of immunoaffinity column (IAC) for OTA extraction from red wine samples had previously been reported by other research groups [129,130]. In this work, the IAC was activated with 5 mL PBS buffer/methanol (20 mM PBS buffer, pH=7.4; 0.55 g $\text{NaH}_2\text{PO}_4\cdot\text{H}_2\text{O}$ + 2.85 g $\text{Na}_2\text{HPO}_4\cdot 2\text{H}_2\text{O}$ + 9 g NaCl dissolved in 1 L of DDW/methanol, 9:1 v/v). Wine samples (3 mL) were passed through a 0.45- μ m filter and diluted with 3 mL of PBS buffer. They were sonicated for 5 min before loading over the IAC. Non-specific interferences were washed off by 1 mL PBS and 1 mL water. The final elution of OTA from the IAC was carried out using 2 mL of methanol. The eluate (100 μ L) was then analyzed by HPLC.

6.3 Results and Discussion

6.3.1 MIPPy-modified Stainless Steel Frits

As shown in Fig. 6-2, the 2- μm stainless steel frit exhibited a dark brown-color after MIPPy-modification. No significant back pressure was measured when a HPLC pump was used to drive water through the MIPPy frit, placed inside a frit housing, at a flow rate of 0.5 mL/min. It suggested the electrochemical imprinting technique successfully formed a thin coat of MIPPy on stainless steel surfaces without clogging the 2- μm porous channels.

Figure 6-2 Image of stainless steel frit: Original (left) and after MIPPy-modification (right)

In the conventional preparation of MIP monoliths, complete removal of the template molecules from the ground particles is generally difficult [122]. In this new development of electrochemical imprinting, the template was expected to be imprinted onto the pore surfaces of a stainless steel frit as a thin coat of MIPPy. Any bleeding caused by the slow diffusion of template molecules from the MIPPy would be minimal. It was expected that the MIPPy interacted with OTA molecules through hydrogen bonding and π - π interactions (see Fig. 1-5 on page 24). A strongly basic reagent ($pK_a > 10$) would be capable of removing the OTA template. Removal of OTA was observed when 1% TEA ($pK_a = 10.78$ [131]) in acetonitrile (or methanol) was tested as the elution solution. As shown in Fig. 6-3, OTA was effectively removed when 20 μ L of 1% TEA in acetonitrile was employed as the reagent in multiple pulsed elutions (PE). This suggested that TEA was not only able to disturb the hydrogen bonding between OTA and MIPPy, but also form an ion-pair complex with OTA in solution. Note that each PE collected all of the eluted OTA in a small volume of 20 μ L, producing a concentration that was relatively easy to monitor. In practice, complete removal of OTA was achieved more quickly by continually flushing the μ SPP device with 1% TEA in acetonitrile at a flow rate of 0.5 mL/hr until no OTA fluorescence could be detected in

the eluate.

Figure 6-3 Removal of OTA template from MIPPy by multiple PEs using 1% TEA in acetonitrile

Complete removal of OTA was achieved by continually flushing the MIPPy-modified 2- μm frit, housed in a μSPP device, with 1% TEA in methanol. At a flow rate of 0.5 mL/hr, the flushing was continued until no OTA fluorescence could be detected in the eluate. A large surface area of MIPPy was hence prepared, together with mass transport (or diffusion) distances shorter than 2 μm , to enhance the selective binding of OTA. No significant hindrance to sample flow due to back pressure

problems was noticed when a syringe pump was used at the tested rates from 0.2-0.5 mL/min.

6.3.2 MIPPy- μ SPP-HPLC

HPLC methods had previously been reported in the literature for the determination of OTA at 0.1-3.0 ppb levels in wine. One typical method involved SPE of OTA sample (100 mL) using a C18 cartridge and an additional preconcentration step (solvent evaporation from 5 mL to 0.4 mL under nitrogen stream) prior to HPLC with fluorimetric detection. The performance of the C18 cartridge compared very favorably with results of other published OTA studies that used immunoaffinity columns or solvent extraction techniques [132,133,134]. In the present work, several different carrier solvents were investigated (including methanol, ethanol, acetonitrile, water, and different ratios of acetonitrile/water) for the pre-concentration of OTA on the MIPPy. MIPPy- μ SPP-HPLC results showed that OTA had strong binding (and adequate % recovery) with the MIPPy in water. However, weak binding was observed in methanol, ethanol and acetonitrile. These binding properties were very favorable for fast elution of OTA after the preconcentration from red wine samples. A 0.1-3.0 mL of sample was pumped through the MIPPy for μ SPP. The eluate was next injected onto the HPLC column for analysis. The recovery of OTA was determined to be consistently 40-50%

when standard solutions of 0.5-ppb and 10-ppb OTA were tested, as shown by a common slope in Fig. 6-4. Based on HPLC analysis results, maximum OTA binding was reached in 6 min when a 10-ppb OTA standard solution was pumped through the MIPPy in a continuous flow at 0.25 mL/min. After pre-concentration, the fluorescence signal for the 10-ppb OTA solution was 12 times higher than that measured for a single 100- μ L aliquot injection of the same solution. The precision of MIPPy- μ SPP-HPLC analysis was good, with relative standard deviations at or better than 9.0 %.

Figure 6-4 Standard calibration curves for MIPPy- μ SPP-HPLC determination of OTA using 100 μ L and 500 μ L of 1% TEA in methanol for OTA elution.

6.3.3 Effects of Flow Rate and Elution Volume on MIPPy- μ SPP-HPLC

Different sample flow rates were investigated over a syringe pumping range from 0.2-1.5 mL/min wherein the MIPPy-modified stainless steel frit exhibited a fairly low back-pressure. The MIPPy- μ SPP-HPLC peak areas in Fig. 6-5 show that OTA was enriched by MIPPy- μ SPP at flow rates up to 0.5 mL/min. At higher flow rates, the HPLC peak area would decrease.

Figure 6-5 Flow rate effects on MIPPy- μ SPP-HPLC peak area, using 5 mL of 5-ppb OTA solution in water/ethanol (9:1 v/v).

The HPLC peak area increased linearly with the mass of OTA that was pumped through the MIPPy-modified frit in each analysis. Figure 6-6 shows the standard calibration curve obtained at a sample flow rate of 0.2 mL/min by varying either the OTA concentration or the sample volume. In one experiment, 100 μ L of 1% TEA/MeOH was used to elute the pre-concentrated OTA from the frit; in another experiment, 500 μ L was used. These results indicated that 100 μ L of 1% TEA/MeOH was adequate for the quantitative elution of up to 15 ng OTA. The use of 500 μ L of 1% TEA/MeOH did not elute any more OTA. 100 μ L was an optimal elution volume in that no further sample enrichment (drying under nitrogen gas and reconstitution) would be necessary.

6.3.4 Selectivity of MIPPy- μ SPP-HPLC for OTA over OTB

Ochratoxin B (OTB) was used as a structural analog for evaluating the selectivity of MIPPy in μ SPP. This compound differs from OTA only in the lack of a chlorine substituent group. It does not occur naturally in red wines, and its toxicity is ten times less than OTA. The MIPPy exhibited a 1.4-times higher selectivity for OTA over OTB in terms of their % recoveries that were determined separately. When these two compounds were applied as a mixture in the sample solution, the % recovery of OTB decreased resulting in a 2.8-times higher selectivity for OTA. As shown in Table 1, PPy-EDMA was evaluated as a control and it exhibited the same selectivity for both OTA and OTB. Last, PPy (without EDMA as a cross-linker) exhibited a slightly reverse selectivity for OTB over OTA. All these results are in support of molecular recognition, as built in by the electrochemical imprinting technique, specifically for OTA. They also suggest the possibility of using OTB as an internal standard in future analysis of red wines for OTA determination. It was observed that the HPLC peak for OTB showed up at 6.5 ± 0.1 min, allowing adequate separation from the OTA peak at 8.2 ± 0.1 min.

Although recoveries with the present method are lower than 10%, selective solid phase pre-concentration of OTA can attain recoveries higher than 95% by using high-capacity stainless steel frits for the MIPPy-modification. Note that recoveries for

OTA and OTB measured on not-imprinted frits are 3-times bigger than the same recoveries measured on imprinted frits. This capacity reduction seems to be related to the imprinting process. One plausible explanation is that the imprinting process decreased the hydrophobicity of MIPPy relative to PPy. This would make non-specific binding of OTA less favorable to occur while the specific binding of OTA was going strong.

Table 6-1 Selectivity of MIPPy- μ SPP-HPLC for OTA over OTB. Concentration = 0.5 ppb; sample volume = 3 mL

	% Recovery OTA	% Recovery OTB	Ratio of % Recoveries (OTA / OTB)
MIPPy- μ SPP-HPLC (OTA or OTB)	8.7 $\pm$ 0.1%	6.0 $\pm$ 0.1%	1.4 $\pm$ 0.1
MIPPy- μ SPP-HPLC (OTA and OTB)	9.0 $\pm$ 0.1%	3.1 $\pm$ 0.1%	2.8 $\pm$ 0.2
PPy-EDMA- μ SPP-HPLC (OTA or OTB)	27 $\pm$ 1%	27 $\pm$ 1%	1.0 $\pm$ 0.1
PPy- μ SPP-HPLC (OTA or OTB)	34 $\pm$ 1%	45 $\pm$ 1%	0.8 $\pm$ 0.1

6.3.5 MIPPy- μ SPP-HPLC Analysis of Red Wines

Figure 6-6 shows a typical chromatogram from the MIPPy- μ SPP-HPLC analysis of three 0.1-ppb-OTA-spiked red wine samples after pre-concentration of OTA from 3.0 mL of each wine. The OTA peak appeared at a retention time of 10.4 min, which was

well separated from the earlier peaks of wine matrix components.

Figure 6-6 Analysis of 0.1 ppb-OTA-spiked red wines by MIPPy- μ SPP-HPLC after enrichment of OTA from 3.0 mL of wine sample. All samples were acidified with 1 M HCl (1% by volume) to provide nearly quantitative recovery of OTA for μ SPP. After washing with 100 μ L of DDW to eliminate polar organics and HCl, the pre-concentrated OTA was eluted with 100 μ L of 1% TEA/MeOH.

Relative to the peak area obtained from direct HPLC analysis of 3-ppb OTA (using a 100- μ L sample loop), the recovery of OTA from the red wine matrix by MIPPy- μ SPP was determined to be 18.9% for an Ontario wine, 92.3% for a home made

wine, 114.3% for a French wine, and 1.7% for a Montreal wine. These results suggested that proper removal of the sample matrix would be needed before MIPPy- μ SPP for a better consistency of % recoveries from different red wine samples. Note that without proper matrix removal, IAC columns also exhibit low recoveries of OTA from red wine samples. The MIPPy could extract OTA at a % recovery 2-3 times higher than the non-imprinted PPy control, as observed in this work for 0.05 ppb OTA and detailed in Chapter 5 for ≥ 1 ppb OTA. More importantly, it extracted only half as much ($50 \pm 5\%$) of the major wine matrix components. These findings proved a significant enhancement of μ SPP selectivity due to the successful electrochemical imprinting. One advantage of such selectivity enhancement was that the OTA retention time could actually be reduced (to 8.4 min) by using a higher pH, % acetonitrile, or flow rate of the HPLC mobile phase, without causing any significant overlap of the OTA peak with the wine matrix component peaks.

MIPPy- μ SPP-HPLC readily afforded a detection limit at the concentration of 0.05 ppb OTA. This compares favorably with a detection limit of 0.2 ppb OTA for direct HPLC analysis. The ultimate merit of MIPPy- μ SPP was assessed by studying the sample volume effects on the OTA detection limit. Hypothetically, the lowest detection limit would be governed by the amount of co-enriched wine matrix components.

Different wine samples were analyzed by the MIPPy- μ SPP-HPLC using external calibration and standard additions. No significant concentration of OTA was detected in the Montreal wine sample. The Ontario, French and home-made wines were determined to contain OTA in the concentration range from 0.1-0.4 ppb. These MIPPy- μ SPP-HPLC analysis results are summarized in Table 6-2. Note that the different wines showed different recoveries. It is commonly known that this happens in red wine analysis for several extraction techniques (not only the MIPPy-based one) and it could be due to different polyphenols contents in each wine. Nevertheless, sample variability should be greatly reduced if variable recoveries are due to the wine matrix only.

Table 6-2 Determination of OTA in different red wines by MIPPy- μ SPP-HPLC using both external calibration and standard additions (from 0.05-1.00 ppb)

Red wine	OTA concentration by external calibration (ppb)	OTA concentration by standard additions (ppb)
Ontario	0.15 $\pm$ 0.05	0.18 $\pm$ 0.05
Home made	0.42 $\pm$ 0.05*	0.17 $\pm$ 0.05*
French	0.29 $\pm$ 0.05	0.14 $\pm$ 0.05
Montreal	Not detectable	Not detectable

* The OTA concentrations for the Home made red wine showed significant difference between the two methods. Further investigation would be required to determine the cause of the discrepancy.

Figure 6-7 Comparison of three standard calibration curves for direct HPLC, IAC-HPLC, and MIPPy- μ SPP-HPLC.

6.3.6 Comparison of MIPPy- μ SPP-HPLC with IAC-HPLC

Figure 6-7 compares three standard calibration curves for direct HPLC, IAC-HPLC, and MIPPy- μ SPP-HPLC. Within the OTA concentration range from 0.1-1.0 ppb, MIPPy afforded a higher sensitivity than IAC-HPLC when 3-mL OTA standard solutions were analyzed. The total extraction time for sample clean-up by MIPPy- μ SPP was 20 min, which is shorter than the 60 min required by IAC columns. Moreover, the MIPPy frit was fairly robust; it could be re-used over 200 real-world red

wine samples until it eventually became clogged. When it came to the analysis of red wines for OTA, the performance of MIPPy was different from IAC as summarized in Table 3. Note that low binding and recovery can be circumvented using an assembling of several electropolymerized frits.

In summary, for OTA analysis from red wine, the MIPPy frits offer several advantages: not as specific as IAC columns, more robust than IAC columns, reusable while IAC columns are not, lower in binding capacity than the IAC columns, half in % recovery compared with IAC columns, and shorter in total extraction time than IAC columns. However, the short-comings are out-weighted by the advantages of MIPPy frits.

Table 6-3 Comparison of MIPPy with IAC for OTA preconcentration from red wine samples

	MIPPy-modified frit	IAC column
Specificity (peaks appearing in HPLC chromatogram)	Other than the OTA peak, 2-3 peaks from wine matrix components appear at early retention times.	Only the OTA peak appears after the early methanol peak.
Robustness	Stable at various temperatures over a wide range of pH; long shelf life; good resistance to organic solvents	Stable at room temperature and neutral pH; short shelf life
Reusability	Good	Poor
Binding capacity	~ 3 ng / frit (multiple frits are feasible)	~ 200 ng / column
Recovery	Varied by matrix components; can reach a maximum of 50% (multiple frits are feasible)	Varied by matrix components; can reach a maximum of 90-100%
Total extraction time	~ 20 min / sample	~ 60 min / sample

6.4 Conclusions

In the presence of OTA, electropolymerization of Py on stainless steel frits has successfully modified them with MIPPy for selective μ SPP. Good selectivity for OTA was afforded by these frits due to the electrochemical imprinting technique. This selectivity is superior to our previous report on PPy frits for μ SPE of OTA [124]. MIPPy- μ SPP-HPLC results clearly demonstrated the capability of MIPPy to pre-concentrate OTA at 0.05 ppb and higher levels in red wine. The frits had sufficient binding capacity to accommodate the presence of abundant red wine matrix components. As validated in this work, the new method can be indifferently used for different red wine matrices, which is potentially valuable to inspection programs and monitoring studies.

Stainless steel frits represent a new spin-off technology that expands the use of MIPs for selectivity and sensitivity enhancement in biochemical and food analyses. The MIPPy-modified frits exhibit molecular recognition properties that are comparable with those reported for conventional MIPs, monoliths specific for OTA [62], and IAC columns. It does not need any time-consuming steps of grinding the bulk polymer and removing the template molecules. The relatively low % recovery of OTA from some red wine samples during the MIPPy- μ SPP would need continuing improvement. One promising approach is the use of high-capacity stainless steel frits for the MIPPy

modification.

Alternatively, commercially available nano-materials can be quite attractive.

The use of carbon nanotube as a substrate for polypyrrole is a common practice now in order to functionalize the surface of CNTs. In the next chapter, a unique idea that combines the new nano-material and MIP technology to overcome the limits on conventional preparation of MIP particles (for packing with sub-micron interstitial spaces) will be demonstrated.

Chapter 7 Molecularly Imprinted Polypyrrole on Carbon Nanotube for Micro Solid Phase Extraction

7.1 Introduction

The particles of an MIP material can be either packed into a micro-column for solid phase extraction (SPE), or possibly glued to the surface of a solid-phase micro-extraction (SPME) fiber. These efforts, while successful for selective extraction in bioanalytical applications, are hampered by difficulties associated with the low amounts of analyte that can be extracted. Packing MIP particles smaller than 10 μm into a micro-column would cause high back-pressures. Similarly, gluing MIP particles smaller than 10 μm onto a SPME fiber could be tricky. New fabrication techniques must hence be developed for molecularly imprinted solid phase extraction (MISPE) and molecularly imprinted solid phase micro-extraction (MISPME).

A microextraction fiber, coated at its tip with PPy as an extraction medium, was capable of concentrating drugs from circulating blood. Indeed, the PPy-coated wire could be autoclaved for sterilization. Wang *et al* demonstrated that conductive PPy coatings can be used for ionization of peptides without the addition of a matrix [135]. Their successful application of PPy coatings as both an extraction phase and a surface to enhance laser desorption and ionization (SELDI) has been developed for both small molecules and peptides.

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 [136]. It was found that CNTs possess a high electro-catalytic effect and a fast electron-transfer rate [137, 138, 139]. Recently, electrochemical studies revealed that the unique properties of CNTs make them very promising in the design of electrochemical sensors and biosensors [140, 141, 142]. In addition to the potential electrochemical applications of CNTs, their high conductance, tensile strength, chemical stability, ultra-small size and poor solubility make them potential candidates for development of novel analytical devices. Multi-walled carbon nanotubes (MWCNTs) have been immobilized on a glassy electrode to serve as a strong accumulation surface for adsorptive stripping voltammetry [143]. Cai *et al* demonstrated the application of MWCNTs for solid phase extraction (SPE) of organic pollutants in water [144].

The desirable merging of CNT (or MWCNT) and conducting polymer properties in composite form presents new opportunities to produce superior materials for novel applications. To date, most reports on electrochemically grown MWCNT-conducting polymer films have focused on those based on PPy since this conducting polymer offers a greater degree of electrochemical processing flexibility than most other commercially available conducting polymers. A uniform coating of PPy onto CNTs was

electrochemically synthesized by several research groups [145, 146, 147]. Nano sized CNT has been considered as the surface-enhancing component for PPy film. An open porous structure with high surface area of PPy-CNT was reported. A glucose sensor was developed, using PPy as glucose oxidase immobilization media, into a gold supported nanotube microelectrode array [148]. Large accessible enzyme loadings were obtained by the use of these three dimensional electrodes within an ultra-thin layer. PPy can also be synthesized on to CNTs using $(\text{NH}_4)_2\text{S}_2\text{O}_8$ as oxidant [149]. Compared to PPy, better thermal stability and higher charge delocalization properties were observed in the resulting PPy-CNT. Nano-porous composite films of multi-walled carbon nanotubes (MWCNTs) and either PPy or poly(3-methylthiophene) were grown using an electrochemical polymerization technique in which the nanotubes and conducting polymer were deposited simultaneously [150]. A biosensing strategy for electrochemical impedance spectroscopy for the measurement of DNA hybridization was developed by the doping of nucleic acid probes within a PPy film onto carboxylic group-functionalized MWCNTs [151]. Oligonucleotide probes were used as the counter anions during the growth of conducting PPy film on a CNT-modified electrode. Recently, Han *et al* electrochemically prepared a polypyrrole/MWCNT 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 MWCNTs. They found the MWCNTs were able to disperse in the film uniformly when mixed with DBSA prior to the adding of pyrrole monomer [152]. The electrochemical growth of MWCNT-conducting polymer composites offers the ability to produce 3-dimensional nano-structured films that combine the redox pseudo-capacitive charge storage mechanism of conducting polymers with the high surface area and conductivity of MWCNTs [153]. Such films should be very useful in the fabrication of micro-SPE (μ SPE) cartridges. It is noteworthy that composites of polyaniline and CNTs prepared similarly by electrodeposition were recently reported by Wu *et al* [154].

In this Chapter, molecularly imprinted polypyrrole (MIPPy) was synthesized in the presence of CNTs. MIPPy is defined as a molecularly imprinted polymer (MIP) that is prepared using pyrrole (Py) as the functional monomer. The ultra-high surface-to-volume ratio and nano-structured surface morphology were utilized to enhance the binding capacity of new MIPPy/CNT-modified stainless steel frits. The aim of this study was to develop a micro-solid phase preconcentration (μ SPP) device for the determination of ochratoxin A (OTA). OTA analysis has been extensively reported in the literature, at low levels (0.01-0.1 ppb) in red wine [155,156]. Due to the high complexity of wine matrices, a selective μ SPP device with minimal dead volume is

required for accurate OTA determination. The use of MIPPy/CNT-modified stainless steel frits in rapid extraction of small OTA amounts was compared with traditional μ SPE cartridges and MIPPy-modified stainless steel frits (without CNT). On-line coupling of μ SPP (for sample purification and analyte concentration during loading onto the injection valve) was chosen for high performance liquid chromatography with fluorescence detection (HPLC-FLD).

7.2 Experimental

7.2.1 Preparation of MIPPy/CNT-modified Stainless Steel Frits

MIPPy/CNT composite films were grown using an electrochemical method in which MIPPy and CNT were simultaneously deposited on a stainless steel frit with a pore size of 0.5 μ m and an active bed volume of 1.5 μ L (Upchurch Scientific, Oak Harbor, WA, USA). The concentrations of Py and TBAP were 0.1 M in the pre-polymerization mixture with acetonitrile as the solvent. 75 μ M OTA 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. CNTs were freely suspended in acetonitrile before polymerization. When partial oxidation was not used, the suspension was temporary in nature. As shown in Fig. 7-1, a platinum counter electrode and a Ag/AgCl reference electrode were used to complete a three-electrode setup for the

electropolymerization in a potentiostatic mode. The applied potential was switched off when MIPPy/CNT with a dark brown color was observed.

Figure 7-1 Electrochemical preparation of MIPPy with carbon nanotubes

Similarly, PPy/CNT-modified stainless steel frits were prepared, in the absence of 75 μ M OTA, to serve as a control for comparison. More details of the instrumentation for electrodeposition of MIPPy can be found in Chapter 5, 6 or in a separate report [55]. After the preparation of MIPPy/CNT-modified stainless steel frits, the OTA template was removed with 1% triethylamine in methanol. Note that the chemical nature of MIPPy/CNT-modified stainless steel frits used in the present work was essentially that of the top coating of MIPPy. The CNTs served as nano-structured fibers on top of the

stainless steel frit, which merely provided a solid substrate for building up the MIPPy/CNT composite material.

7.2.2 SEM Images of MIPPy/CNT-modified Stainless Steel Frits

Scanning electron microscopy (SEM) images were obtained by using JSM-5600LV SEM (JEOL, Japan). Stainless steel frit and MIPPy/CNT-modified frits were adhered onto SEM stage by a double-sided carbon tape for examination without carbon or gold sputtering. No damage of the polymer or charge accumulation was observed when the SEM images were taken at an accelerating voltage of 5 to 10 kV.

7.2.3 MIPPy/CNT- μ SPP-HPLC Determination of OTA

Samples were acidified with 1% by volume of 1 M HCl to provide nearly quantitative recovery of OTA for μ SPP. After washing the device with 100 μ L of DDW to eliminate polar organics and HCl, the pre-concentrated OTA was pulse-eluted with 100 μ L of 1% TEA in MeOH. The mobile phase transported the extracted analyte from the MIPPy/CNT-modified frit to the HPLC column. For HPLC analysis of the eluate, 20:80 v/v acetonitrile-ammonia buffer (20 mM $\text{NH}_4\text{Cl}/\text{NH}_3$, 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/CNT device and MIPPy control were calculated from

OTA peak areas measured in MIPPy/CNT- μ SPP-HPLC analysis versus direct HPLC analysis of the same 1.0-ppb OTA standard solution. After each μ SPP-HPLC analysis, 1% TEA in methanol was used in a 200- μ L aliquot for the regeneration of MIPPy/CNT. The MIPPy/CNT was finally washed with 100 μ L of 1 M HCl (1% by volume) for surface re-conditioning.

After MIPPy/CNT- μ SPP-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.

7.2.4 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 *et al* [157]. Briefly, one pump was used to elute an acidic mobile phase (water-acetonitrile-acetic acid, 98:2:1, 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 the same flow rate. The breakthrough volume was measured by multiplying the flow rate by the frontal time (when fluorescence signal reach half height of the mobile phase containing 50 ppb OTA)

between non-modified frit and modified frit. Note that the fluorescence detection condition was adjusted to 330 nm for excitation and 460 for emission to accommodate a better sensitivity for OTA in the acidic medium. After each run the modified frit was washed extensively with 1% TEA/methanol.

7.3 Results and Discussion

7.3.1 Preparation of MIPPy/CNT-modified Stainless Steel Frits

Given the bearing that the nanostructure of CNT-conducting polymer composite films has on their physicochemical properties, it is important to investigate how the film structure can be controlled via manipulation of the electrochemical growth conditions. The CNT content of the pre-polymerization dispersion was one of the crucial factors to be considered. When growing composite films of CNTs and MIPPy in the present work, a polymerization electrolyte was used in co-operate with 0.1 M pyrrole monomer and 0.05% by weight (or 0.5 mg/mL) of suspended CNTs in acetonitrile. There was no need to oxidize the suspended nanotubes using a 3:1 ratio of sulfuric acid and nitric acid refluxed at 130°C for 1 h as a surface functionalization technique. The resultant MIPPy/CNT films apparently showed good mechanical stability for μ SPP applications.

7.3.2 SEM of MIPPy/CNT-modified Stainless Steel Frits

Figure 7-2 displays typical SEM images of (a) a stainless steel frit (low

magnification), (b) a stainless steel frit before coating, (c) a MIPPy film deposited on a stainless steel frit, and (d) a MIPPy/CNT film deposited on a stainless steel frit. Electrodeposition of MIPPy with CNTs enabled them to be immobilized easily and stably on the stainless steel electrode. The nanotube loading in the MIPPy/CNT film was dictated largely by nanotube availability in the proximity of the growing film, as previously reported by Hughes *et al* [150]. The SEM image in (c) reveals that a homogeneous MIPPy film was obtained over the porous stainless steel frit. For the MIPPy/CNT composite film in (d), a second phase in which the CNTs were coated by only a few nanometers of MIPPy becomes apparent. The CNTs, with a diameter ranging from 30 to 80 nm, are randomly distributed on the porous stainless steel frit. As can be seen in Fig. 7-2(e) under high magnification, most of the CNTs are in the form of small bundles or single tubes. Such small bundles and single tubes well dispersed on the stainless steel substrate are believed to be very beneficial for the μ SPP performance, since most of their MIPPy-modified surfaces are accessible to OTA molecules. The electrodeposition also gave rise to low loadings of largely agglomerated CNTs in some areas. When dopant anions such as perchlorate were present in the polymerization electrolyte, the porosity of the PPy/CNT composite film produced was expected to be significantly reduced [150].

(a) SS frit

X250

(b) SS frit

X2,500

(c) MIPPy

X2,500

(d) MIPPy-CNT

X2,500

(e) MIPPy-CNT

X10,000

Figure 7-2 SEM images of (a) and (b) stainless steel frit (0.5 μm pores); (c) MIPPy-modified stainless steel frit; (d) and (e) MIPPy/CNT-modified stainless steel frit.

7.3.3 High Binding Capacity via Surface Area Enhancement

The electrochemical preparation of MIPPy/CNT-modified stainless steel frits is characterized by not only a large absolute surface area, but also a large surface-to-volume ratio, allowing for more efficient μSPP of OTA. It was observed that an increase in the

extraction rate and amount were obtained using this frit geometry. The higher surface-to-volume ratio resulted in fast extraction and desorption, all occurring within a very compact size of the frit with an active bed volume of 1.5 μL . Determination of specific surface area of the MIPPy/CNT-modified frit was failed to obtain by the commonly used B.E.T. technique due to the lack of proper adapter for the frit. Alternatively, a standard solution of OTA (50 ppb) was employed in frontal chromatography to determine the binding capacity of each and every frit. Frontal chromatography is a well-established measurement technique that takes both surface area and sub-surface porosity into account, yielding one single data [158,159]. Typical results are summarized in Table 7-1 for the binding capacities of frits that were modified by PPy, MIPPy, PPy/CNT, and MIPPy/CNT. The compositions of their pre-polymerization solutions are shown in Table 7-2. Note that MIPPy (or PPy/CNT) was studied as the corresponding control polymer without the presence of CNTs (or OTA template). Thus, the capacity (or the selectivity) provided by MIPPy/CNT can be properly attributed to the CNTs (or the molecular imprinting). In addition, a set of 8 different MIPPy/CNT-modified stainless steel frits yielded a relative standard deviation (RSD) of 3.6% in their binding capacity measurements. This RSD result indicated a good reproducibility of their electrochemical preparation.

Table 7-1 Binding capacities of frits (with 0.5 μm pores) modified by PPy, MIPPy, PPy/CNT, and MIPPy/CNT

Frit modification	PPy	MIPPy	PPy/CNT	MIPPy/CNT
Binding capacity (ng OTA)	312	573	437	2272
Uncertainty = ± 0.5 ng				

Table 7-2 Pre-polymerization solutions

	PPy	MIPPy	PPy/CNT	MIPPy/CNT
Pyrrole	0.1 M	0.1 M	0.1 M	0.1 M
TBAP	0.1 M	0.1 M	0.1 M	0.1 M
EDGMA	0.2 M	0.2 M	0.2 M	0.2 M
OTA		75 μM		75 μM
CNT			0.05% by weight	0.05% by weight

7.3.4 MIPPy/CNT- μ SPP-HPLC Analysis of Red Wine

Red wine samples are often monitored for the presence of specific compounds of interest. One general problem in the HPLC-FLD determination of OTA would be the interferences from fluorescent matrix components (such as flavonoids) that were usually present in red wines. If these components are selectively removed before introduction of a red wine sample to the HPLC instrument for analysis, the discrimination ability should be greatly enhanced. 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 SPE between OTA and structurally related compounds, a MIPPy/CNT-modified stainless steel frit was designed and fabricated. This frit had an internal volume that was optimal to achieve efficient desorption of the OTA analyte into a suitable eluate plug for HPLC. High selectivity was achieved by using the frit on-line for cleanup of red wine samples.

Each 100 μ L of sample was manually loaded from a syringe into the frit over approximately 20 s. The HPLC separation was performed in accordance with the literature report, using 20:80 v/v acetonitrile-ammonia buffer (20 mM $\text{NH}_4\text{Cl}/\text{NH}_3$) as the mobile phase at an optimum pH of 9.2 and a flow rate of 1 mL/min. From the chromatogram shown in Fig. 7-3, it can be seen that the interference of wine matrix components with

respect to 1 ppb of spiked OTA ($t_R = 8.8 \pm 0.1$ min) became practically negligible. However, using a sample volume of 100 μ L red wine for pre-concentration, it was only possible to determine OTA (by fluorescence detection) down to a detection limit ($S/N=3$) of 0.08 ppb, or a quantification limit ($S/N=10$) of 0.27 ppb.

Figure 7-3 Analysis of 100 μ L Red Wine

The MIPPy/CNT-modified stainless steel frit displayed a good combination of high binding capacity with excellent selectivity for μ SPE of OTA in red wine. When the frit was calibrated using standard OTA solutions, it was shown to produce a linear response and constant recovery (nearly 100% binding). Experimental results indicated that the frit was a fast and quantifiable means of trapping OTA in red wine. Its fast binding kinetics was explored for the cleanup of larger red wine samples (>100 μ L) to

increase the sensitivity of MIPPy/CNT- μ SPP-HPLC analysis. Figure 7-4 shows the OTA peak area responded to multiple 100- μ L loadings of the red wine sample spiked with 1 ppb OTA. From 1x100 μ L to 5x100 μ L, the calibration curve exhibits a linear response with a correlation coefficient (R^2) of 0.987. The detection limit was thus progressively improved by the increasing OTA peak area.

Figure 7-4 Analysis of Red Wine up to 500 μ L

Eventually up to 30x100 μ L of red wine (containing 1 ppb OTA) was analyzed, and the strong OTA peak area is plotted in Fig. 7-5. Hence using a sample volume of 3 mL red wine for pre-concentration, it was possible to determine OTA down to a detection

limit of 12 ppt, or a quantification limit of 41 ppt. Since the 3 mL of red wine loaded a total of 3 ng OTA onto the MIPPy/CNT-modified stainless steel frit, special investigations were pursued to determine the extent of the carryover in the extraction phase.

Figure 7-5 Analysis of Red Wine up to 3000 μ L

As shown in Fig. 7-6, the second pulsed elution (with 100 μ L 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 third pulsed elution yielded an OTA peak area as little as 1% of the first pulsed elution. These observations signify that a second pulsed

elution must always be made to clean out any residual OTA from the MIPPy/CNT-modified stainless steel frit. After that, even without making a third pulsed elution, the extent of carryover would be equivalent to 1% of 1 ppb (= 0.01 ppb) OTA. This especially low analyte concentration is already at the detection limit of MIPPy/CNT- μ SPP-HPLC for red wine analysis. A blank determination showed no detectable OTA peak at 8.8 ± 0.1 min.

Figure 7-6 Analysis of 3000 μ L red wine

A comparison was made with red wine analysis using a MIPPy-modified stainless steel frit (without carbon nanotubes) or commercially available immunoaffinity columns (IACs) for sample cleanup. Figure 7-7 shows the standard calibration curves

for the three different methods compare with that for direct HPLC analysis of the same red wine. The regression equations of these methods (with blank subtraction) are:

Direct HPLC $y = (119556 \pm 3151) x \quad r^2 = 0.9986$

IAC-HPLC $y = (307829 \pm 15702) x \quad r^2 = 0.9948$

MIPPy- μ SPP-HPLC $y = (506479 \pm 30649) x \quad r^2 = 0.9927$

MIPPy/CNT- μ SPP-HPLC $y = (496148 \pm 11496) x \quad r^2 = 0.9984$

Figure 7-7 The standard calibration curves for the IAC-HPLC, MIPPy- μ SPP-PE-HPLC and MIPPy/CNT- μ SPP-PE-HPLC methods compare with that for direct HPLC analysis.

It is obvious to note that MIPPy/CNT- μ SPP-HPLC and MIPPy- μ SPP-HPLC

offered the highest sensitivity for OTA determination in red wine. These two methods had almost identical sensitivities, based on the slopes of their regression equations. However, MIPPy/CNT- μ SPP-HPLC offered a larger linear dynamic range (up to 15 ppb OTA at least) due to its enhanced binding capacity for OTA. More comparison between MIPPy- μ SPP-HPLC and IAC-HPLC can be found in Chapter 6.

One of the main findings of this work is the improvement of the capacity of the MIPPy/CNT-modified stainless steel frit. Such capacity is really necessary to determine OTA in red wine samples. From our knowledge, OTA has been detected in red wine at 0.01-0.1 ppb, which means that using a sample volume of 3 mL, a MIP capacity of 0.3 ng would be necessary. However, we need a ten-, hundred- or thousand-times higher capacity for OTA analysis because red wines may contain a great abundance of matrix components that can readily bind onto the MIPPy-CNT surfaces in a non-specific mechanism. In other words, it is always important to have the extra capacity that ensures quantitative binding of all OTA molecules in the wine sample.

7.3.5 Stability of MIPPy/CNT-modified Stainless Steel Frits

The new MIPPy/CNT-modified stainless steel frit seemed to be stable and robust under ordinary lab operation conditions. From the observation, its binding capacity remained relatively constant over the first 15 days, but decreased to about 80% of the

original value after one month (used more than 80 times). Good long-term stability can be resulted from the stability of the classical technology of cross-linking the carbon nanotubes with PPy through EGDMA. When not in use, the frit was stored (either dry in air or wet in HPLC mobile phase) at room temperature. Leakage of OTA from the frit was monitored over storage time periods ranging from hours to days, as demonstrated by the PE data in Fig. 7-8. In good laboratory practice, a couple of pulsed elutions can readily clean out any OTA leakage prior to using the frit for MIPPy/CNT- μ SPP-HPLC analysis of red wine samples.

Figure 7-8 Time-dependent leaking of OTA from MIPPy/CNT-modified stainless steel frit.

7.4 Conclusions

The MIPPy/CNT-modified stainless steel frit was developed as a device that would be inexpensive to fabricate for on-line implementation of μ SPP-HPLC. A composite film of MIPPy and CNTs was grown using electrochemical polymerization, in which the conducting polymer and nanotubes were simultaneously deposited. The resultant frit exhibited a significant concentration capacity to better handle complex red wine matrices. Application of this new but simple nano-technology would lead to the development of novel extraction procedures and new sample preparation devices. More efforts can be focused on how to control the three-dimensional network of CNTs in different stainless steel devices. Different variables affecting the preparation of the modified frits are being optimized systematically. As PPy is an inert extraction phase that is biocompatible, MIPPy/CNT-modified stainless steel frits can potentially be used for pharmacokinetic, clinical, forensic, food safety, and environmental health analyses. Future applications could see better quality and more cost-effective analyses in these industries.

Chapter 8 Ion-Pairing HPLC Analysis of Red Wines by Multiple Pulsed Elutions of Ochratoxin A from Molecularly Imprinted Polypyrrole Modified Stainless Steel Frit with Carbon Nanotubes

8.1 Introduction

The occurrence of ochratoxin A (OTA) in red wine was first reported by Zimmerli *et al* in 1995 [119]. Since then, the development of analytical methods generally involved extraction of OTA by liquid–liquid extraction, clean-up on an immunoaffinity column (IAC), and determination by HPLC with fluorescence detection. An accurate and precise method was developed for determination of OTA in red, rosé, and white wine by Visconti *et al* [60]. After simple dilution with water containing polyethylene glycol and NaHCO₃, red wine samples were cleaned-up using IAC and analyzed by reversed-phase HPLC with fluorescence detection. This method was validated in a collaborative study. It has also been adopted by AOAC International and is being considered for adoption by the European Commission of Standardization (CEN) [160].

Nano-sized CNTs have been developed as the surface-enhancing component for PPy film to produce an open porous structure with high surface area [161]. Such films should be very useful in the fabrication of μ SPE cartridges. The ultra-high surface-to-volume ratio and nano-structured surface morphology were utilized to enhance

the binding capacity of these new MIPPy/CNT-modified stainless steel frits. The new MIPPy/CNT- μ SPP-HPLC results described in Chapter 7 clearly demonstrated a significant enhancement of selective binding capacity for OTA at levels below 1 ppb even in the presence of abundant red wine matrix components. In this chapter, due to the high complexity of wine matrices, the use of differential pulsed elution (DPE) or multiple pulsed elution (MPE) to eliminate some matrix interferences for accurate OTA determination is described. On-line coupling of μ SPE (for sample purification and analyte pre-concentration during loading onto the injection valve) with DPE or MPE was demonstrated for successful HPLC-FLD determination of OTA in three red wines from France, Canada and Bulgaria.

8.2 Experimental

The preparation and characterization of MIPPy/CNT modified frit was described in detail in Chapter 7. HPLC was operated under the optimized conditions as described in Chapters 6, and 7.

8.3 Results and Discussion

8.3.1 MIPPy/CNT- μ SPP-PE-HPLC-FLD Analysis of Red Wine

To enhance the binding differences in μ SPE between OTA and red wine matrix components, a MIPPy/CNT-modified stainless steel frit was fabricated based on the molecular imprinting principle. High selectivity was achieved by using the frit for cleanup of red wine samples on-line prior to HPLC analysis. Each 100 μ L of sample was manually loaded from a syringe into the frit over approximately 20 s. Pulsed elution (PE) coupled to HPLC separation was then performed in accordance with the conditions in Chapter 7. 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 of 0.08 ppb, or a quantification limit of 0.27 ppb. One obvious limitation was OTA peak broadening due to the PE chemistry.

8.3.2 Investigation of Pulsed Elution Chemistry

Standard solutions of 10 ppb OTA were prepared in different solvents for a systematic investigation of the PE chemistry. These solutions were analyzed by direct HPLC, and the OTA retention behaviour would simulate those obtained from actual PE with the corresponding solvents. As shown in Fig. 8-1, 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 in either the mobile phase (retention time = 6.5 min) or with 3% TEA in the mobile phase (retention time = 9.5 min). Presumably, three different ion-pairs were formed in these preparations: $[\text{NH}_4^+ \text{---} \text{OTA}^{2-} \text{---} \text{TEA}^+]$, $[\text{NH}_4^+ \text{---} \text{OTA}^{2-} \text{---} \text{NH}_4^+]$, and $[\text{TEA}^+ \text{---} \text{OTA}^{2-} \text{---} \text{TEA}^+]$. These results suggested that it would be best to use 1% TEA in the mobile phase for PE in red wine analysis by MIPPy/CNT- μ SPP-PE-HPLC-FLD. We may call the chemistry of $[\text{NH}_4^+ \text{---} \text{OTA}^{2-} \text{---} \text{TEA}^+]$ formation dual ion-pairing.

Figure 8-1 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 $\text{NH}_4\text{Cl}/\text{NH}_3$, pH = 9.2).

8.3.3 Differential Pulsed Elution and Multiple Pulsed Elution

In our study, one French red wine and one Canadian red wine did not pose any problem with OTA determination by MIPPy/CNT- μ SPP-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. 8-2. Obviously, analytical

selectivity must be enhanced for the purpose of eliminating all interference due to matrix components in the Bulgarian red wine [162]. One plausible approach was differential pulsed elution (DPE), which had been established in the previous works in our lab [163, 164, 165, 166].

Figure 8-2 MIPPy/CNT- μ SPE-PE-HPLC-FLD analyses of three red wines from France, Ontario (Canada) and Bulgaria, using 1% TEA in mobile phase for PE

DPE involved an intermediate wash step that would remove much of the wine matrix components from the MIPPy/CNT-modified stainless steel frit. Several organic

solvents were evaluated for their DPE effectiveness, by using 100 μ L of each solvent to wash the MIPPy before PE-HPLC-FLD analysis. As summarized in Fig. 8-3, acetonitrile was bad for eluting the bound OTA, but good for keeping peaks 1 and 3 (retention time = 2.39 min). Acetonitrile/mobile phase (1:1 v/v) was good for eluting all the peaks, including OTA. THF was bad for eluting peak 1 and OTA, but good for keeping peak 3. Dichloromethane and methanol were good for eluting the OTA, but bad for peaks 1 and 3. Ideally, DPE must eliminate all the wine matrix components quantitatively while keeping a good amount of OTA for HPLC-FLD detection.

Figure 8-3 Evaluation of organic solvents for their DPE effectiveness on OTA, by using 100 μ L of each solvent for washing the MIPPy/CNT-modified stainless steel frit prior to PE-HPLC-FLD analysis

In a new approach, 1% TEA in the mobile phase was used to perform DPE. The top chromatogram in Fig. 8-4 resulted from the MIPPy/CNT-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/CNT-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, the third chromatogram was obtained. This result came as a nice surprise 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/CNT-modified stainless steel frit. Further data analysis was carried out to subtract the second chromatogram from the first one, yielding a blank-subtracted chromatogram to reveal essentially the OTA peak. As can be seen in Fig. 8-5, its signal-to-noise ratio is only slightly better than the chromatogram obtained from the 2nd PE. This comparison clearly suggests that the 2nd PE provided a good opportunity to quantify OTA, after using the 1st PE for elimination of the wine matrix component. Note that the second chromatogram, required to serve as a blank for subtraction from the first one, is hardly available in real wine analysis. The new approach, now called double pulsed elution (DoPE), is uniquely based on both the strong molecular recognition property of MIPPy and the high OTA binding capacity of MIPPy/CNT-modified stainless steel frit.

Figure 8-4 Analysis of Bulgarian red wine, before and after spiking at 10 ppb OTA, by MIPPy/CNT- μ SPE-PE-HPLC-FLD using 1% TEA in mobile phase for multiple PE. Mobile phase = 20:80 v/v acetonitrile/ammonia buffer (20 mM $\text{NH}_4\text{Cl}/\text{NH}_3$, pH = 9.2).

Figure 8-5 Comparison of a blank-subtracted chromatogram (bottom) with the chromatogram obtained from 2nd PE (top).

8.3.4 Optimization of Pulsed Elution Chemistry

One limitation of the above DoPE approach (using 1% TEA in the mobile phase) was a small blank signal due to OTA bleeding from the MIPPy/CNT-modified stainless steel frit. In search of a better method for red wine analysis by PE-HPLC-FLD, the chemistry of pulsed elution was further optimized. A range of 0%-3%TEA in the mobile phase was evaluated for the 1st PE, and HPLC chromatograms obtained from the analysis of a 10-ppb OTA standard solution are shown in Fig. 8-6. These new results are very interesting in that the % TEA 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 pulsed elution.

Figure 8-6 HPLC chromatograms from analysis of 10-ppb OTA standard solution by MIPPy/CNT- μ SPE-PE-HPLC-FLD using 0%-3%TEA in mobile phase for 1st PE

8.3.5 Multiple Pulsed Elutions 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 = 6.92 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 interference 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: $[\text{NH}_4^+ - \text{OTA}^{2-} - \text{NH}_4^+] \leftrightarrow [\text{NH}_4^+ - \text{OTA}^{2-} - \text{TEA}^+] \leftrightarrow [\text{TEA}^+ - \text{OTA}^{2-} - \text{TEA}^+]$). 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/CNT- μ SPP-PE-HPLC-FLD using 2% TEA in the mobile phase. Figure 8-7 and 8-8 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 and the 3rd PE, as well as the summation of all three PEs, were used for repeating the standard additions

calculation. All these final results (for the original concentration of OTA in the Bulgarian red wine) are listed in Table 8-1.

Table 8-1 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)
1 st PE	13100	259649	396748	786995	0.89
2 nd PE	3875	92115	105878	222854	1.53
3 rd PE	7195	55552	39161	107860	2.82
1 st + 2 nd + 3 rd PEs	24170	407316	541787	1117709	1.18

Figure 8-7 Analysis of Bulgarian red wine, before and after spiking at 10 ppb OTA, by MIPPy/CNT- μ SPE-PE-HPLC-FLD using 2% TEA in mobile phase for multiple PEs: 1st PE chromatogram.

Figure 8-8 Analysis of Bulgarian red wine, before and after spiking at 10 ppb OTA, by MIPPy/CNT- μ SPE-PE-HPLC-FLD using 2% TEA in mobile phase for multiple PEs: 2nd PE chromatogram.

Based on the statistics presented in Fig. 8-9, the determination of OTA by MIPPy/CNT- μ SPP-PE-HPLC-FLD was best done using the peak area obtained from the 1st PE. Advantages of this new approach are rapid analysis (time $\sim$ 10 min), high sensitivity (slope = 37766), and good correlation ($R^2 = 0.9936$). An OTA concentration of 0.89 ppb was determined to be a conservative level in this difficult-to-analyze red wine.

Figure 8-9 Standard addition curves for OTA determination in Bulgarian red wine by MIPPy/CNT- μ SPE-PE-HPLC-FLD, using peak areas obtained from multiple PEs

8.3.6 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* [62]. 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/CNT- μ SPP-PE-HPLC analysis of a new red wine sample over the next 30 min. Hence, it was not necessary to adapt the concept of analyte mimicking (with OTB) as the direct imprinting procedure ensured the best possible recognition sites.

Finally, the new MIPPy/CNT-modified stainless steel frit seemed to be stable and robust under ordinary lab operation conditions. Should physical pore blocking eventually happen, the frit can easily be replaced by a new one (which costs \$3 each to purchase and modify with MIPPy/CNT).

8.4 Conclusions

The MIPPy/CNT-modified stainless steel frit was utilized as a μ SPE device for on-line HPLC-FLD determination of OTA in three different red wines. The frit possessed a high binding capacity that better handled the complex wine matrices. The OTA retention behavior was governed by the formation of three different ion-pairs: $[\text{NH}_4^+ \text{---} \text{OTA}^{2-} \text{---} \text{NH}_4^+]$, $[\text{NH}_4^+ \text{---} \text{OTA}^{2-} \text{---} \text{TEA}^+]$, and $[\text{TEA}^+ \text{---} \text{OTA}^{2-} \text{---} \text{TEA}^+]$. Two new techniques based on DoPE and MuPE were developed successfully for the elimination of wine matrix interferences. More importantly, the % TEA in the PE solution was found to have a scalable effect on the OTA retention time. The Bulgarian red wine was best analyzed by MIPPy/CNT- μ SPE-PE-HPLC-FLD using 2% TEA in the PE solution. A conservative OTA concentration of 0.89 ppb was finally determined in this difficult-to-analyze wine.

Chapter 9 Conclusions and Future Work

9.1 Sensor Development

In this thesis research, a novel combination of SPR device and electrochemical imprinting of PPy has been demonstrated to be an effective way for sensor development. Advantages are many in terms of ease of operation, short preparation time, low consumption of imprint molecules, and real-time monitoring of the imprinting process. The MIPPy modified SPR sensor responds to OTA molecules within a linear working range from 50-500 ppb. Future work should focus on the development of MIPPy-SPReeta sensors for other mycotoxins (such as fumonisin B₁ and zearalenone) using the electrochemical imprinting technique. Sequential versus parallel sensing configurations would be evaluated using multiple SPReeta devices for different mycotoxins. Another promising direction is to integrate the MIPPy with electrochemical sensors for mycotoxin analysis. For example, the changes in the resistive and capacitive elements within the MIPPy during interaction with target analytes can be determined by the measurement of impedance spectroscopy [167]. By using an extended frequency range (0.1-100 kHz) to monitor impedance functions, it should be possible to fine tune the sensor response to take real time measurements of the binding events.

9.2 MIPPy for SPE

In molecularly imprinted solid phase extraction (MISPE) applications, stainless steel frits have been successfully modified by MIPPy. Good selectivity (due to better affinity constant) for OTA was afforded by these frits as a result of the electrochemical imprinting to a level superior to non-imprinted PPy frits for μ SPE. MIPPy- μ SPE-HPLC results clearly demonstrated the capability of MIPPy to pre-concentrate OTA at 0.05 ppb and higher levels in red wine. Modification of stainless steel frits represents a new spin-off technology that expands the use of MIPs for selectivity and sensitivity enhancement in biochemical and food analyses. The MIPPy-modified frits exhibit molecular recognition properties that are comparable with those reported for conventional MIPs, monoliths specific for OTA, and IAC columns. It does not need any time-consuming steps of grinding the bulk polymer. Future work should be focused on the improvement of MIPPy- μ SPE selectivity by imprinting different kinds of target molecules, altering the N-substitution of Py, changing the doping ions, modifying the mobile phase, tailoring the choice of cross-linkers, and developing new chemistry for differential elution of the matrix components.

The MIPPy/CNT-modified stainless steel frit was proved effectively increased the total number of available specific binding sites within the frit. The composite film of

MIPPy and CNTs was grown using electrochemical polymerization, during which the conducting polymer and nanotubes were simultaneously deposited. The resultant MIPPy/CNT was equipped with a significant concentration capacity (and selective recognition for OTA) to better handle complex red wine matrices.

The latest results obtained in our lab show that the selectivity of MIPPy/CNT modified frits can be tailored by using different templates. A comparison of % recoveries of ethynylestradiol (EE2), estradiol (E2), and OTA from EE2-, E2-, and OTA-MIPPy/CNT modified frits are shown in Fig. 9-1. Using the ratio of %recovery results between the target molecule and any other molecule as an alternative indicator of selectivity, it strongly suggested that the better selectivities of different MIPPy-modified frits toward their target molecules were the benefit of molecularly imprinting. For example, under this definition, the PPy had a native selectivity = 1 for OTA over EE2 (i.e., it showed no selectivity). In comparison, OTA-MIPPy offered a better selectivity = 4 for OTA over EE2. This was an exciting finding because it would lead to a universal imprinting method that is applicable to a wide range of target (imprint) molecules. Application of this simple nano-technology would readily be transferred to the development of novel extraction procedures and new sample preparation devices.

Future work is expected to focus on how to control the three-dimensional

network of CNTs in different stainless steel devices, such as preparing the MIPPy/CNT inside a syringe needles, or on-line SPE device for the nano electrospray tips. As PPy is an inert extraction phase that is biocompatible, MIPPy/CNT-modified stainless steel frits can potentially be used for pharmacokinetic, clinical, forensic, food safety, and environmental health analyses. Future applications could see better quality and more cost-effective analyses in these industries.

Figure 9-1 % Recoveries of Ethynylestradiol (EE2), Estradiol (E2), and Ochratoxin A (OTA) from EE2-, E2-, and OTA- MIPPy/CNT modified frits. Data from a non-imprinted PPy/CNT frit is included for comparison.

References

- [1] K. Haupt, K. Mosbach, *Chem. Rev.* 100 (2000) 2495-2504.
- [2] L.I. Andersson, *J. Chromatogr B.* 745 (2000) 3-13.
- [3] P.S. Reddy, T. Kobayashi, M. Abe, N. Fujii, *Eur. Polym. J.* 38 (2002) 521-529.
- [4] S.A. Piletsky, E.V. Piletskaya, T.A. Sergeyeva, T.L. Panasyuk, A.V. El'skaya, *Sens. Actuators B* 60 (1999) 216-220.
- [5] W.M. Yeh, K.C. Ho, *Anal. Chim. Acta* 542 (2005) 76-82.
- [6] C.I. Lin, A.K. Joseph, C.K. Chang, Y.D. Lee, *J. Chromatogr. A* 1027 (2004) 259-262.
- [7] M.C. Blanco-Lopez, M.J. Lobo-Castanon, A.J. Miranda-Orrdieres, P. Tunon-Blanco, *Biosens. Bioelectron.* 18 (2003) 353-362.
- [8] T.L. Panasyuk, V.M. Mirsky, S.A. Piletsky, O.S. Wolfbeis, *Anal. Chem.* 71 (1999) 4609-4613.
- [9] B. Sellergren, *Molecularly Imprinted Polymers—Man-Made Mimics of Antibodies and Their Application in Analytical Chemistry*, Elsevier, Amsterdam, 2001.
- [10] J.O. Mahony, K. Nolan, M.R. Smyth, B. Mizaikoff, *Anal. Chim. Acta* 534 (2005) 31-39.
- [11] K. Karim, F. Breton, R. Rouillon, E. Piletska, A. Guerreiro, I. Chianella, S. A. Piletsky, *Adv. Drug Delivery Rev.* (2005) article in press.
- [12] L. Wu, K. Zhu, M. Zhao, Y. Li, *Anal. Chim. Acta*, 549 (2005) 39-44.
- [13] W.M. Mullett, E.P.C. Lai, *J. Pharm. Biomed. Anal.* 21(4) (1999) 835-843.
- [14] P.A.G. Cormack, A.Z.J. Elorza, *Chromatogr. B*, 804 (2004) 173-182.
- [15] M.J. Whitcombe, M.L. Vulfson, *Chromatographia*, 47 (1998) 457-464.
- [16] I.A. Nicholls, K. Adbo, H.S. Andersson, P.O. Andersson, J. Ankerloo, J. Hein-Dahlstrom, P. Jokela, J.G. Karlsson, L. Olofsson, J. Rosengren, S. Shoravi, J. Svenson, S. Wikman, *J. Mol. Recogn.* 11 (1998) 79-82.
- [17] V.S. Pande, Y.A. Grosberg, T. Tanaka, *Physica D*. 107 (1997) 316-321.
- [18] G. Wulff, *Angew. Chem. Int. Ed. Engl.* 34 (1995) 1812-1832.

-
- [19] T. Takeuchi, A. Dobashi, K. Kimura, *Anal. Chem.*, 72 (2000) 2418-2422.
- [20] I. Chianella, M. Lotierzo, S.A. Piletsky, I.E. Tothill, B.N. Chen, K. Karim, A.P.F. Turner, *Anal. Chem.* 74 (2002) 1288-1293.
- [21] S.A. Piletsky, K. Karim, E.V. Piletska, C.J. Day, K.W. Freebairn, C. Legge, A.P.F. Turner, *Analyst* 126 (2001) 1826-1830.
- [22] D. Pavel, J. Lagowski, *Polymer*, 46 (2005) 7528-7542.
- [23] D. Pavel, J. Lagowski, *Polymer*, 46 (2005) 7543-7556.
- [24] J. O'Mahony, A. Molinelli, K. Nolan, M.R. Smyth, B. Mizaikoff, *Biosens. Bioelectron.* 20 (2005) 1884-1893.
- [25] J. Haginaka, *Trends Anal. Chem.* 24 (2005) 407-415.
- [26] E. Luzi, M. Minunni, S. Tombelli, M. Mascini, *Trends Anal. Chem.* 22 (2003) 810-818.
- [27] J.L. Naffin, Y. Han, H.J. Olivos, M.M. Reddy, T. Sun, T. Kodadek, *Chem. Biol.* 10 (2003) 251-259.
- [28] S.Y. Feng, E.P.C. Lai, E. Dabek-Zlotorzynska, S. Sadeghi, *J Chromatogr. A* 1027 (2004) 155-160.
- [29] S.N. Zhou, E.P.C. Lai, J.D. Miller, *Anal. Bioanal. Chem.* 378 (2004) 1903-1906.
- [30] S.G. Wu, E.P.C. Lai, P.M. Mayer, *J Pharm. Biomed. Anal.* 36 (2004) 483-490.
- [31] M. Liu, Z. Zenf, H. Fang, *J Chromatogr. A* 1076 (2005) 16-26.
- [32] H.J. Liang, T.R. Ling, J.F. Rick, T.C. Chou, *Anal. Chim. Acta* 542 (2005) 83-89.
- [33] K.C. Ho, W.M. Yeh, T.S. Tung, J.Y. Li, *Anal. Chim. Acta*, 542 (2005) 90-96.
- [34] N. Pérez, M.J. Whitcombe, E.N. Vulfson, *Macromolecules* 34 (2001) 830-836.
- [35] H.H. Yang, S.Q. Zhang, F. Tan, Z.X. Zhuang, X.R. Wang, *J. Am. Chem. Soc.* 127(5) (2005) 1378-1379.
- [36] J. Matsui, K. Akamatsu, S. Nishiguchi, D. Miyoshi, H. Nawafune, K. Tamaki, N. Sugimoto, *Anal. Chem.* 76 (2004) 1310-1315.
- [37] W.Y. Chen, C.S. Chen, F.Y. Lin, *J. Chromatogr. A.* 923 (2001) 1-6.
- [38] D.A. Spivak, R. Simon, J. Campbell, *Anal. Chim. Acta*, 504 (2004) 23-30.

-
- [39] E. Turiel, A. Martin-Esteban, *Anal. Bioanal. Chem.* 378 (2004) 1876-1886.
- [40] Z.H. Liu, Y.L. Xu, C. Yan, R.U. Gao, *J. Chromatogr. A* 1087 (2005) 20-28.
- [41] L. Ye, K. Mosbach, *React. Funct. Polym.* 48 (2001) 149-157.
- [42] A.G. Mayes, K. Mosbach, *Anal. Chem.* 68 (1996) 3769-3774.
- [43] M.C. Blanco-Lopez, M.J. Lobo-Castanon, A.J. Miranda-Ordieres, P. Tunon-Blanco, *Trends Anal. Chem.* 23-1 (2004) 36-48.
- [44] A. Ersoz, V.G. Gavalas, L.G. Bachas, *Anal. Bioanal. Chem.* 372 (2002) 786-790.
- [45] H. Shiigi, M. Kishimoto, H. Yakabe, B. Deore, T. Nagaoka, *Anal. Sci.* 18 (2002) 41-44.
- [46] A. Ramanaviciene, A. Ramanavicius, *Biosens. Bioelectron.* 20 (2004) 1076-1082.
- [47] B.S. Ebarvia, S. Cabanilla, F. Sevilla, *Talanta* 66 (2005) 145-152.
- [48] V. Stefov, L. Pejov, *J. Mol. Struct.* 649 (2003) 231-234.
- [49] M.A. Muñoz, M. Galán, L. Gómez, C. Carmona, P. Guardado, M. Balón, *Chem. Phys.* 290 (2003) 69-77.
- [50] J.L. Sessler, G. Berthon-Gelloz, P.A. Gale, S. Camiolo, E.V. Anslyn, P. Anzenbacher, H. Furuta, G.J. Kirkovits, V.M. Lynch, *Polyhedron* 22 (2003) 2963-2983.
- [51] B. Deore, Z. Chen, T. Nagaoka, *Anal. Chem.* 72 (2000) 3989-3994.
- [52] H. Okuno, T. Kitano, H. Yakabe, M. Kishimoto, B.A. Deore, H. Siigi, T. Nagaoka, *Anal. Chem.* 74 (2002) 4184-4190.
- [53] H. Shiigi, D. Kijima, Y. Ikenaga, K. Hori, S. Fukazawa, T. Nagaoka, *J. Electrochem. Soc.* 152 (2005) 129-134.
- [54] J.C.C. Yu, E.P.C. Lai, *React. Funct. Polym.* 63 (2005) 171-176.
- [55] J.C.C. Yu, S. Krushkova, E.P.C. Lai, E. Dabek-Zlotorzynska, *Anal. Bioanal. Chem.* 382 (2005) 1534-1540.
- [56] Y. Saito, K. Jinni, *J. Chromatogr. A* 1000 (2003) 53-67.
- [57] K.M. Kim, J.W. Hur, S.I. Jung, A.S. Kang, *Electrochim. Acta* 50 (2004) 858-867.
- [58] H. Lord, R. Grant, M. Walles, B. Incledon, B. Fahie, J. Pawliszyn, *Anal. Chem.* 75 (2003) 5103-5115.

-
- [59] E.H. Soufleros, C. Tricard, E.C. Bouloumpasi. *J. Sci. Food Agric.* 83 (2003) 173-179.
 - [60] A. Visconti, M. Pascale, G. Centonze, *J. Chromatogr. A* 864 (1999) 89–101.
 - [61] X. Zhang, E. T. Kang, K.G. Neoh, K.L. Tan, D.Y. Kim, C.Y. Kim, *J. Appl. Polym. Sci.* 60 (1996) 625-636.
 - [62] N.W. Turner, E.V. Piletska, K. Karim, M. Whitcombe, M. Malecha, N. Magan, C. Baggiani, S.A. Piletsky, *Biosen. Bioelectron.* 20 (2004) 1060-1067.
 - [63] C. Baggiani, G. Giraudi, A. Vanni, *Bioseparation* 10 (2001) 389-394.
 - [64] J. Homola, S.S. Yee and G. Gauglitz, *Sens. Actuators, B, Chem.* 54 (1999), 3
 - [65] H. Raether, *Phys. Thin Films*, 74 (1977) 237-244
 - [66] Z. Salamon, H. A. Macleod, G. Tollin, *Biochim. Biophys. Acta* 1331 (1997) 117–129
 - [67] http://www.aigproducts.com/surface_plasmon_resonance/spr.htm.
 - [68] R.J. Whelan, T. Wohland, L. Neumann, B. Huang, B.K. Kobilka, R.N. Zare, *Anal. Chem.* 74 (2002) 4570-4576.
 - [69] M. Suzuki, F. Ozawa, W. Sugimoto, S. Aso, *Anal. Bioanal. Chem.* 372 (2002) 301-304.
 - [70] Q. Huang, L. Gao, *J. Mater. Chem.* 13 (2003) 1517–1519.
 - [71] J. Wu, X. Yu, H. Lord, J. Pawliszyn, *Analyst* 125 (2000) 391-394.
 - [72] S. Sadki, P. Schottland, N. Brodie, G. Sabouraud, *Chem. Soc. Rev.* 29 (2000) 283-293.
 - [73] A. Ersoz, J.C. Ball, C.A. Grimes, L.G. Bachas, *Anal. Chem.* 74 (2002) 4050-4053.
 - [74] J.O. Iroh, W. Su, *J. Appl. Polym. Sci.* 85 (2002) 2757-2763.
 - [75] U. Sree, Y. Yamamoto, B. Deore, H. Shiigi, T. Nagaoka, *Synth. Met.* 131 (2002) 161-165.
 - [76] J.E.G. de Souza, F.L. dos Santos, B.B. Neto, C.G. dos Santos, M.V.B. dos Santos and C.P. de Melo, *Sens. Actuators B* 88 (2003) 246-259.
 - [77] J.N. Barisci, G.G. Wallace, M.K. Andrews, A.C. Partridge, P.D. Harris, *Sens. Actuators B* 84 (2002) 252-257.

-
- [78] W.M. Mullett, E.P.C. Lai, J.M. Yeung, *Methods* 22 (2000) 77-91.
- [79] X. Kang, Y. Jin, G. Cheng, S. Dong, *Langmuir* 18 (2002) 1713-1717.
- [80] K. Gaus, E.A.H. Hall, *Anal. Chim. Acta*, 470 (2002) 3-17.
- [81] L.E. Bailey, D. Kambhampati, K.K. Kanazawa, W. Knoll, C.W. Frank, *Langmuir*, 2002, 18, 479-489.
- [82] X. Kang, G. Cheng, S. Dong, *Electrochem. Commun.* 3 (2001) 489-493.
- [83] P. Guedon, T. Livache, F. Martin, F. Lesbre, A. Roget, G. Bidan, Y. Levy, *Anal. Chem.* 72 (2000) 6003-6009.
- [84] J.E. Garland, K.A. Assiongbon, C.M. Pettit, D. Roy, *Anal. Chim. Acta* 475 (2003) 47-58.
- [85] A. Otto, *Z. Phys.* 216 (1968) 398-410.
- [86] D. Cossement, F. Plumier, J. Delhalle, L. Hevesi, Z. Mekhalif, *Synth. Met.* 138 (2003) 529-536.
- [87] D. Kim, D. Lee, W.K. Paik, *Bull. Korean Chem. Soc.* 27 (1996) 707-712.
- [88] M.M.J. Ayad, *J. Appl. Polym. Sci.* 53 (1994) 1331-1337.
- [89] A. Cameron, D. Louise, H. Wayne, *Tetrahedron* 59 (2003) 2025-2057.
- [90] B. Dolly, S.J. Kenneth, *Curr. Opin. Chem. Biol.* 7 (2003) 434-442.
- [91] C.J. Percival, S. Stanley, A. Braithwaite, M.I. Newton, G. McHale, *Analyst* 127 (2002) 1024-1026.
- [92] M. Quaglia, E. De Lorenzi, C. Sulitzky, G. Massolinia, B. Sellergren, *Analyst* 126 (2001) 1495-1498.
- [93] S.A. Piletsky, S. Subrahmanyam, A.P.F. Turner, *Sensor Rev.* 21 (2001) 292-296.
- [94] K. Taniwaki, A. Hyakutake, T. Aoki, M. Yoshikawa, M.D. Guiver, G.P. Robertson, *Anal. Chim. Acta* 489 (2003) 191-198.
- [95] E. P.C. Lai, A. Fafara, V. A. VanderNoot, M. Kono, B. Polsky, *Can. J. Chem.* 76 (1998) 265-273.
- [96] A. Kugimiya, T. Takeuchi, *Biosens. Bioelectron.* 16 (2001) 1059-1062.
- [97] P. Li, Y. Huang, J. Hu, C. Yuan, B. Lin, *Sensors* 2 (2002) 35-40.

-
- [98] L.X. Wang , X.G. Li , Y.L. Yang, *React. Funct. Polym.* 47 (2001) 125–139.
- [99] R.A. Khalkhali , W.E. Price , G.G. Wallace, *React. Funct. Polym.* 56 (2003) 141–146.
- [100] D.S. Minehan, K.A. Marx, S.K. Tripathy, *J. Macromol. Sci. A* 38 (2001) 1245-1258.
- [101] F. Chen, G. Shi, M. Fu, L. Qu, X. Hong, *Synth. Met.* 132 (2003) 125-132.
- [102] J. Wu, J. Pawliszyn, *J. Chromatogr. A* 909 (2001) 37-52.
- [103] G. Liljegren, J. Pettersson, K. E. Markides, L. Nyholm, *Analyst* 127 (2002) 591-597.
- [104] J. Wu, Z. Mester, J. Pawliszyn, *Anal. Chim. Acta.* 424 (2000) 211-222.
- [105] H. Bagheri, A. Mohammadi, *J. Chromatogr. A* 1015 (2003) 23-30.
- [106] J. Wu, H. Lord, J. Pawliszyn, *Talanta* 54 (2001) 655-672.
- [107] J.C.C. Yu, E.P.C. Lai, *Synth. Met.* 143 (2004) 253-258.
- [108] L. Qu, G. Shi, J. Yuan, G. Han and F. Chen, *J. Electroanal. Chem.* 561 (2004) 149-156.
- [109] Alltech Chromatography Sourcebook Catalog 550 572-573, (2004).
- [110] K. M. Li, M. R. Thompson, I. S. McGregor, *J. Chromatogr. B* 804 (2004) 319-326.
- [111] P. Viberg, S. Nilsson, K. Skog, *Anal. Bioanal. Chem.* 378 (2004) 1729-1734.
- [112] L. Monaci, F. Palmisano, *Anal. Bioanal. Chem.* 378 (2004) 96-103.
- [113] D. Jornet, O. Busto , J. Guasch, *J. Chromatogr. A* 882 (2000) 29-35.
- [114] H. Valenta, *J. Chromatogr. A* 815 (1998) 75-92.
- [115] A.M. Domijan, M. Peraica, M. Miletic-Medved, A. Lucic, R. Fuchs, *J. Chromatogr. B* 798 (2003) 317–321.
- [116] E. González-Peñas, C. Leache, M. Viscarret, A. Pérez de Obanos, C. Araguás, A. López de Cerain, *J. Chromatogr. A* 1025 (2004) 163–168.
- [117] C. Dall'Asta, G. Galaverna, A. Dossena, R. Marchelli, *J. Chromatogr. A* 1024 (2004) 275–279.
- [118] <http://biology.clc.uc.edu/scripts/pHResl.pl>

-
- [119] B. Zimmerli, R. Dick, J. Chromatogr. B 666 (1995) 85-99.
- [120] J. M. Sáez, Á. Medina, J. V. Gimeno-Adelantado, R. Mateo, M. Jiménez, J. Chromatogr. A 1029 (2004) 125-133.
- [121] A. Aresta, N. Cioffi, F. Palmisano, C. G. Zambonin J. Agric. Food Chem. 51 (2003) 5232-5237.
- [122] J. Jodlbauer, N.M. Maier, W. Lindner, J. Chromatogr. A 945 (2002) 45-63
- [123] H. Kataoka, TrAC, 22 (2003) 232-244.
- [124] J.C.C. Yu, E.P.C. Lai, Anal. Bioanal. Chem. 381 (2005) 948-952.
- [125] H. Korri-Youssoufi, F. Garnier, A. E. Kassmi, P. Godillot, P. Srivastava Synth. Met. 84 (1997) 169-170.
- [126] N.M. Maier, G. Buttinger, S. Welhartizki, E. Gavioli, W. Lindner, J. Chromatogr. B 804 (2004) 103-111
- [127] J.L. Gong, F.C. Gong, Y. Kuang, G.M. Zeng, G.L. Shen, R.Q. Yu, Anal. Bioanal. Chem. 379 (2004) 302-307.
- [128] H. H. Weetall, K. R. Rogers, Talanta 62 (2004) 329-335.
- [129] J. Blesa, J. M. Soriano, J. C. Moltó and J. Mañes, J. Chromatogr. A 1054 (2004) 397-401.
- [130] M. Antonio, N. Carmine, T. Luca, D. L. Pietro, J. Wine Res. 14 (2003) 115-120.
- [131] <http://www.waters.com/watersdivision/pdfs/triethyl.pdf>
- [132] I. Stefanaki, E. Foufa, A. Tsatsou-Dritsa, P. Dais, Food Addit. Contam. 20 (2003) 74-83.
- [133] G.S. Shephard, A. Fabiani, S. Stockenström, N. Mshicileli, V. Sewram, J. Agric. Food Chem. 51 (2003) 1102-1106.
- [134] E.H. Soufleros, C. Tricard, E.C. Bouloumpasi, J. Sci. Food Agric. 83 (2003) 173-179.
- [135] Y. Wang, M. Walles, B. Thompson, S. Nacson, J. Pawliszyn, Rapid Commun. Mass Spectrom. 18 (2004) 157-162.
- [136] S. Iijima, Nature 354 (1991) 56-58.
- [137] R.S. Chen, W.H. Huang, H. Tong, Z.L. Wang, J.K. Cheng, Anal. Chem. 75 (2003)

- [138] R.R. Moore, C.E. Banks, R.G. Compton, *Anal. Chem.* 76 (2004) 2677-2682.
- [139] K.P. Gong, M.N. Zhang, Y.M. Yan, L. Su, L.Q. Mao, S.X. Xiong, Y. Chen, *Anal. Chem.* 76 (2004) 6500-6505.
- [140] J. Wang, M. Musameh, *Anal. Chem.* 75 (2003) 2075-2079.
- [141] S. Hrapovic, Y. Liu, K.B. Male, J.H.T. Luong, *Anal. Chem.* 76 (2004) 1083-1088.
- [142] M.G. Zhang, A. Smith, W. Gorski, *Anal. Chem.* 76 (2004) 5045-5050.
- [143] J. Wang, S.B. Hocevar, B. Ogorevc, *Electrochem. Commun.* 6 (2004) 176-179.
- [144] Y. Cai, G. Jiang, J. Liu, Q. Zhou, *Anal. Chem.* 75 (2003) 2517-2521.
- [145] G.Z. Chen, M.S.P. Shaffer, D. Colby, G. Dixon, W. Zhou, D.J. Fray, A.H. Windle, *Adv. Mater.* (2000) 522-527.
- [146] J.H. Chen, Z.P. Huang, D.Z. Wang, S.X. Yang, J.G. Wen, Z.F. Ren, *Appl. Phys. A* 73 (2001) 129-131.
- [147] J.H. Chen, Z.P. Huang, D.Z. Wang, S.X. Yang, W.Z. Li, J.G. Wen, Z.F. Ren, *Synth. Met.* 125 (2002) 289-294.
- [148] M. Gao, L. Dai, G.G. Wallace, *Electroanalysis* 13 (2003) 1089-1094.
- [149] J. Fan, M. Wan, D. Zhu, B. Chang, Z. Pan, S. Xie, *Synth. Met.* 102 (1999) 1266-1267.
- [150] M. Hughes, G.Z. Chen, M.S.P. Shaffer, D.J. Fray, A.H. Windle, *Compos. Sci. Technol.* 64 (2004) 2325-2331.
- [151] Y. Xu, Y. Jiang, H. Cai, P.G. He, Y.Z. Fang, *Anal. Chim. Acta* 516 (2004) 19-27.
- [152] G. Han, J. Yuan, G. Shi, F. Wei, *Thin Solid Films* 474 (2005) 64-69.
- [153] J.J. Gooding, *Electrochim. Acta* 50 (2005) 3049-3060.
- [154] M. Wu, G.A. Snook, V. Gupta, M. Shaffer, D.J. Fray, G.Z. Chen, *J. Mater. Chem.* 15 (2005) 2297-2303.
- [155] J.M. Sáez, A. Medina, J.V. Gimeno-Adelantado, R. Mateo, M. Jiménez J. *Chromatogr. A* 1029 (2004) 125-133.
- [156] C. Brera, J.M. Soriano, F. Debegnach, M. Miraglia, *Microchem. J.* 79 (2005) 109-113.

-
- [157] C. Baggiani, C. Giovannoli, L. Anfossi, C. Tozzi, *J. Chromatogr. A* 938 (2001) 35-44.
- [158] A. Tejeda-Mansir, R.M. Montesinos, R. Guzmán, *J. Biochem. Biophys. Methods*, 49 (2001) 1-28.
- [159] J. Gutenwik, B. Nilsson, A. Axelsson, *J. Chromatogr. A* 1048 (2004) 161-172.
- [160] A. Visconti, M. Pascale, G. Centonze *J. AOAC Int* 84 (2001) 1818-1827.
- [161] J. Wang, J. Dai, T. Yarlagadda, *Langmuir*. 21 (2005) 9-12.
- [162] A. Kulomaa, H. Sirén, M.L. Riekkola, *J. Chromatogr. A*, 781 (1997) 523-532.
- [163] W.M. Mullett, E.P.C. Lai, *J. Pharm. Biochem. Anal* 21 (1999) 835-843.
- [164] W.M. Mullett, E.P.C. Lai, *Microchem. J.* 61 (1999) 143-155.
- [165] W.M. Mullett, E.P.C. Lai, B. Sellergren, *Anal. Commun.* 36 (1999) 217-220.
- [166] W.M. Mullett, M.F. Dirie, E.P.C. Lai, H. Guo, X. He *Anal. Chim. Acta* 414 (2000) 123-135.
- [167] S.M. Park, J.S. Yoo, *Anal. Chem.* 75 (2003) 455A-461A