Analysis for Ochratoxin A by Molecularly Imprinted Solid Phase Extraction and Pulsed Elution

by

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ABSTRACT

Non-imprinted polymers were prepared from several functional monomers and a cross-linker. N-phenylacrylamide (PAM) yielded a cross-linked polymer with the highest affinity for ochratoxin A (OTA) in a variety of media. With water flowing at 0.5 mL/min, the condition of solid phase extraction was optimized using OTA in methanol/acetic acid (99:1 v/v) to achieve nearly 100% binding. Pulsed elution (PE) using methanol/triethylamine (99:1 v/v) was good for the quantitative desorption of OTA.

An OTA molecularly imprinted polymer (MIP) was synthesized using PAM. A molecularly imprinted solid phase extraction and pulsed elution (MISPE-PE) method was developed for the rapid analysis of wheat extracts for OTA. The MISPE–PE peak area was linearly related (R^2 = 0.9973) to the mass of OTA. This method with fluorescence detection afforded a detection limit of 5.0 ng/mL. Recovery of OTA from wheat extracts was 103±3%.

A rapid analytical method for the determination of ochratoxin A (OTA) in red wines was developed using online molecularly imprinted solid phase extraction, pulsed elution and ion-pair high performance liquid chromatography (MISPE-PE-HPLC) with fluorescence detection. An alkaline mobile phase (CH_3CN/NH_4OH, 15:85, v/v, pH = 9.8) for HPLC was employed to enhance OTA fluorescence, reproduce the short retention time of 3.65 min, and achieve a good separation of OTA from wine matrix components. This new method offered a detection limit of 0.05 ng/mL, a recovery of 89±4% from red wines, and a fairly linear calibration curve (R^2 = 0.9966) up to 100 ng/mL. Spartan software was used to predict the interactions between OTA and functional monomers using the atomic charges and the heats of formation.

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GLOSSARY

4-VP	4-Vinylpyridine
AIBN	2,2'-Azobisisobutyronitrile
BMA	*t*-Butyl methacrylate
CT	Citrinin
DDW	Distilled deionized water
DEAE-MA	[2-(Diethylamino)ethyl]methacrylate
DMAP-MAM	N-[3-(Dimethylamino)propyl] methacrylamide
DPE	Differential pulsed elution
EDMA	Ethylene glycol dimethacrylate
FI-SPE	Flow injection-solid phase extraction
FPE	Final pulsed elution
HOAc	Acetic acid
HPLC	High performance liquid chromatography
IAC	Immunoaffinity columns
MAAd	Methacrylamide
MeOH	Methanol
MIPs	Molecularly imprinted polymers
MISPE	Molecularly imprinted solid phase extraction
MISPE-PE	Molecularly imprinted solid phase extraction and pulsed elution

MISPE-PE-HPLC	Molecularly imprinted solid phase extraction, pulsed elution and ion-pair high performance liquid chromatography
NIPs	Non-imprinted polymers
NISPE	Non-imprinted solid phase extraction
NISPE-PE	Non-imprinted solid phase extraction and pulsed elution
OTA	Ochratoxin A
OTB	Ochratoxin B
PAM	N-phenylacrylamide
PE	Pulsed elution
QSAR	Quantitative structure-activity relationships
SPE	Solid phase extraction
TEA	Triethylamine
TFA	Trifluoroacetic acid
TFMAA	2-(Trifluoromethyl)acrylic acid
TOF	Tim-of-Flight
TRIM	1,1,1-Trimethylolpropane trimethacrylate
VI	1-Vinylimidazole

Publications

Simon N. Zhou, Edward P.C. Lai, J. David Miller, and Jianjun Li, "Analysis for Ochratoxin A in Red Wines by online Molecularly Imprinted Solid Phase Extraction, Pulsed Elution and Ion-pair High Performance Liquid Chromatography", Journal of Chromatography A, (submitted).

Simon N. Zhou, Edward P. C. Lai and J. David Miller, "Analysis of Wheat Extracts for Ochratoxin A by Molecularly Imprinted Solid Phase Extraction and Pulsed Elution", Analytical and Bioanalytical Chemistry, 378 (2004) 1903.

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Conference Presentations

Oral Presentation

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Poster Presentations

N.S. Zhou, E.P.C. Lai and J.D. Miller, "Analysis for Ochratoxin A in Wines by Online Molecularly Imprinted Solid Phase Extraction and Ion-pair High Performance Liquid Chromatography", poster # ANP 1317, 87th Canadian Chemical Conference, London, Ontario, May 29-June 1, 2004.

S.N. Zhou, E.P.C. Lai and David Miller, "Analysis of Ochratoxin A by Molecularly Imprinted Solid Phase Extraction - Pulsed Elution - High Performance Liquid Chromatography", 6th Annual Chemistry & Biochemistry Graduate Research Conference, Concordia University, November 14-15, 2003.

S.N. Zhou, E.P.C. Lai, J.D. Miller, Development of Functional Polymers for Rapid Solid Phase Extraction of Ochratoxin A on Micro-columns with Fluorescence Detection", poster # AE.6.P099, 39th IUPAC Congress and 86th CSC Conference, Ottawa, Ontario, August 10-15, 2003.

S.N.S. Zhou, E.P.C. Lai and D. Miller, "Development of Functional Polymers for Rapid Solid Phase Extraction of Ochratoxin A on a Micro-column with Pulsed Elution and Fluorescence Detection", poster # 157P, 49th International Conference on Analytical Sciences and Spectroscopy, Ottawa, Ontario, June 1-4, 2003.

CHAPTER I

INTRODUCTION

1.1 Molecularly imprinted polymers

1.1.1 History of molecularly imprinted polymers

The idea of molecular specificity that could be tailored in sorbents has been around for a long time. In 1931, Polyakov [1, 2] demonstrated that molecular specificity could be imprinted into silica gel by pretreating silicic acid with organic adsorbates before polycondensation. Later in 1949, Dickey [3, 4] performed more extensive investigations. Dickey precipitated silicic acid in the presence of alkyl orange dyes and found that after drying the hydrogel and removing as much of the excess imprinting agent as possible, the resulting xerogel had a greatly increased adsorption capacity for the particular dye present during gelation. In addition, the natural selectivity of silica gel for one dye in a series of azo dyes could be reversed by this pretreatment procedure. Modern molecular imprinting has its roots in Europe with the extensive investigations of Wulff and co-workers [5, 6], and Ramström and colleagues [7]. Sellergren [8] and a host of others have brought molecular imprinting to the forefront of selective chromatographic sorbents with potential applications in widely different fields such as selective sorbents in SPE for trace analysis, sorbents for scavenging purposes, and polymeric selectors for preparative chiral separations.

1.1.2 Concept of molecular imprinting

Molecularly imprinted polymers (MIPs) are highly stable polymers that possess recognition sites within the polymer matrix that are adapted to the three-dimensional (3-D) shape and functionalities of an analyte of interest [9, 10]. These molecularly imprinted polymers can be produced in a covalent or a non-covalent manner. In covalent imprinting, the print molecule — the so-called template — is chemically coupled with one of the building blocks of the

polymer, and, after the polymerization, the resulting bond must be cleaved to obtain free selective binding sites. The most common approach is non-covalent imprinting, which relies upon self-assembly between the template and carefully selected functional monomers. Polymerisable monomers are chosen so that self-assembly between template molecule and monomer(s) occurs [11]. Addition of cross-linker, porogen and radical initiator to this complex leads to the formation of a macroporous polymer network. After polymerization is complete, the template is extracted and cavities left behind which are complementary to the template molecule with regards to their geometric and electronic topology. The process of MIP binding the template molecule is called molecularly imprinted solid phase extraction (MISPE). Figure 1 depicts this entire synthesis process schematically.

The selective interactions between the template and the monomers are based upon hydrogen bonds as well as ionic and hydrophobic interactions. In general, the most selective binding occurs in organic solvents in which hydrogen bonding will strongly contribute to interactions with the analyte, although some templates are also well-recognized in aqueous media [12, 13]. For that reason, most polymers are produced in organic solvents, such as toluene, methylene chloride, chloroform and acetonitrile. Polymerization can yield either a monolith [14] that must be ground and sieved into the desired particle size range or monodisperse particles [15]. The latter approach is seldom used for producing molecularly imprinted polymers because it is generally less applicable. It is good to emphasize that both the morphology and the number of functional groups of the template (i.e. analyte) play important roles in selective recognition [16, 17].

To date, MIPs have been produced for recognizing templates as diverse as pharmaceuticals [18], sugars [19], peptides [20, 21], nucleotides [22], proteins [23], crystals [24] and even whole cells [25, 26, 27].
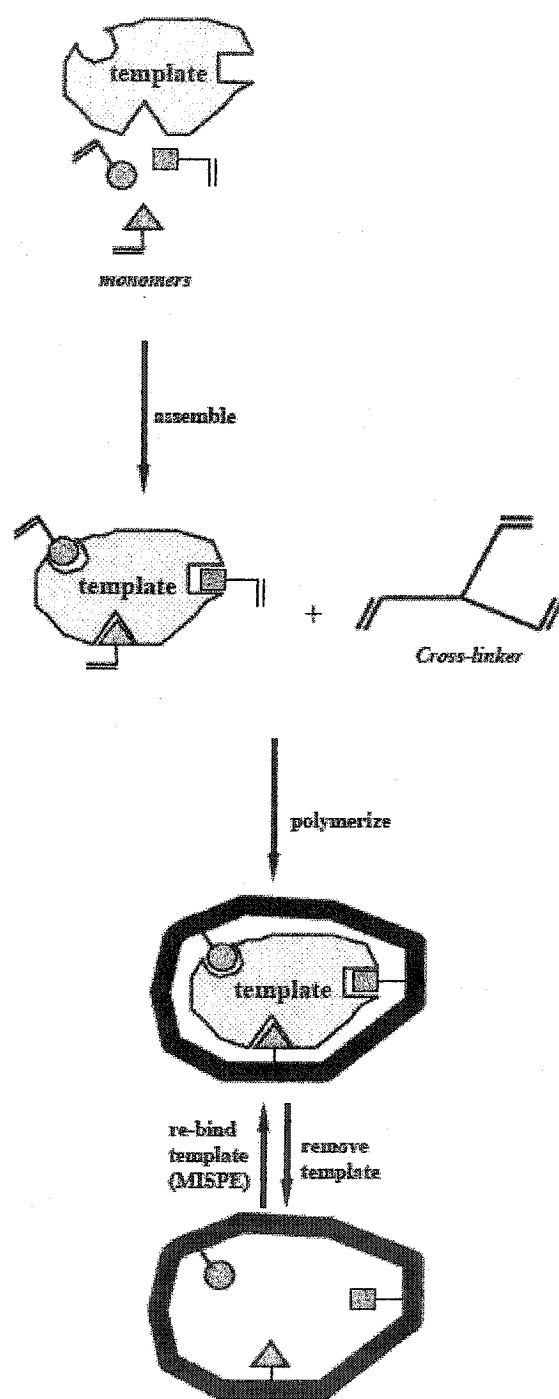
Figure 1. Principle of molecular imprinting

1.1.3 Process of making a molecularly imprinted polymer

Firstly, the print molecule is dissolved in the porogen together with functional monomers, which form solution interactions and pre-arrangement complexes with the print molecule [28]. The number and quality of the interactions is controlled by the specific functionalities maintained by the print molecule and the functional monomers. In most cases, ionic bonds may be formed as well as hydrogen bonds. In the following step, a large amount of crosslinking monomer such as ethylene glycol dimethacrylate (EDMA) is added together with the initiator 2,2'-azobisisobutyronitrile (AIBN). Since free-radical polymerization is inhibited by the presence of oxygen, the solution is normally purged with nitrogen at this stage. The radical polymerization is subsequently initiated by raising the temperature to 60°C or irradiating with UV light, thus starting the homolytic cleavage of AIBN to nitrogen and isobutyronitrile radicals. This radical formation then triggers the polymerization of the functional monomers and the crosslinker, which leads to the formation of a rigid polymer. The prepared MIP is subjected to a work-up scheme designed for the relevant application. If the polymer is made as a solid monolith, pulverization is normally performed, by grinding and sieving, resulting in particles with a required size. Before the MIP may be used for rebinding studies, one step remains - extraction of the original print molecule from the polymer matrix. A number of extraction protocols have been developed for different imprinting procedures. In most cases, washing the MIP with excessive amounts of methanol is appropriate. Finally, after drying, the molecularly imprinted polymer is ready for use. The process of making MIP is shown in Figure 2.
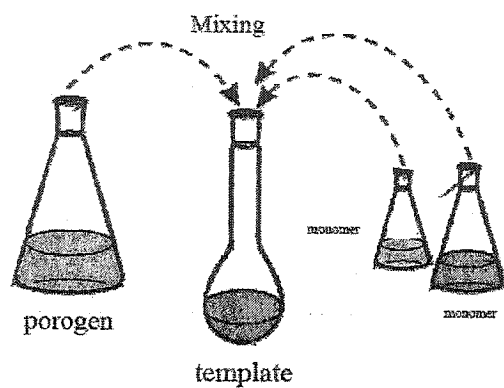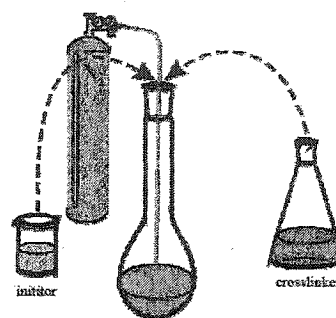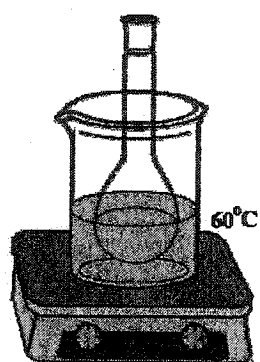
Figure 2. Process of making a molecularly imprinted polymer [28]

1.1.4 Molecularly imprinted solid phase extraction and pulsed elution

In analytical separation science, molecularly imprinted polymers have been applied in several analytical techniques, such as liquid chromatography, capillary electrochromatography and capillary electrophoresis, immunoassay, chemical sensors and solid phase extraction (SPE), which is a technique for extracting and purifying analytes in solution [1]. The basic concept for the application of MISPE is that the chromatographic parameters are tuned such that the MIP column traps only the analytes, or a group of structurally related compounds, whereas the other interfering components in the sample matrix were washed without retention. Sellergren in 1994 reported the first molecularly imprinted polymer used for solid-phase extraction [8]. The high selectivity of the polymer allowed pentamidine (a drug used to treat AIDS-related pneumonia) to be sufficiently enriched even when present in low concentration in a diluted human urine sample.

A benefit of MIPs is the possibility to prepare sorbents with selectivity pre-determined for a particular substance, or group of structural analogues. The most useful application is solid phase extraction for cleanup of environmental and biological samples [29]. Major advantages of MIPs in analytical method development include low material costs, as well as their chemical and physical stability. Advanced MIP technology enables the preparation of polymers with functionalities that recognize specific guest molecules in a wide range of SPE applications [30, 31, 32, 33, 34, 35, 36, 37, 38].

In 1998, Mullet and Lai firstly introduced pulsed elution (PE) combined with MISPE [30]. Pulsed elution is a technique that uses a small volume (< 50 µL) of strong solvent or solution to elute the analyte rapidly. In their work, the method made use of a MIP column for the on-line enrichment of theophylline and an injection of a small plug of methanol to produce a

rapid pulsed desorption of the analyte. The sample was applied in chloroform since in this solvent a complete binding of the analyte and good selectivity were observed. The matrix constituents and the potential interferences (e.g. other drugs) which were not recognized by the binding sites were rapidly eluted with chloroform. Then, a rapid and quantitative recovery of theophylline was accomplished in a pulsed format through injection of 20 µL of methanol. In this way, the eluted analyte could be detected using columns (0.8 mm i.d., 80 mm long), which offer the advantages of low consumption of solvents and fast analysis time [32]. With the application of 20-µL pulsed elution solution it was demonstrated that the non-specifically bound analytes could be removed and nicotine could be quantitatively recovered over a linear dynamic range of 1-1000 µg/L and with a detection limit of 1.8 µg/mL.

The method of MISPE-PE was further applied to other chemical analyses including 4-aminopyridine, cephalexin and metformin. In these works, MISPE selectivity trials were performed on MIP micro-columns under isocratic mobile phases such as 100% chloroform or acetonitrile. Pulsed elution was achieved with 20 µL aliquots of methanol or methanol containing an acidic modifier. MISPE-PE kept the advantage of low consumption of solvents and fast analysis time of less than 5 min [31, 32, 33, 34, 35, 36, 37, 39].

1.1.5 Characteristics of molecularly imprinted polymers

Apart from the more obvious recognition properties of molecularly imprinted polymers, their physical and chemical characteristics are highly appealing. These materials exhibit high physical and chemical resistance towards various external degrading factors. Thus, molecularly imprinted polymers are remarkably stable against mechanical stress, elevated temperatures and high pressures, resistant against treatment with acid, base or metal ions and stable in a wide

range of solvents. The storage endurance of the polymers is also very high: storage for several years at ambient temperature leads to no apparent reduction in performance. Further, the polymers can be used repeatedly, in excess of 100 times during periods of years without loss of the "memory effect". In comparison with natural, biological recognition sites, which are often proteins, these properties are highly advantageous [40].

1.2 Ochratoxin A

1.2.1 Introduction to ochratoxin A

Ochratoxin A (OTA), shown in Fig. 3, is a mycotoxin with nephrotoxic, carcinogenic, teratogenic and immunosuppressive properties [41]. A number of descriptive studies have found a correlation between the geographical distribution of Balkan endemic nephropathy and a high incidence of mortality from urinary tract tumors. In the only study in which OTA was measured, levels were higher in the blood of patients with Balkan endemic nephropathy and/or urinary tract tumors than in unaffected people; no distinction was made between the two diseases [42].
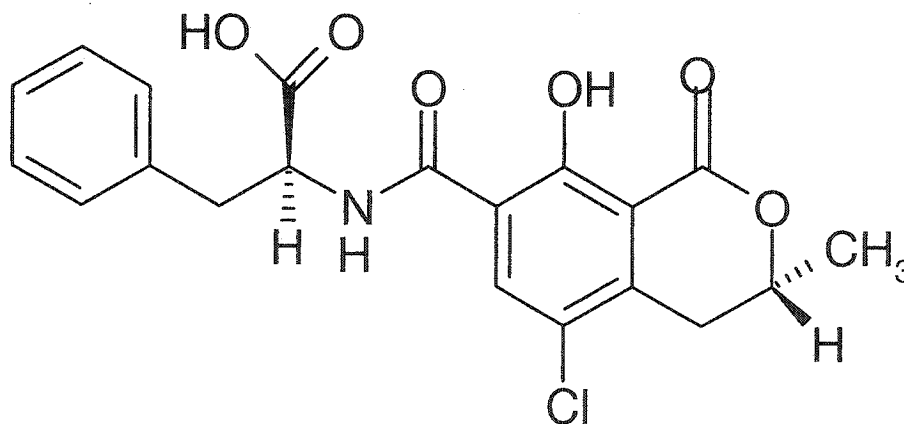
Figure 3. Structure of ochratoxin A (OTA)

OTA is a secondary metabolite produced by several *Aspergillus* and *Penicillium* species, which was first reported in 1965 [43]. Since *Aspergillus* and *Penicillium* species grow well on a variety of substrates under different conditions of moisture, pH and temperature, the natural occurrence of OTA in human food and animal feed is widespread and has been reported world-wide [44, 45, 46, 47]. Cereals and derived products are assumed to be the major dietary source

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of OTA. In addition, other products of vegetable origin, as nuts, beans, coffee, cocoa, spices, dried fruits and beer may contain OTA. The intake of OTA by contaminated feed may lead to residues in the blood, the kidney and the liver of pigs and of poultry and to a lesser extent in muscle tissue, adipose and eggs [47]. Thus, products of animal origin can contribute to the OTA-intake of humans. Therefore, the availability of reliable and sensitive methods for the determination of ochratoxin A is highly desirable to protect consumers from exposure to the mycotoxin.

1.2.2 Current analytical methods for ochratoxin A

OTA (M.W.: 403.8) is shown in Fig. 3, containing 7-carboxy-5-chloro-8-hydroxy-3,4-dihydro-3R-methylisocoumarin that is linked through the 7-carboxy group to L-β-phenylalanine by an amide bond [41]. Physical data of OTA are published by IUPAC [48]. It is a colorless, crystalline compound, soluble in polar organic solvents, slightly soluble in water and soluble in diluted aqueous bicarbonate solutions. For the analytical procedures, the character of OTA as a weak acid is important. The pK_a value for of the carboxyl group of the phenylalanine part is given as 4.4 [49], and the pK_a of the phenolic hydroxyl group as 7.3 [49] or a 7.05 [50].

Ochratoxin A is stable and resistant to heat; its solution in ethanol can be stored in the refrigerator for more than a year without loss [51]. Wood *et al* reported problems with the stability of OTA in methanol [52]. In the H. Valenta's laboratory, no OTA decomposition was observed in concentrated solutions in methanol over a period of some years when stored at −18 °C [41]. It is recommended to protect OTA solutions from light since decomposition occurs on exposure for several days to fluorescent light [47]. An alternative to the storage of OTA

standard solutions is to evaporate the solvent, store the toxin as a film in the glass vial and reconstitute it only when needed [47].

The major aspects of ochratoxin A analysis included sampling and sample storage, extraction, clean-up, detection and determination, and confirmation [47].

As with other moulds and mycotoxins, it is crucial that sampling is carried out in a way that ensures that ochratoxin A in the analytical sample is truly representative of the consignment. Failure to do this may invalidate the subsequent analysis [53]. In most cases, samples should be stored at -18°C. No study was found in the literature on the influence of the storage conditions on the OTA amount in naturally contaminated animal or human tissues or fluids. The stability of OTA in spiked cow's milk at very low concentration levels of 0.01–0.05 ng/ml was tested during storage at -18°C over a period of 6 weeks; no decrease of the OTA concentration was observed [54].

In most cases for ochratoxin A extraction, the method included a liquid–liquid extraction (chloroform–0.1 M sodium hydrogen carbonate), in which the OTA was retained in the high pH aqueous layer, followed by a second liquid–liquid extraction, in which the pH of the aqueous phase was lowered to 2.0 and the ochratoxin A was driven back into chloroform. The chloroform was evaporated to dryness and the analyte was reconstituted in the required solvent [55].

Sample cleanup is an essential part in analytical methods for mycotoxins. Generally, it is necessary to obtain low detection limits and to protect the high performance liquid chromatography (HPLC) column. The methods most frequently used in the extract cleanup are liquid–liquid and solid–liquid extraction; however, in the case of mycotoxins, the most important development in the field of clean-up methods until now is the use of immunoaffinity columns (IAC) [41]. The extract is poured onto a column filled with immobilized antibodies against the

specific mycotoxin. Other compounds in the sample are washed off by water or an aqueous buffer and the toxin is eluted by methanol or methanol-buffer. Many commercial IACs are available for ochratoxin A and most of the current analytical methods for the determination of OTA use IACs as a sample clean-up tool. Some of these methods maintain a previous process of liquid–liquid extraction with organic solvents as chloroform [56]. However, some authors have reported the direct application of food samples diluted with phosphate buffer onto the immunoaffinity column [57, 58] in order to reduce or eliminate toxic solvents in the analytical process.

Reversed phase HPLC with fluorescence detection is the most widely used procedure for separation and determination of OTA. A very low detection limit can be reached, due to the fact that OTA has natural fluorescence [41]. Generally speaking, HPLC methods can be sorted into two categories, i.e. ion-suppression chromatography and ion-pairing chromatography.

For the ion-suppression chromatography method, general conditions for the HPLC analysis have not changed in the recent years. RP-HPLC on C_{18} columns was used in nearly all methods. Columns with the standard size 250 x 4 or 4.6 mm were most often employed. As OTA is a weak acid, pKa is 3.5. The mobile phase in RP-HPLC must be acidic to avoid strong tailing and unspecific adsorption to the column. Mixtures of acetonitrile or methanol with diluted acetic or phosphoric acid were used as the mobile phase in most cases [41]. Acetonitrile was preferred, as acetonitrile-water mixtures have a lower viscosity and better separation efficiency than methanol-water mixtures [59]. Isocratic elution was used in most cases and, usually, the fluorescence detector is set to $\lambda_{ex} = 330$ nm and $\lambda_{em} = 460$ nm.

Ion-pair chromatography was employed in several reports with an increased sensitivity [60, 61, 62, 63]. C. Dall'Asta *et al* [63] carried out systematic studies in order to establish the

chromatographic conditions compatible with the variation of the mobile phase pH value. The classical mobile phase CH_3CN/H_2O (1:1 v/v), acidified with 1% CH_3COOH, was compared with the new one CH_3CN/ammonia buffer (NH_4Cl 20 mM, pH 9.8). These studies show that the use of an alkaline mobile phase in HPLC determination allows a 10-fold fluorescence enhancement.

1.2.3 Limitation of current methods for ochratoxin A

Sample treatment for OTA determination involves liquid-liquid extraction and/or solid-phase extraction (SPE) [64]. Liquid-liquid extraction is necessary for some samples. This approach has the disadvantage of intensive laboriousness, high solvent consumption, difficulty to automate, and low recoveries due to emulsion formation. Solid-phase extraction is preferred due to simplicity in operation and reduced solvent requirements, which is a concern of environmental conservation. However, SPE is less selective regarding molecular recognition.

IACs have the advantage that OTA is bound specifically to the antibody, thereby permitting almost total removal of the matrix. The benefits also include operational simplicity, rapidity, and reduction of the use of organic, potentially toxic solvents [64]. However, it is not without reason that immunoaffinity columns are used less widely for many different compounds because a selective antibody must be developed for each analyte including OTA. Moreover, the major disadvantage of the method is the high cost of the columns as well as the insufficient stability of the antibody. Developing a specific antibody is a long and arduous process that can take as long as 12 months. The column should not be used in case of denaturation of the antibodies, so operational and storage conditions are strictly required [65, 66, 67]. Furthermore, in the case of toxins additional complications arise because of their inherent toxicity to living species. Hence the technological difficulty arises in the antibody preparation when animal or

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hybridome technology is applied. Zimmerly and Dick [68] tried to solve this problem of high cost by reusing the columns, but others' experience showed that reused columns give some problems in the reproducibility of results [69]. Another problem with IACs is that they may not be completely specific because the ethyl ester of OTA is also retained, and yet another problem is the possibility that they may contain ochratoxin A as contaminant [70].

Reversed phase HPLC with fluorescence detection is the most widely used procedure for separation and determination of OTA in foods, feeds and biological fluids. But, in reported methods, the retention time for OTA is long, about 10 to 20 minutes.

The maximum level for ochratoxin A in raw wheat, barley and rye is 5 g/kg [71]. The guideline values are 0.5 μg/L and 0.2 μg/L for wines and beers, respectively [72]. Only a limited number of analytical methods for OTA have yet been validated [73]. The % recovery for wheat analysis is 91% at the spiking level of 5 ng/g and the within-lab %RSD is 9.7-12.5% [74]. For red wine analysis, the % recovery is 84.3-93.1% at the spiking level of 0.2-3.0 ng/mL and the within-lab %RSD is 6.5-10.8% [75].

1.3   Analysis for ochratoxin A using molecularly imprinted polymers

An alternative technology is to use molecularly imprinted polymers, which is currently being extensively evaluated by academic and industrial research groups. The design of MIP materials for OTA extraction is an interesting topic, and two other research groups have already characterized the interaction between OTA and MIPs [76, 77]. However, no agreement was obtained between two papers in the case of the choice of monomer(s). Justus Jodlbauer *et al* has successfully synthesized two monomers whose high affinity and good selectivity were proved by both theory and experiment. However, those procedures of synthesizing monomers were quite

complicated and hard to apply in a common analytical lab. The design of an OTA-MIP might be simplified by using commercially available monomers. Although Claudio Baggiani's group also made an OTA-MIP using methacrylic acid as the monomer, these conclusions were totally different from Justus Jodlbauer *et al.* Methacrylic acid should not display appreciable affinity for ochratoxin A according to the experience of J. Jodlbauer *et al.* A big doubt is remaining.

In the case of OTA-MIP applications, Norbert M. Maier *et al* [64] has successfully applied their MIP to wine samples. They used the separation method, ion-suppression HPLC, which offered a retention time of 10 minutes. The retention time was long and should be shortened in order to decrease the total analysis time. Moreover, the off-line procedure might not be ideal regarding the long time (2 hours) of sample treatment.

1.4   Objectives

The project was initialized by the hypothesis that mycotoxin molecularly imprinted polymers could be synthesized and the technique of molecularly imprinted solid-phase extraction and pulsed elution might be able to apply to mycotoxin analysis. The objectives of this thesis are to synthesize less expensive material, stable molecularly imprinted polymers with high affinity and selectivity for ochratoxin A for the development a fast analytical method, which can be applied to real-world samples. The objectives are divided into four main parts.

The first part is to prepare non-imprinted polymers (NIPs) from several functional monomers and 1,1,1-trimethylolpropane trimethacrylate (TRIM). Flow injection analysis should be able to test the affinity of these non-imprinted polymers for OTA in organic solution by non-imprinted solid phase extraction (NISPE) on different micro-columns. Multiple loading injections of OTA through a micro-column would find a NIP with the highest affinity for OTA in

a variety of media. The binding capacity and binding constant would be calculated for the NIP. A mechanism might be proposed for the OTA binding, pulsed elution. The optimal conditions should be obtained regarding binding and pulsed elution.

The second is to synthesize ochratoxin A molecularly imprinted polymer using a monomer with high affinity for OTA, and then develop a molecularly imprinted solid phase extraction and pulsed elution (MISPE-PE) method for the rapid analysis of wheat extracts for OTA. Newly synthesized MIP particles could be slurry-packed into a micro-column for selective solid phase extraction of OTA. A total binding capacity of OTA should be determined for an amount of MIP particles. Optimal conditions should be obtained for rapid analysis of OTA. The detection limit and recovery could be gotten.

The third one is to design online molecularly imprinted solid phase extraction, pulsed elution and high performance liquid chromatography, which could be applied to analysis of real samples. The newly designed instrumentation should be able to separate OTA peak from matrix peaks. A ppb-level detection limit and a recovery of > 80% should be obtained.

The final one is to use Spartan software to predict the interactions between OTA and functional monomers using the atomic charges and the heats of formation.

CHAPTER II

EXPERIMENTAL PROCEDURES

2.1 Materials

Ochratoxin A (OTA), trifluoroacetic acid (TFA) and triethylamine (TEA) were obtained from Sigma–Aldrich (Mississauga, ON). Piperidine was purchased from Fischer (Alpharetta, GA). Acetonitrile, chloroform, methanol and n-hexane were HPLC-grade solvents obtained from Fisher (Fair Lawn, NJ). Acetic acid (HOAc) was purchased form Anachemia (Toronto, ON). The ammonium hydroxide (28%) was from Anachemia (Champlain, NY).

N-phenylacrylamide (PAM), *t*-butyl methacrylate (BMA), methacrylamide (MAM), 2-(trifluoromethyl) acrylic acid (TFMAA), 4-vinylpyridine (4-VP), N-[3-(dimethylamino)propyl]methacrylamide (DMAP-MAM), 1-vinylimidazole (VI), [2-(diethylamino)ethyl]methacrylate (DEAE-MA), N-[3-(dimethylamino)propyl]methacrylamide (DMAP-MAM), 1,1,1-trimethylolpropane trimethacrylate (TRIM), 2,2'-azobisisobutyronitrile (AIBN) were obtained from Sigma-Aldrich (Mississauga, ON)

18-MΩ-cm distilled deionized water (DDW) was supplied from a Millipore Milli-Q water system (Bedford, MD).

Wheat extracts were supplied by the Canadian Food Inspection Agency in Winnipeg. Red wine samples, made in Italy, France or Australia, were purchased at local supermarkets (Ottawa, Canada).

2.2 Preparation of ochratoxin A standards

A stock solution of 100 μg/ml of OTA in methanol was prepared and the concentration was verified spectrophotometrically (MW: 403.8, $\varepsilon_{333nm} = 5500$ $M^{-1}cm^{-1}$) [78]. OTA solutions in amber vials were dried under a stream of nitrogen. Then the vials were stored at 0°C. Prior to each experiment, OTA was dissolved in the solution according to analytical requirement.

2.3   Preparation and recognition of non-imprinted polymer

2.3.1 Preparation of non-imprinted polymers and micro-columns

Chloroform (4.5 mL), functional monomers (2 mmol or 0.292 g for PAM), cross-linker (TRIM, 10 mmol or 3.384 g) and initiator (AIBN, 0.12 mmol or 0.026 g) were mixed in a glass vial. The molecular structures of PAM, TRIM and AIBN are shown in Fig. 4. The mixture was degassed in an ultrasonic bath for 10 min, and then purged with nitrogen gas for 5 min. The vial was capped and placed under 365-nm UV light for 24 h to achieve polymerization. The general reaction equation is shown in Fig. 5. The formed polymer bulk was crushed and ground with a mortar and pestle. Sieved particles from 38 to 65 μm were collected. Fine particles were removed by rinsing with methanol.

A slurry of the 38-65 μm non-imprinted polymer particles in methanol was manually injected from a syringe through a plastic connection tubing to a stainless steel column (0.8 mm inner diameter, 55 mm length). After the column was fully packed with non-imprinted polymer particles, the mobile phase was pumped through for 0.5 h to achieve uniformly tight packing of the micro-column. The column was installed in a non-imprinted solid phase extraction – pulsed elution (NISPE-PE) system.
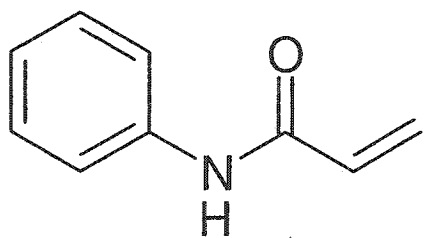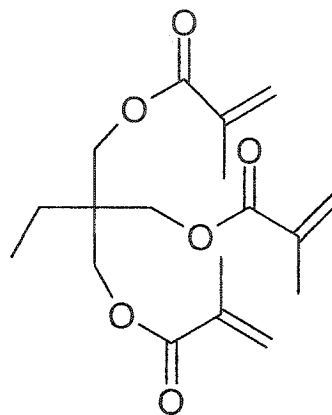
N-Phenylacrylamide (PAM)   1, 1,1-Trimethylolpropane trimethacrylate (TRIM)
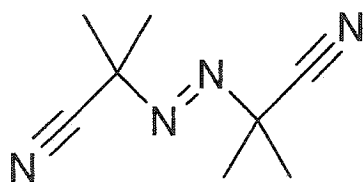
2,2'-Azobis(2-methylpropionitrile)

(AIBN)

Figure 4. Molecular structure of PAM, TRIM and AIBN
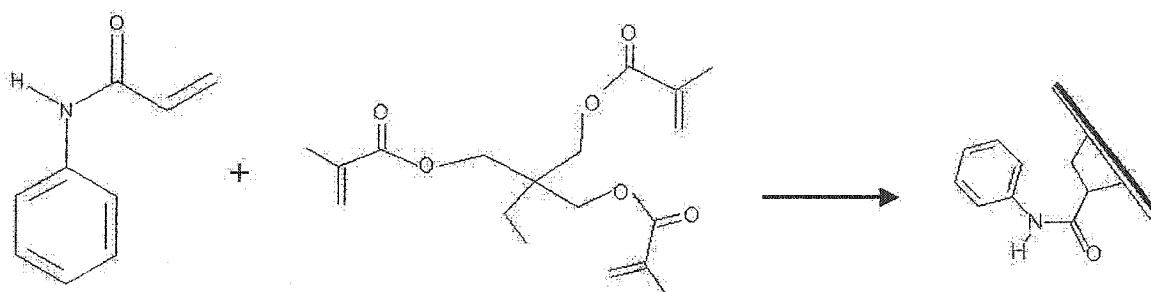
Figure 5. Reaction equation of synthesizing a non-imprinted polymer

2.3.2 NISPE-PE instrumentation

Figure 6 illustrates non-imprinted solid phase extraction and pulsed elution (NISPE-PE) set-up used for testing the OTA affinity of new non-imprinted polymers. It consisted of an Eldex 9600 solvent delivery system (San Carlos, CA), a Valco Cheminert VICI C2XL injector valve (Houston, TX), a Perkin-Elmer 204-A fluorescence spectrophotometer (Torrance, CA), and a Spectra Physics 42900 integrator (San Jose, CA). A similar set-up has been reported in detail elsewhere [36].
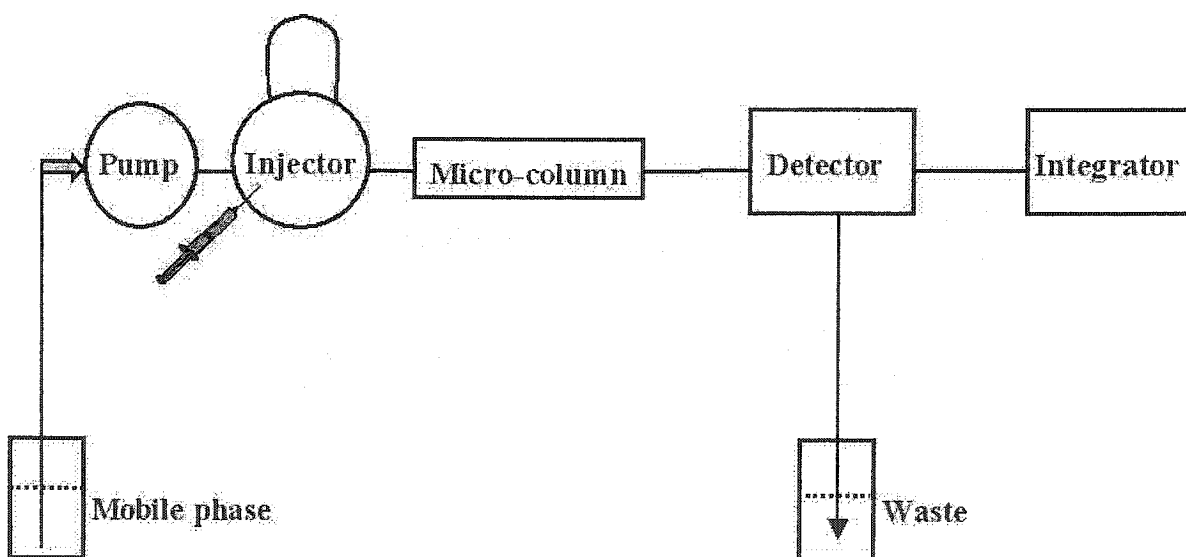
Figure 6. NISPE-PE set-up for testing monomers' affinity for OTA

2.3.3 Multiple loading injections

Chloroform was used as the mobile phase at a flow rate of 0.5 mL/min. A 5.0-μL sample loop was employed to perform multiple loading injections of a 2.0-μg/mL OTA solution. Detection of OTA in the eluate was detected by fluorescence at λ_{ex} = 333 nm and λ_{em} = 460 nm. All experiments were performed at room temperature (22^0C).

Using the saturation breakthrough peak area, two important properties of each non-imprinted polymer could be calculated:

(a) % binding of first loading injection = [(saturation breakthrough peak area – first injection breakthrough peak area) / saturation breakthrough peak area] x 100%,

(b) Binding capacity = [mass of OTA in one loading injection x Σ (% binding of all loading injections)] / mass of non-imprinted polymer in micro-column.

2.3.4 Testing % binding

Different solvents (n-hexane, chloroform, acetonitrile, methanol and water) were used as the mobile phase. A 2.0-μg/mL OTA solution (in the same solvent as the mobile phase) was injected, and the breakthrough peak area was recorded. After 60 min, the mobile phase was changed to methanol. The peak area of eluted OTA was recorded.

2.3.5 Rapid screening methods

A single injection of 2.0-μg/mL OTA in acetonitrile was made. Next, 3% trifluoroacetic acid in methanol was injected three times to perform pulsed elution (PE). The breakthrough peak area and PE peak areas were measured by fluorescence detection at λ_{ex} = 333 nm and λ_{em} = 460 nm (under the acidic conditions) [41]. 3% piperidine in methanol was injected twice to re-condition the micro-column.

A single injection of 2.0-μg/mL OTA in methanol/acetic acid (99:1 v/v) was made. Next, methanol/triethylamine (99:1 v/v) was injected three times to perform PE. The breakthrough peak area and PE peak areas were measured by fluorescence detection at λ_{ex} = 385 nm and λ_{em} = 445 nm (under the alkaline conditions).

2.3.6 Stepped elution

Water was employed as the mobile phase. A 2.0-μg/mL OTA solution in water was injected. The breakthrough peak area was recorded to determine the % binding. After 5 min, the mobile phase was changed to a mobile phase B such as acetonitrile/water (1:1 v/v) in order to elute OTA. The elution times of OTA was recorded to determine the strength of elution.

2.3.7 Analysis of wheat extracts

Wheat extracts were obtained from the Winnipeg Food Inspection Agency, Grains Canada. The extraction method involved a biphasic extraction of a 20-g wheat sample with 0.1 M H_3PO_4 and chloroform in a 250-mL Teflon centrifuge bottle. The sample was polytroned for 5 min at 10000 rpm, and centrifuged at 4°C for 10 min at 3000 rpm. The organic layer was

25

filtered and 25 mL of the extract was applied to a 7-g Chem Elute column, which had its pH modified with 1% $NaHCO_3$. The column was rinsed with chloroform and then eluted with a mixture of chloroform and formic acid. The sample was taken to near dryness on a rotary evaporator and then dried under nitrogen. 5mL of a methanol/water (1:1 v/v) was added, and the sample was sonicated, vortexed and filtered. The entire eluate was transferred to an autosampler vial for HPLC analysis; not all of this was used. Figure 7 shows the preparation of wheat extracts. The balance was transferred to another vial for delivery to our lab if the OTA result turned out to be negative.

In our lab, 1.0 mL of delivered wheat extract was transferred to a glass vial and dried under nitrogen. The residue was dissolved in 1.0 mL of methanol/acetic acid (99:1 v/v) and spiked with 100.0 ng of OTA. The spiked wheat extract sample was injected to NISPE-PE instrument system to perform sample loading. After 1 min, methanol/TEA (99:1 v/v) was injected three times to perform PE. PE peak areas were measured by fluorescence detection at $\lambda_{ex} = 385$ nm and $\lambda_{em} = 445$ nm (under the alkaline condition).

wheat sample

$CHCl_3 + 0.1$ M H_3PO_4

Organic phase

1% $NaHCO_3$

Chem Elute Column

$CHCl_3$, dried
$CHCl_3/CH_3COOH$

Eluate

Dried and re-dissolved

instrument

Aqueous layer

discard
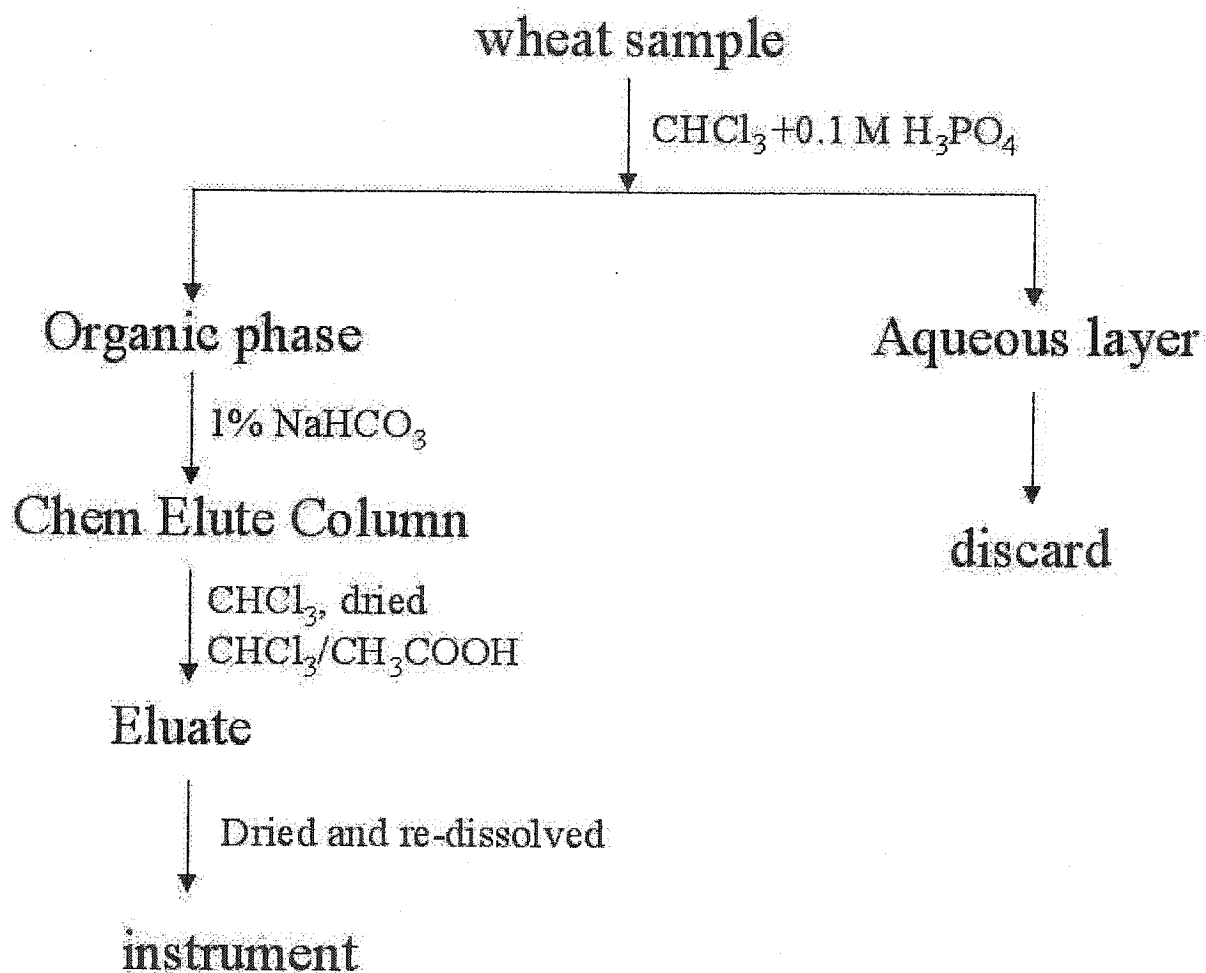
Figure 7. Preparation of wheat extracts

2.4 Molecularly Imprinted Solid Phase Extraction and pulsed elution

2.4.1 Preparation of MIP and micro-column

Chloroform (0.5 mL), print molecule (OTA, 0.012mmol or 4.9 mg), functional monomer (PAM, 0.048 mmol or 7.1 mg), cross-linker (TRIM, 0.24 mmol or 0.08 mL) and initiator (AIBN, 0.0029 mmol or 0.6 mg) were mixed in a glass vial. The molecular structures of OTA, PAM and TRIM are shown in Fig. 3 and 4, respectively. The mixture was degassed in an ultrasonic bath for 10 min, and then purged with nitrogen gas for 5 min. The vial was capped and placed under 365-nm UV light for 24 h to achieve polymerization. The solid MIP bulk was crushed and ground with a mortar and pestle. Fine particles were removed by several sedimentations in methanol.

A slurry of the MIP particles in methanol was manually injected from a syringe through a plastic connection tubing to a stainless steel column (0.8 mm inner diameter, 40 mm length). After the micro-column was fully packed with MIP particles, the mobile phase was pumped through for 30 min to achieve uniformly tight packing. This micro-column, containing approximately 20 mg of MIP particles, was connected to the MISPE-PE setup (as detailed in 2.4.2 below). The micro-column was washed extensively with 5% $NaHCO_3$ in DDW, until constant peak areas were attained from multiple pulsed elutions (PE) with methanol/TEA (99:1 v/v).

A control polymer (or NIP) was also prepared using the same synthesis conditions as described above, but without the addition of OTA.

2.4.2 MISPE-PE instrumentation

Figure 8 illustrates the MISPE-PE setup used for evaluating the OTA affinity of the newly prepared MIP micro-column. It consisted of an Eldex 9600 solvent delivery system (San Carlos, CA), a Valco Cheminert VIGI C2XL injector valve (Houston, TX), a Perkin-Elmer 204-A fluorescence spectrophotometer (Torrance, CA), and a Spectra Physics 42900 integrator (San Jose, CA). A similar setup has been used for non-imprinted polymers.
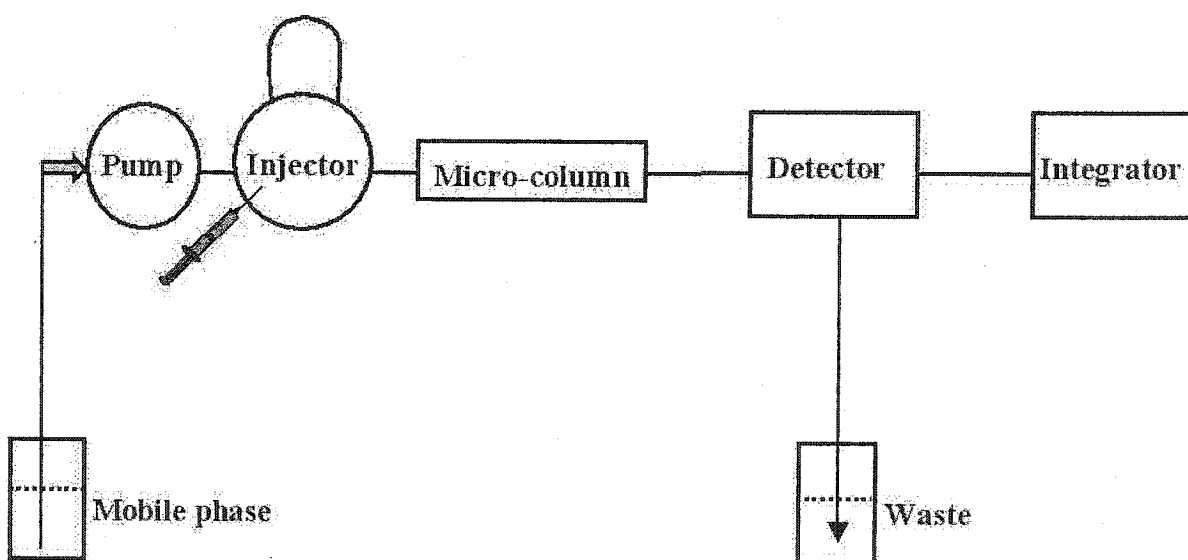

Figure 8. MISPE-PE instrumentation

2.4.3 Testing % binding

DDW was used as the mobile phase at room temperature ($23\pm1^{\circ}C$). 20 μL of OTA solution in methanol/acetic acid (99:1 v/v) was injected to perform sample loading. The breakthrough peak area was measured by fluorescence detection at λ_{ex} = 333 nm and λ_{em} = 460 nm (under the acidic condition). After 1 min, methanol/TEA (99:1 v/v) was injected three times to perform PE. PE peak areas were measured by fluorescence detection at λ_{ex} = 385 nm and λ_{em} = 445 nm (under the alkaline condition). Comparing the breakthrough peak area with the PE peak area (blank-corrected), a % binding result was calculated.

2.4.4 Mass spectrometry to confirm binding and pulsed elutions

Mass spectrometer was employed as a detector to confirm binding and pulsed elutions. The instrumentation was the same as Figure 8. A micro-column was packed with MIP particles and 20μL sample loop was used. Electrospray ionization mass spectrometry (ESIMS) was performed with a PE/Sciex Qstar Time-of-Flight (TOF) spectrometer in negative ion mode. Full-scan spectra were acquired over the range m/z 300-500 (cycle time, 1.0000 sec). The electrospray voltage was –5.2 kV. A solution of 0.1 ppm OTA in methanol/acetic acid (99:1 v/v) was loaded to the micro-column. Pulsed elutions were used methanol/triethylamine (99:1 v/v). Both OTA and PE solutions were injected via a syringe pump into the running eluent, water, at a flow rate of 10 μL/min. Data acquisition was controlled using the software, Analyst QS.

2.4.5 Analysis of wheat extracts

The wheat extract sample was analyzed as described in Section 2.3.7 above.

2.5 Online molecularly imprinted solid phase extraction, pulsed elution and ion-pair high performance liquid chromatography (MISPE-PE-HPLC)

2.5.1 MISPE-PE-HPLC instrumentation

Figure 9 illustrates the MISPE-PE-HPLC setup used for analysis for OTA. It consisted of two Shimadzu LC-6A solvent delivery systems (Kyoto, Japan), a Valco Cheminert VIGI C2XL injector valve (Houston, TX), Phenomenex Synergi 250 mm X 4.6 mm C18 column (Torrance, CA), a Perkin-Elmer 204-A fluorescence spectrophotometer (Torrance, CA), and a Spectra Physics 42900 integrator (San Jose, CA).

After OTA was loaded, a PE solution was injected to elute OTA from the MIP column. When the eluted OTA reached the 100 mL sample loop, the switching valve was turned. Then the HPLC mobile phase carried the eluted OTA from the sample loop to the C18 column for separation.
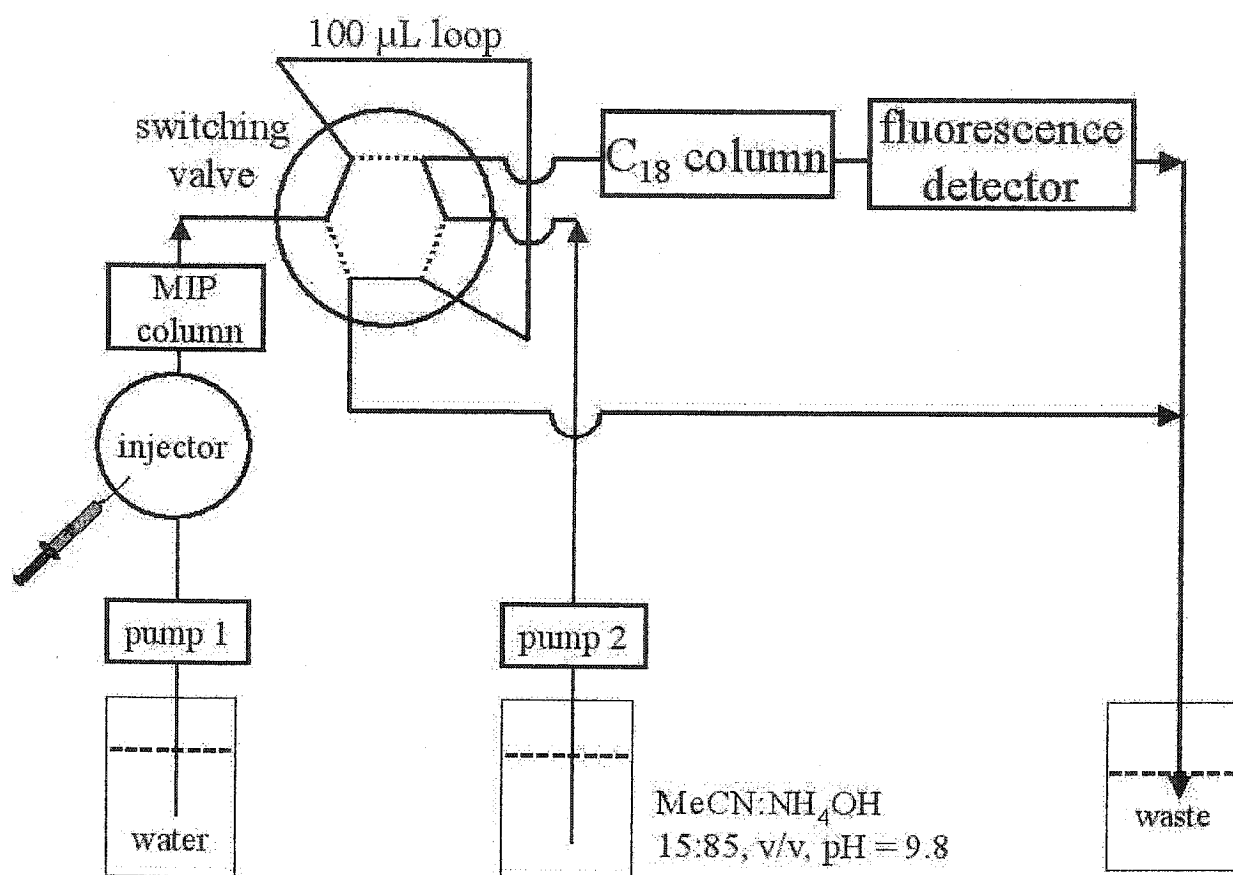
Figure 9. MISPE-PE-HPLC instrumentation

2.5.2 Wine sample preparation

Wine samples were cleaned up using phenylsilane SPE cartridges, which were purchased from J. T. Baker (Phillipsburg, NJ). The cartridges were conditioned with 10 mL of methanol and 5 mL of water, and care was taken to not let the column run dry. Then, 2 mL of original wine sample or spiked sample was passed through the column. The column was washed with 5 mL of water, and then dried in air. Finally, OTA was eluted with 5 mL of methanol/acetic acid (99.5:0.5 v/v). The eluate was dried under a stream of nitrogen at ambient temperature and reconstituted in 2 mL methanol/acetic acid (99:1 v/v). The solution was injected into MISPE-PE-HPLC system.

2.5.3 Analysis using MISPE-PE-HPLC

Water was employed as the mobile phase to perform MISPE-PE at the flow rate of 0.5 mL/min. The eluted OTA was transferred from the MISPE-PE system to HPLC by an injection valve. A mixture of water/acetonitrile / (85:15 v/v, adjusted to with pH = 9.8 ammonium hydroxide) was employed for HPLC system at the flow rate of 1.0 mL/min. OTA was detected at an excitation wavelength of λ_{ex} = 380 nm and an emission wavelength of λ_{em} = 440 nm.

Wine samples or OTA standards were dissolved in methanol/HOAc (99:1 v/v). MISPE-PE was performed by injecting a wine sample or OTA standard followed by a pulsed elution, 20 µL methanol/TEA (99:1 v/v). After 7 second, the switch valve was turned so the eluted OTA or matrices from wines could be transferred from the valve to the C18 column for separation.

2.6   Molecular modeling

Molecular modeling was performed using the Spartan ES software package (Wavefunction Inc., Irvine, CA, USA). The method of equilibrium geometry with semi-empirical AM1 was employed to calculate atomic charges (Mulliken charge) and the heats of formation.

CHAPTER III

RESULTS AND DISCUSSION

3.1 Non-imprinted polymers

3.1.1 Approach to synthesize non-imprinted polymers

Synthesis of molecularly imprinted polymers is the first step for any molecularly imprinted polymer research. There were two challenges. Firstly, a monomer with high affinity for OTA should be found. Secondly, to synthesize an OTA-MIP would need OTA but the price of OTA is pretty high; a purchase of 25 milligrams OTA requires about $700. 4 mg of OTA (about $ 112) can be used to synthesize only 60 mg of OTA-MIP.

To solve the problem, the approach of making non-imprinted polymers (NIPs) was first taken. A NIP was synthesized under the same conditions as molecular imprinting, but without addition of a template. Figure 10 shows the reaction of making a non-imprinted polymer (NIP). When monomers were mixed with a cross-linker, a NIP was formed under certain conditions. This NIP was good for testing the affinity of OTA for the monomer in a solid state.
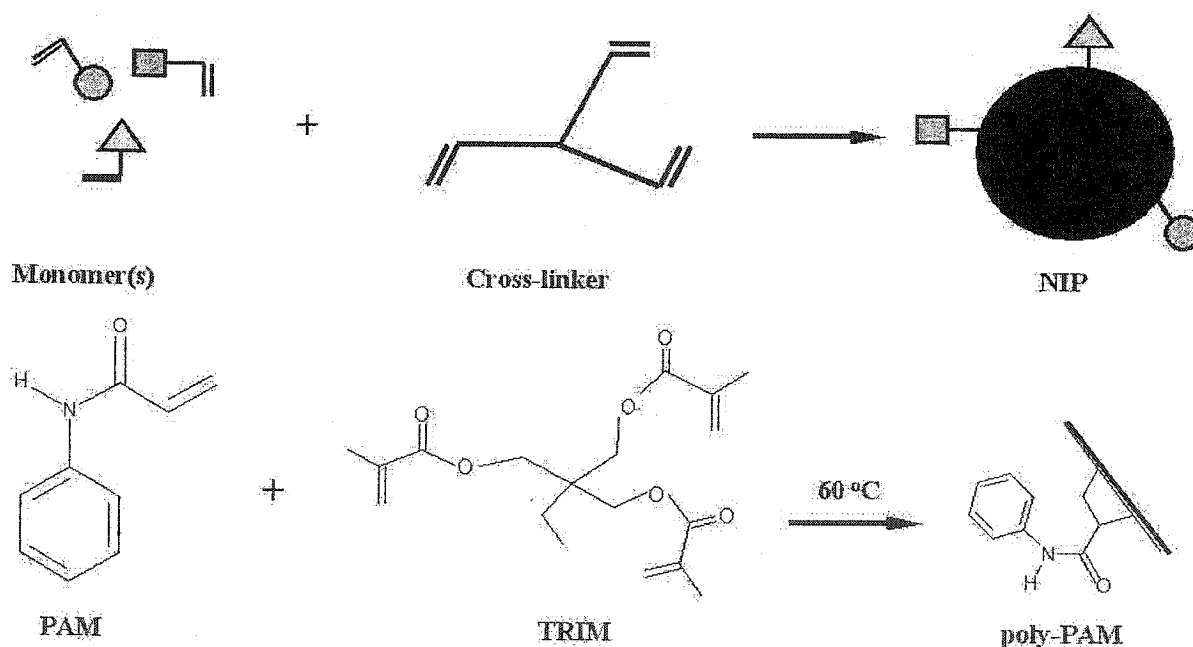
Figure 10. Reaction of making a non-imprinted polymer (NIP)

3.1.2 Choice of functional monomers

Typical functional monomers used are carboxylic acids (acrylic acid, methacrylic acid, vinylbenzoic acid), sulphonic acids (acrylamido-methylpropanesulphonic acid), and heteroaromatic (weak) bases (vinylpyridine, vinylimidazole). The structural features of OTA suggest that the molecule be capable of forming intermolecular complexes with appropriate functional monomers via non-covalent intermolecular interactions. The acidic functionality of OTA effectively interacts with functional monomers that offer basic centers for ion-pairing and hydrogen bonding interactions. The amide bond linking the naphthol unit with the amino acid may bind to the functional monomers by hydrogen bonding and dipole-dipole interactions. The aromatic domains can stabilize intermolecular complexes via π-π interactions and hydrophobic association.

Guided by these considerations, several non-imprinted polymers were prepared using the monomers listed in Figure 11. Other research groups had employed these monomers to make MIPs except N-phenylacrylamide (PAM), which had never been used in the MIP field.

3.1.3 Multiple loading injections

Non-imprinted polymers can be packed in micro-columns for on-line SPE to achieve the rapid screening. The NIP micro-column was set up for flow injection analysis. After OTA was injected to the column, fluorescence detector (in Fig. 6) was able to detect the amount of OTA that did not bind on (and break through) the micro-column. A breakthrough peak area was recorded on the integrator. Multiple loading injections were performed to determine the monomer's affinity for OTA. It was found that the breakthrough peak area increased after each loading injection. When OTA binding onto the micro-column reached saturation, the

breakthrough peak area would stay constant as illustrated in Fig. 12. Comparing the first breakthrough peak area with the saturation one, the % binding data for the first injection was obtained. The greater the % binding data, the higher affinity the monomer had. It was observed that the higher pK_a a functional monomer had, the higher its % binding would be for the first loading injection. PAM was found to afford the highest OTA binding of 94%, which is shown in Table 1. A binding capacity of 112 ng of OTA (per 30 mg of PAM) and a binding constant (K_{eq}) of 15 ± 3 were also determined.
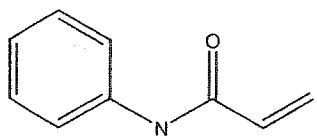
N-Phenylacrylamide (PAM)
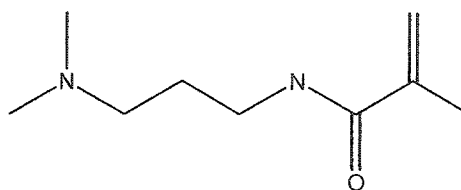
N-[3-(Dimethylamino)propyl]methacrylamide
(DMP-MAAd)
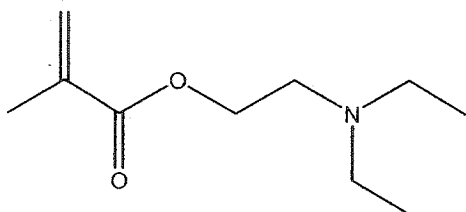
2-(Diethylamino)ethyl methacrylate
(DE-MA)
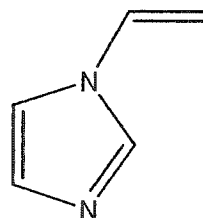
1-Vinylimidazole (VI)
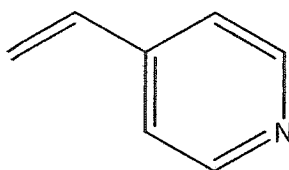
4-Vinylpyridine (4-VP)
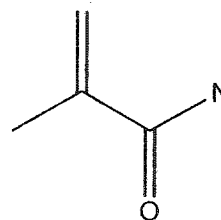
Methacrylamide (MAAd)
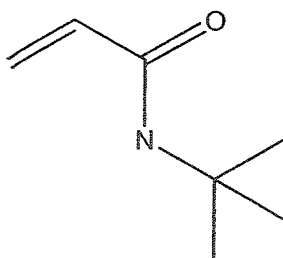
N-tert-Butylacrylamide (N-BAAd)

Figure 11. Monomers used to make non-imprinted polymers

Table 1. Comparison of affinity of non-imprinted polymers for OTA

Monomers used to prepare non-imprinted polymer (mmol)*	pK$_a$ of monomers (±0.1)	% Binding of first injection compared to saturation
TFMAA (2)	2.1	0%
DMAP-MAAd (2)	9.4	33%
DEAE-MAAd (0.5)	8.8	45%
BMA (1.5)	Not found	
DEAE-MA (0.5)	8.8	47%
PAM (0.5)	13.6	
MAAd (1)	6.1	
VI (2)	6.1	47%
DEAE-MAAd (2)	8.8	50 %
MAAd (1)	6.1	85%
4 –VP (1)	5.4	
PAM (2)	13.6	94% *

With 10 mmol of TRIM and 0.12 mmol of AIBN in 4.5 mL CHCl$_3$

* The NIP with highest % binding would indicate the best functional monomer for making a MIP.
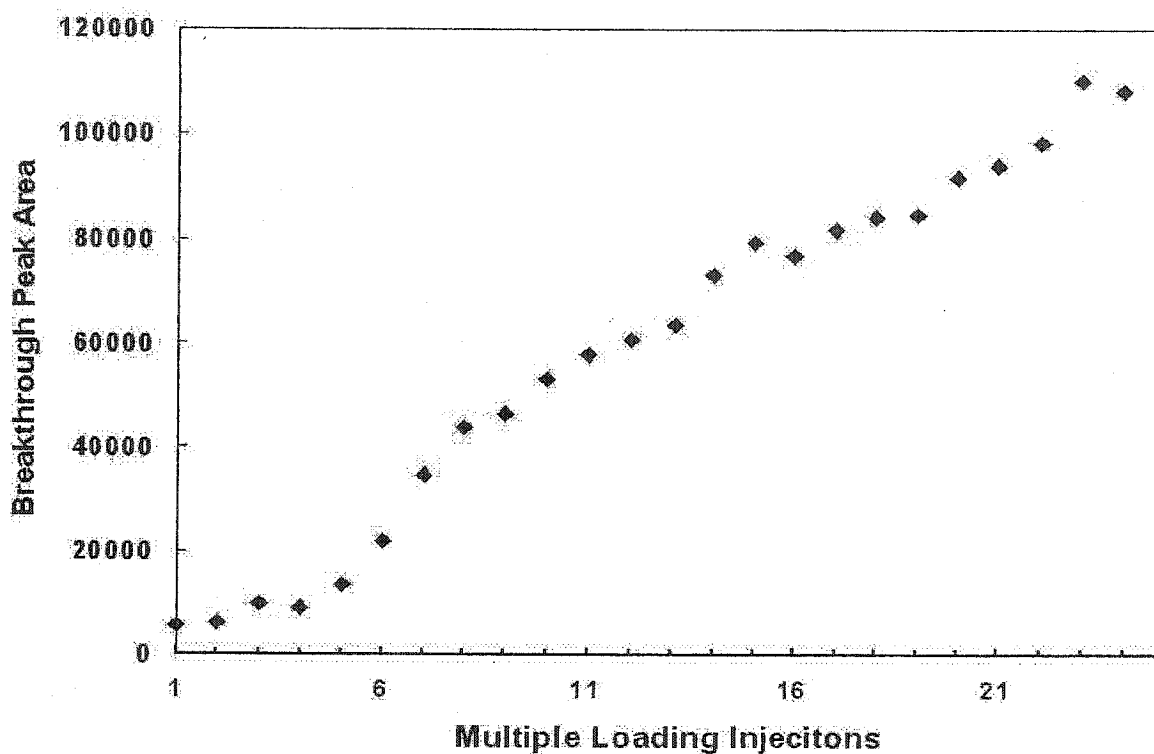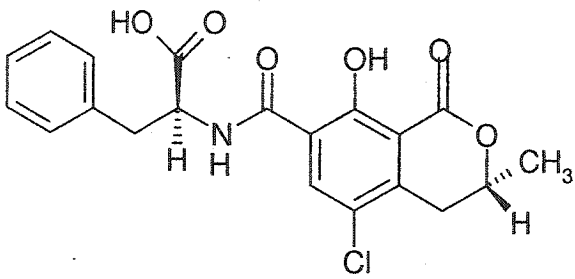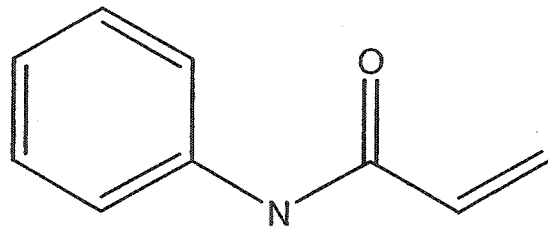
Figure 12. Binding isotherm for multiple 20-µL loading injections of 2-µg/mL OTA solution in $CHCl_3$ onto the PAM micro-column (packed with 30 mg of particles), using $CHCl_3$ as the mobile phase at 0.5 ml/min with fluorescence detection at $\lambda_{ex} = 333$ nm and $\lambda_{em} = 460$ nm.

3.1.4 Properties of PAM

OTA is a weak organic acid molecule (pK_a = 3.46) that possesses both hydrophobic and hydrophilic properties. The compound is soluble in polar organic solvents and slightly soluble in water. According to the theory of quantitative structure-activity relationships (QSAR) [79], N-phenylacrylamide (PAM) is a strong base that can pair up with OTA as a weak acid. The structure of PAM, as shown in Table 2, consists of a benzene ring that can offer π-π interaction. This molecule also has an amide functional group that can form a hydrogen bond with OTA. Thus, the theoretical prediction agrees with the experimental results presented in Table 1. Note in Table 1 that substitution of PAM with DEAE-MA and MAM decreased the % binding of first injection from 94% to 47%.

Table 2.   QSARs analysis between OTA and PAM

OTA

PAM

Structural characters	OTA	PAM	Interactions
Strength of acid or base	Weak acid pKa = 3.46	Strong base pKa = 13.64	Ion-pairing
Functional groups	Carboxylic and amine groups	Amide group	Hydrogen bonding
Aromatic group	Benzene rings	Benzene ring	π-π interaction

3.1.5 Solvent effects on % binding and retention time

When ochratoxin A was dissolved in the same solvent as the mobile phase, OTA % binding was studied on the PAM micro-column. According to the results summarized in Table 3, the non-imprinted polymer exhibited high affinity for OTA in most solvents except methanol. A plausible explanation is offered by the mechanism illustrated in Fig. 13. The hydrogen bonds between OTA and the amide functional groups of PAM are strong in n-hexane, chloroform and acetonitrile. Since these aprotic organic solvents are weak hydrogen bond donors or acceptors themselves, they could not break the hydrogen bonds between OTA and PAM. Although acetonitrile is more polar than chloroform, a slightly higher % binding was observed in acetonitrile due to its lower viscosity. With a viscosity of 0.38 (relative to water) for acetonitrile and 0.57 for chloroform, it would be easier for OTA molecules in acetonitrile to diffuse towards the PAM particles than in chloroform. Figure 14 has demonstrated the diffusion procedure of OTA from the mobile phase to the surface of polymer.

Table 3. Solvent effects on % binding and retention time when OTA dissolved in the same solvent as the mobile phase

	Mobile phase	% Binding	Retention time (min)
Increasing ↓ Solvent ↓ Polarity ↓	n-Hexane	~ 100	∞
	Chloroform	~ 92	∞
	Acetonitrile	~ 95	∞
	Methanol	0	0
	Water	~ 100	∞
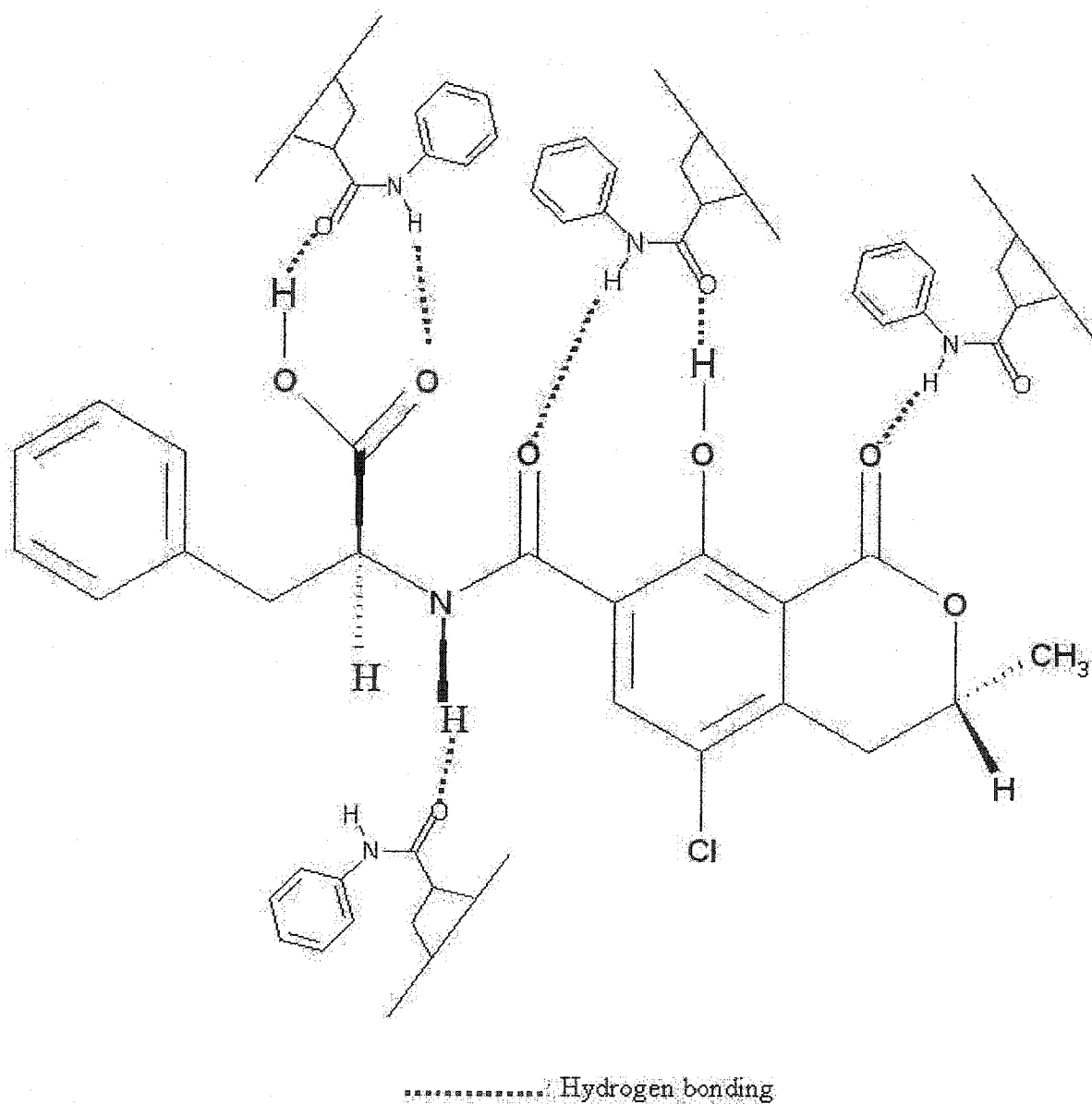
Figure 13. Binding mechanism for OTA and PAM non-imprinted polymer. The hydrogen bonds may not be all present at the same time.
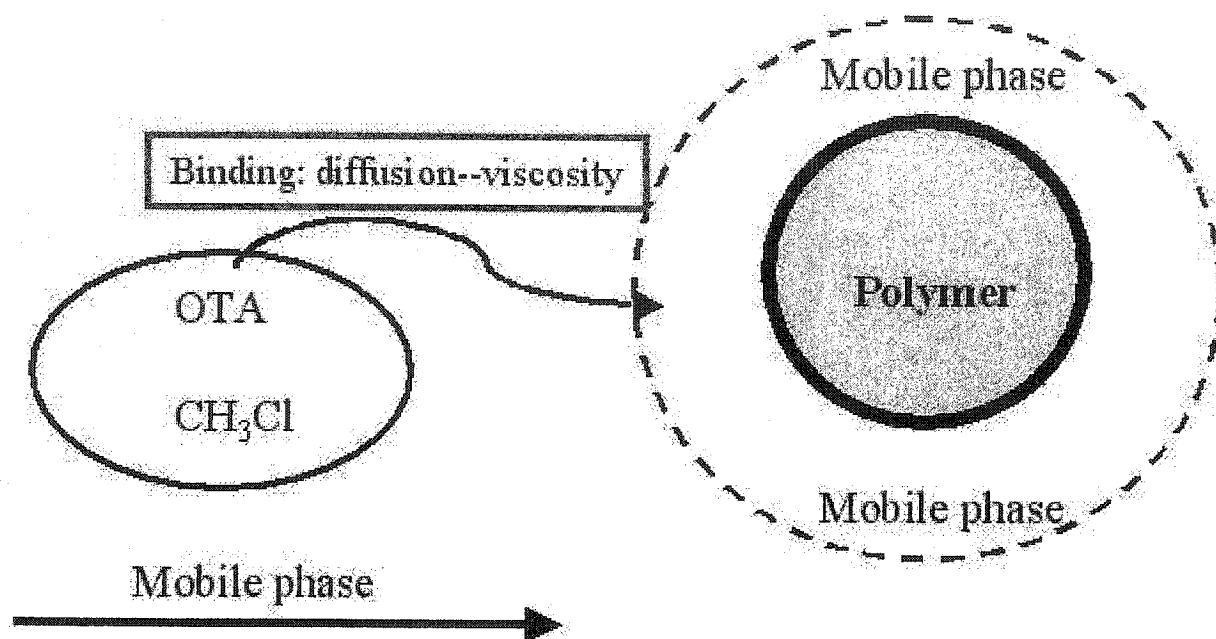
Figure 14. Diffusion procedure of OTA from the mobile phase to the surface

of polymer

No binding was found when methanol was employed as the mobile phase. It indicated that methanol had strong interaction with OTA. The methyl group in methanol offered hydrophobic interaction with OTA, and its hydroxyl group formed hydrogen bonds with OTA. Figure 15 shows these interactions. Moreover, methanol is a protic solvent that acts as a hydrogen bond donor and acceptor, which competed with OTA for the same binding sites on the NIP surface. Hence, no interactions between OTA and PAM were able to form.
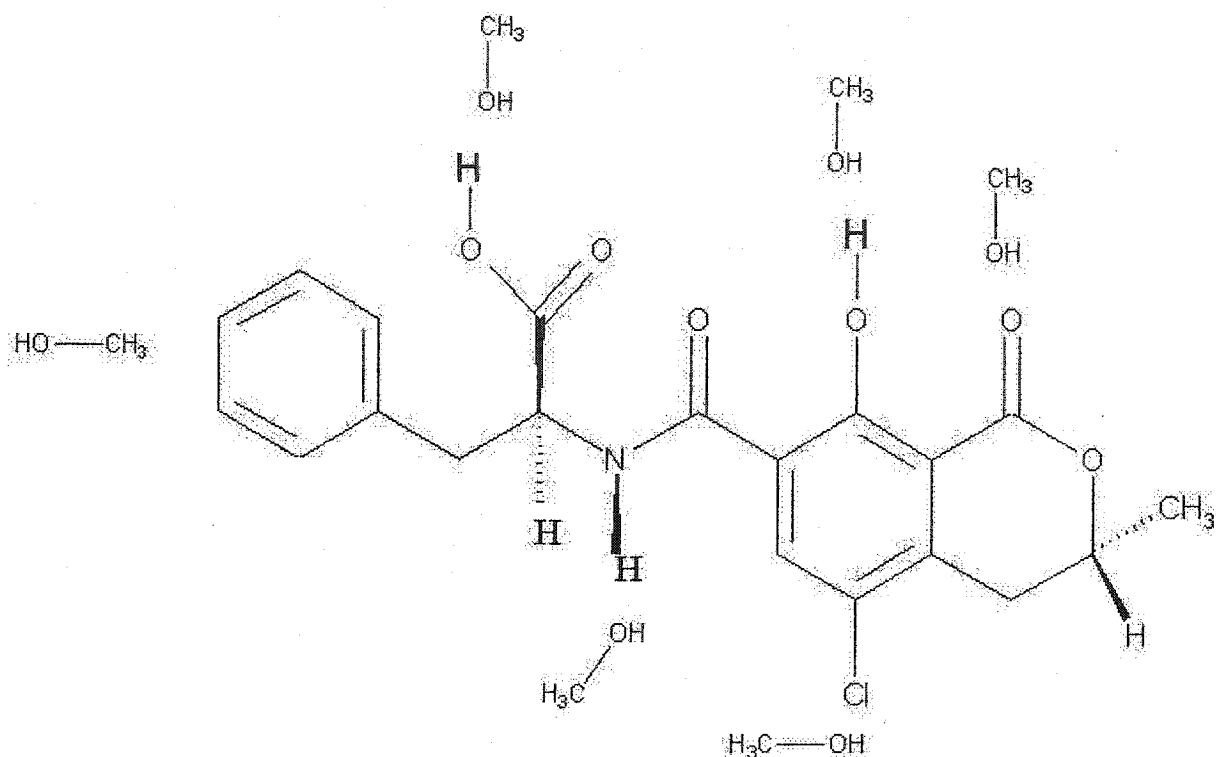
Fig. 15. Interactions between OTA and methanol

When water was employed as the mobile phase, a high % binding was also observed. Apparently, OTA possesses an extensive hydrophobic character. Hydrophobic interaction between OTA and the phenyl functionality of PAM played a dominant role in the strong binding. In all the mobile phases studied except methanol, OTA exhibited a retention time of infinity after the initial breakthrough. This further proved the high affinity of poly(PAM-*co*-TRIM) for OTA.

When ochratoxin A was dissolved only in chloroform and different solvents were employed to test OTA % binding, it was interesting to find the results were a little different from the previous one. Table 4 shows that OTA % binding was related to both the polarity and viscosity of the mobile phase. Higher OTA % binding was observed when the mobile with the low polarity and viscosity such as n-hexane was employed.

Table 4. Solvent effects on % binding and retention time when OTA dissolved only in chloroform

	Mobile phase	% Binding	Retention time (min)	Viscosity (relative to water)
Increasing	n-Hexane	~ 100	∞	0.31
	Chloroform	~ 94	∞	0.57
Solvent	Acetonitrile	~ 95	4	0.38
	Methanol	82	3	0.59
Polarity	Acetonitrile-water (1:1 v/v)	28	2	0.90
	Water	0	0	1.0

3.1.6 Pulsed elutions

Generally speaking, eluting the analyte from the column can be achieved with a solvent or mixtures that break or disrupt the strong and multiple interactions of the analyte with the polymer. This solvent can be methanol that contains a larger amount of a modifier, such as acetic acid or triethylamine, or mixtures of water with either methanol or acetonitrile. The elution volumes can be less than 1 mL, but researchers have reported using volumes of as much as 5 mL, depending upon the elution and binding strengths [80, 30, 81]. Among elution techniques, pulsed elution (PE) is good because it only uses a small amount (about 20 μL) of polar solvent or solution to elution the analytes that are retained in a micro-column. It can be employed to develop a rapid and quantitative analytical method.

Based on the results of last section, acetonitrile and water were chosen as two optimal solvents for the development of rapid NISPE-PE screening methods for OTA determination.

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These common solvents are extensively used in SPE and high-performance liquid chromatography (HPLC). OTA can exist in two interchangeable forms: the associated form (or molecule) is soluble in organic solvents while the dissociated form (or ion) dissolves in water [41], as illustrated in Fig. 16. Rapid screening methods were hence designed based on these solubility properties.
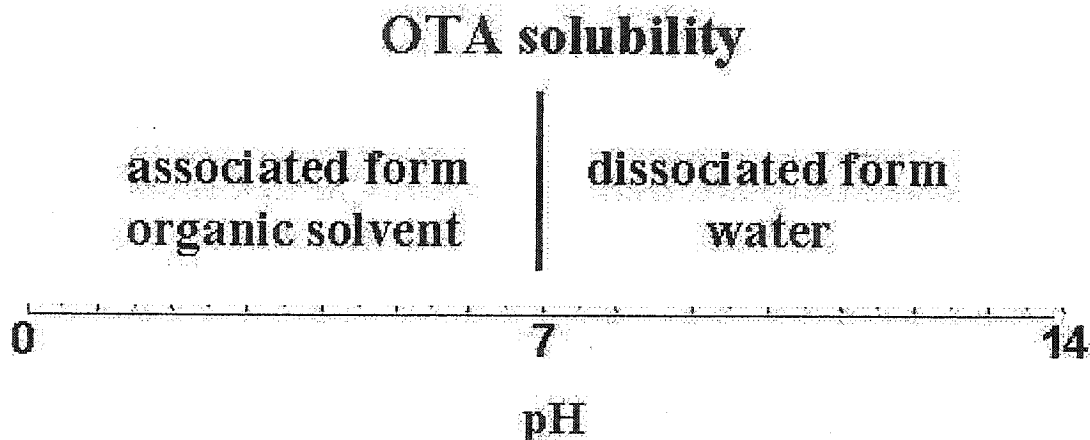
Figure 16. Ochratoxin A solubility

When acetonitrile was employed as the mobile phase, PE of the extracted OTA was found to be effective using 3% TFA in methanol [38]. Different organic and inorganic acids were also evaluated for PE, and very similar OTA recovery results were obtained. Essentially these acids broke up the hydrogen bonds between OTA and poly(PAM-co-TRIM), releasing the OTA back into the mobile phase. The elution procedure is shown in Fig. 17. One complication, however, arose probably due to protonation of the amide functional groups. A basic solution, 3% piperidine in methanol, was therefore needed to re-condition the micro-column after the PE. Otherwise, the micro-column would lose its high affinity for OTA.
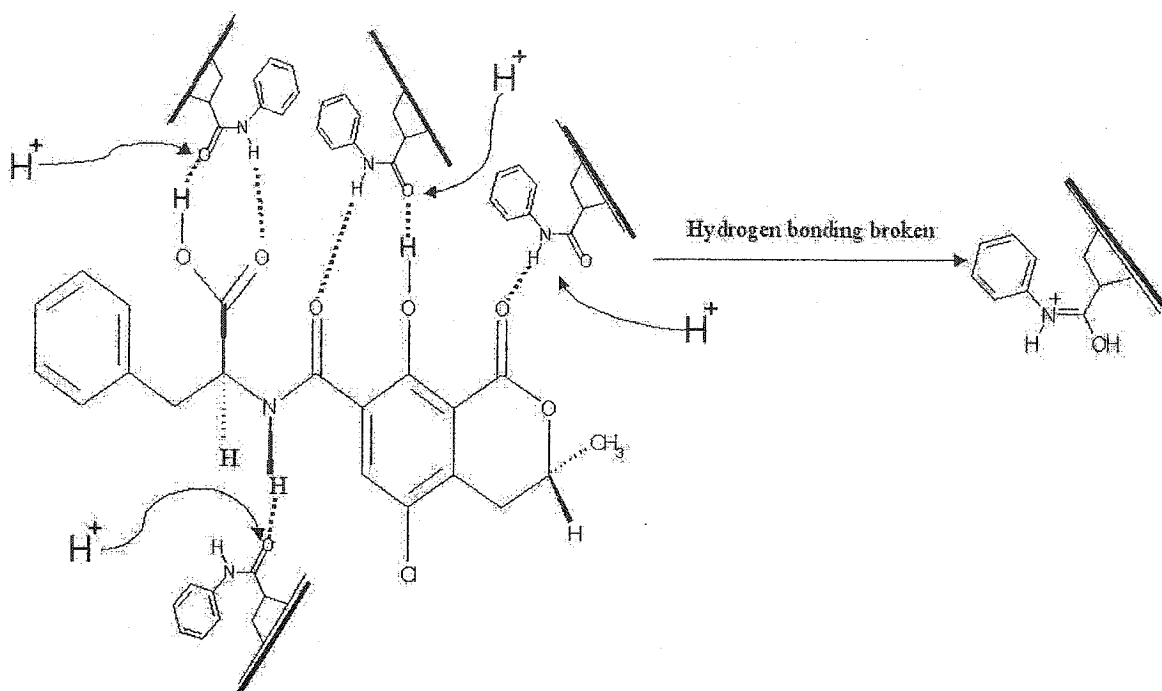
Figure 17. Elution procedure with an acidic compound

When water was employed as the mobile phase, PE of the extracted OTA was found to be effective using methanol/TEA (99:1 v/v). Although 5% $NaHCO_3$ in water or 5% $NaHCO_3$ in methanol/water (2:8 v/v) could achieve PE, broad peaks were observed. When methanol/ TEA (99:1 v/v) was employed for PE, a sharp peak was obtained. One plausible explanation is that this solution was able to wet the hydrophobic NIP particles easily. Figure 18 is a chart when water was employed as the mobile phase for NISPE-PE. No OTA was detected during the loading step. It proved approximate 100% binding. The first pulsed elution produced a big peak which indicated that most of the OTA was eluted from the micro-column.
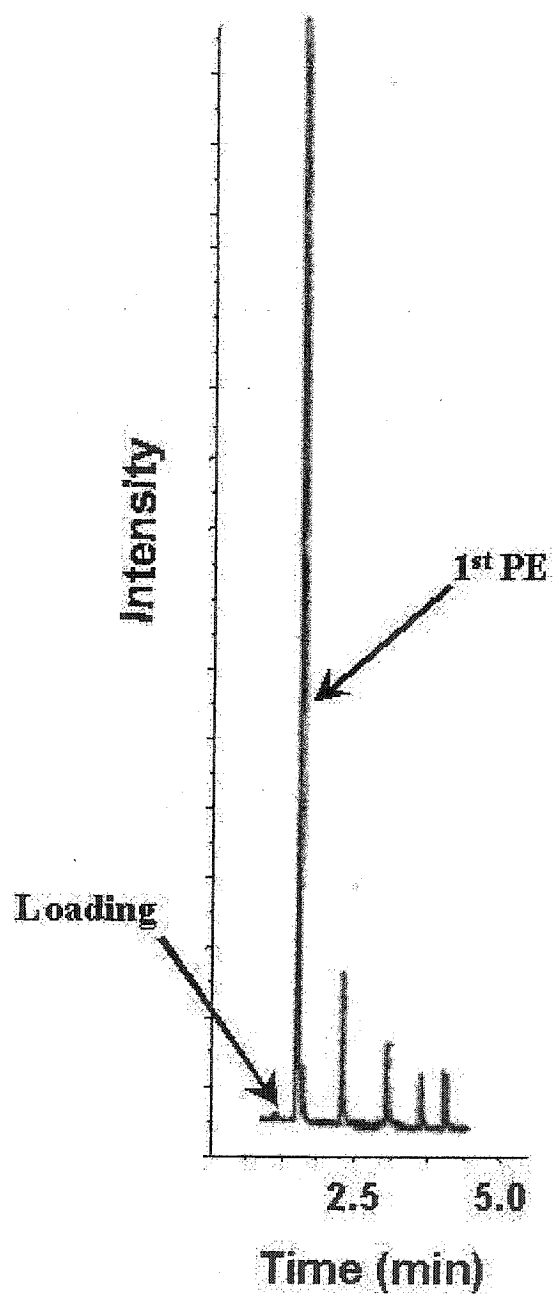
Figure 18. NISPE-PE chart when H_2O was used as the mobile phase, OTA (2.0 ng) in MeOH/HOAc (99:1 v/v) as the loading solution and MeOH/TEA (99:1 v/v) as the pulsed elution solution.

3.1.7 Water as the optimal mobile phase

Based on the above results, water was chosen as the optimal solvent because there were several advantages. First, water is easily available without the high cost and organic toxicity. Second, OTA exhibited enhanced fluorescence sensitivity under the basic conditions of PE with methanol/TEA (99:1 v/v), which can be observed in Fig. 19 and Fig. 20. Third, the ~100% binding in water was higher than the 95% in acetonitrile. Hence, a rapid screening method was successfully developed employing water as the mobile phase.
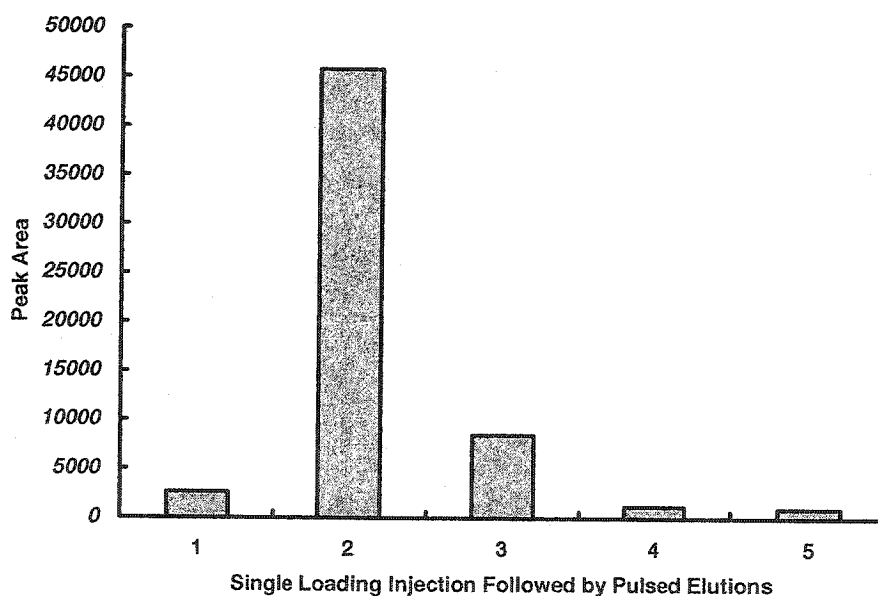
Figure 19. Peak areas of an NISPE-PE when chloroform was used as the mobile phase, OTA (40.0 ng) in CH$_3$CN for loading, and MeOH/HCl (99.9:0.1 v/v) for PE.
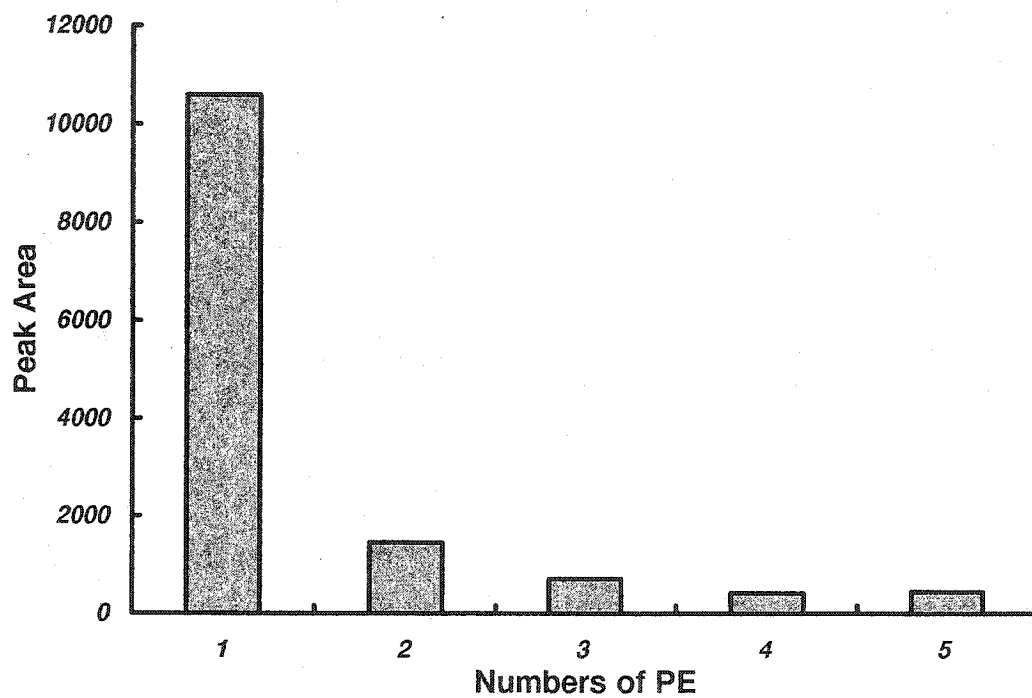
Figure 20. Peak areas of PE when H_2O was the mobile phase, OTA (2.0 ng) in MeOH/HOAc (99:1 v/v) as loading solution and MeOH/TEA (99:1 v/v) as pulsed elution solution

3.1.8 NISPE-PE mechanism—acid-base chemistry

After the bindings and pulsed elutions were tested using different acids and bases, the mechanism was summarized as acid-base chemistry. It was interesting to find the mechanism was totally different comparing water with organic solvents such as acetonitrile.

When an organic solvent such as acetonitrile was employed as the mobile phase, the assumed mechanism of binding and elution was shown in Fig. 21.
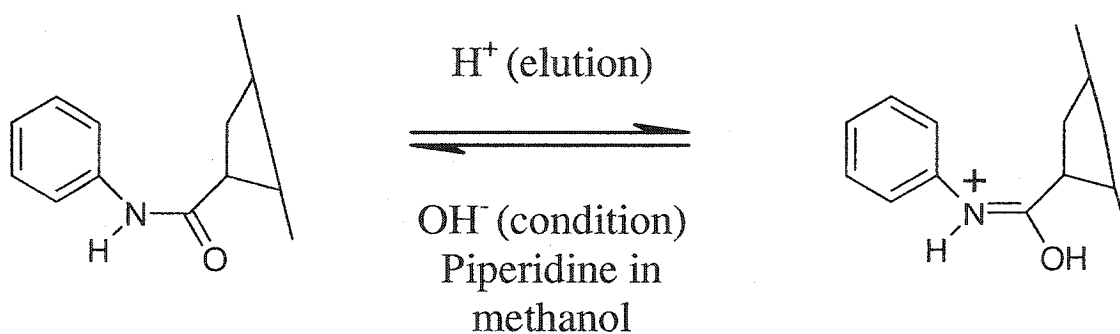
Figure 21. Mechanism of binding and elution when acetonitrile was the mobile phase
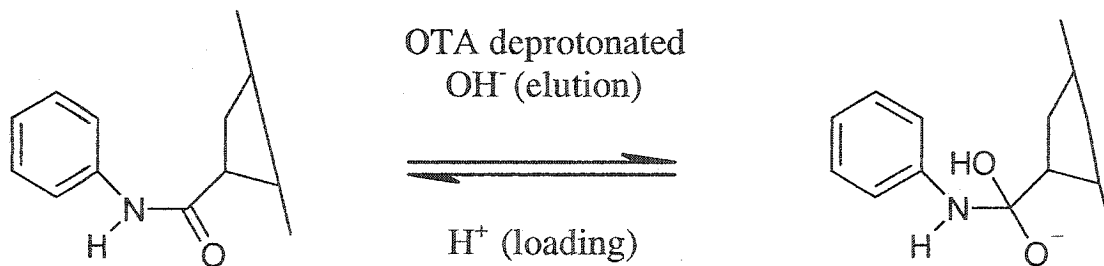
Figure 22. Mechanism of binding and elution using water as the mobile phase

In water, the acid-base requirements of PE were expected to be totally different than the case of acetonitrile. The PE mechanism is shown in Fig. 22.

3.1.9 Stepped elution

The feasibility of poly(PAM-*co*-TRIM) for SPE followed by stepped elution was next investigated. Mobile Phase A such as water was used as the initial mobile phase for loading an OTA sample. Here, SPE on the micro-column could provide analyte enrichment and sample matrix cleanup. After the breakthrough peak was recorded, a different mobile phase was employed to elute the bound OTA under HPLC conditions. Different solvents were evaluated as the HPLC mobile phase, including methanol, methanol/acetic acid (99:1 v/v) and acetonitrile/water (1:1 v/v). The last solvent mixture proved to be the strongest for OTA elution, at a retention time of only 11 min. One plausible explanation is that acetonitrile/water (1:1 v/v) combined hydrophobic and hydrophilic properties in a fine balance. This mixture could annihilate both the hydrogen bonds and hydrophobic interactions between OTA and poly(PAM-*co*-TRIM) effectively. One advantage of this stepped elution approach is that useful solvents can be readily found from the SPE and HPLC literature. It is interesting to note that poly(PAM-*co*-TRIM) may be used on a large industrial scale for the removal of OTA from food products, and also for solid phase extraction.

3.1.10 Poly(PAM-*co*-TRIM) as a stationary phase

Poly(PAM-co-TRIM) can potentially be used as the stationary phase for high performance liquid chromatography because it has two functional groups, i.e. an amide group and a benzene ring. The amide functional group can form hydrogen bonds with certain

55

functional groups in analyte molecules, so the polymer is promising as the stationary phase for normal phase HPLC. The benzene ring affords hydrophobic interactions with nonpolar groups. As a result, the polymer can possibly be used as the stationary phase for reverse phase HPLC. Proposed findings are shown in Figure 23.

Benzene ring
hydrophobic interaction
Reverse phase HPLC

Amide functional group
Hydrogen bonds
Normal phase HPLC
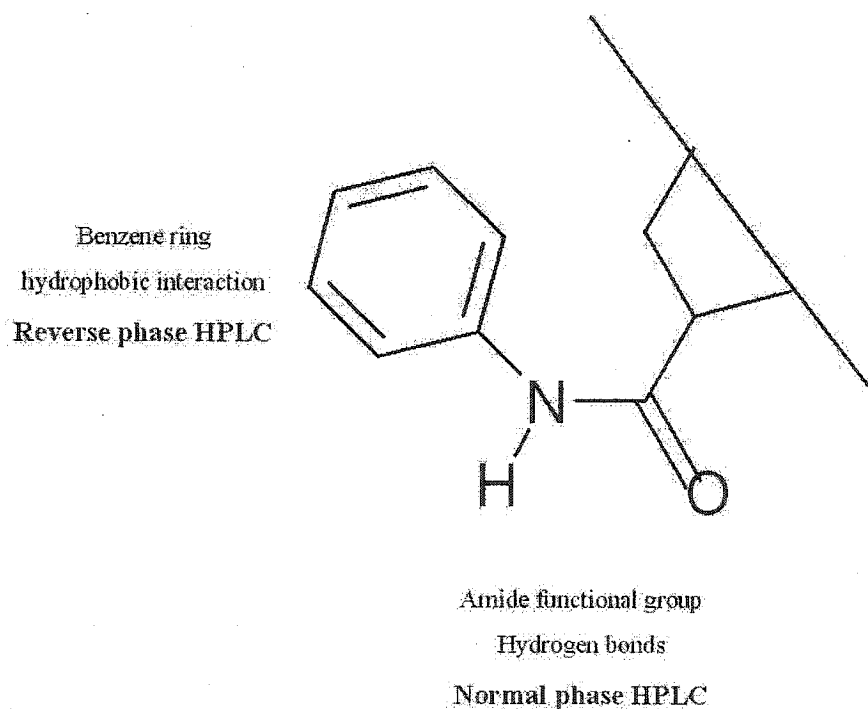
Figure 23. Potential use of poly(PAM-*co*-TRIM) as the stationary phase for high performance liquid chromatography

3.1.11 Calibration curve from non-imprinted polymer

A standard calibration curve for determination of ochratoxin A is shown in Fig. 24, using water as the mobile phase, OTA in MeOH/HOAc (99:1 v/v) as the loading solution and MeOH/ TEA (99:1 v/v) for PE. A fairly good linear dynamic range ($R^2 = 0.9923$) was achieved to the

mass of OTA loaded on the micro-column up to 40 ng (or 2.0 µL/mL of OTA in 20 µL of sample injected). The detection limit was found to be 7.8 ng/mL (or 0.16 ng in 20 µL of sample injected) for ochratoxin A, based on three standard deviations of the blank signal.

Table 5 shows the results of OTA recovery for wheat extracts. Wheat extracts spiked with OTA offered a recovery of 125±5%, which was not ideal. It demonstrated other matrix components in the wheat extract bound onto the control polymer particles due to poor selectivity of NIP. There was no difference regardless of wheat types.

Table 5. Recovery of OTA using NIP for wheat extracts

Sample (number)	Spiked OTA (ng)	Recovery (%)	STD (%)	%RSD
Canadian Western Red Spring Wheat (T50)	5.0	118.6	5.1	4.1%
		126.3		
		128.2		
Canadian Western Amber Durum Wheat (T17)	10.0	120.7	4.8	3.8%
		124.9		
		130.3		
Canadian Eastern White Winter Wheat (T23)	20.0	121.1	4.8	3.8%
		128.7		
		130.1		
Average		125.4	4.9	3.9%
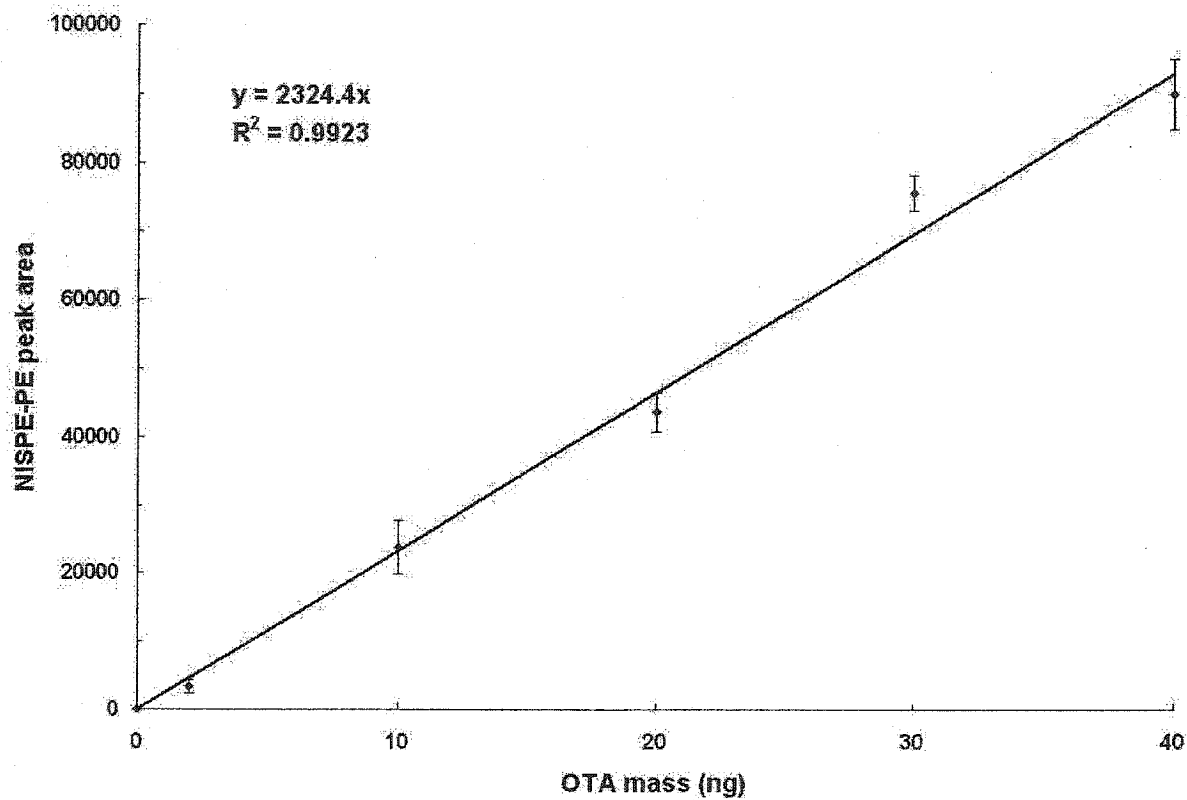
Figure 24. Calibration curve of non-imprinted polymer for determination of ochratoxin A, using H_2O as the mobile phase, OTA in MeOH/HOAc (99:1 v/v) for loading and MeOH/TEA (99:1 v/v) for PE.

3.1.10 Matrix interference and cross-reactivity

Since OTA is a naturally occurring fungal toxin in cereals, beans, groundnuts, spices, dried fruits, coffee, milk, beer and wine, the NISPE-PE method was applied to one of these matrices to evaluate matrix interference. It was found that wheat extracts did not affect the binding efficiency of poly(PAM-co-TRIM) in water as the mobile phase. When an OTA-spiked wheat extract sample was injected, no significant OTA breakthrough peak area was observed. This indicated that 100% binding was achieved for OTA. The wheat extract matrix did not seem to decrease either the binding efficiency or binding capacity of the micro-column. Interference from other food matrices would be tested in the future work. The specificity and cross-reactivity of poly(PAM-co-TRIM) to other mycotoxins was also considered. PAM is a basic functional monomer containing a benzene ring. If a mycotoxin molecule does not consist of a carboxylic or amine group, no significant cross-reactivity with the non-imprinted polymer would be expected. Any mycotoxin that is an acidic compound with benzene ring(s) would potentially engage in cross-reactivity. Table 6 summarizes a quantitative structure activity relationship (QSAR) prediction for the commonly reported mycotoxins. Ochratoxin B (OTB) and citrinin (CT) are the most likely to engage in cross-reactivity with poly(PAM-co-TRIM) during NISPE. Since these two mycotoxins also exhibit native fluorescence to produce interfering PE signals, the present micro-column should best be suited for pre-concentrating trace levels of OTA, OTB and CT from a complex food matrix for subsequent HPLC analysis [19]. In order to achieve specificity for OTA, a molecularly imprinted polymer (MIP) based on TRIM and PAM is the next investigation in this study.

Table 6. QSAR prediction of potential interference from common mycotoxins

Mycotoxin	pK_a	Benzene rings	Potential interference
Aflatoxin B_1	~ 7	1	No
Aflatoxin B_2	~ 7	1	No
Aflatoxin G_1	~ 7	1	No
Aflatoxin G_2	~ 7	1	No
Aflatoxin M_1	10.8 ± 0.2	1	No
Aflatoxin M_2	12.2 ± 0.2	1	No
Alternariol	7.2 ± 0.2	2	No
Alternariol monomethyl ether	~ 7	2	No
Chaetoglobosin A	12.2 ± 0.7	1	No
Citrinin	1.5 ± 0.2	0	Significant
Cyclopiazonic acid	4.5 ± 1.0	1	Slight
Deoxynivalenol	11.9 ± 0.7	0	No
Diacetoxyscirpenol	~ 7	0	No
Ergot alkaloids	7 - 9	1	No
Fumonisin B_1	3.7 ± 0.2	0	No
Fumonisin B_2	3.7 ± 0.2	0	No
Fumonisin B_3	3.7 ± 0.2	0	No
Gliotoxin	12.9 ± 0.1	0	No
3-Hydroxypyridinium	~ 7	0	No
HT-2 toxin	13.3 ± 0.2	0	No

(Continued)

Moniliformin	~ 7	0	No
Mycophenolic acid	4.6 ± 0.2	1	Slight
Nivalenol	11.8 ± 0.7	0	No
Ochratoxin B	3.5 ± 0.1	1	Significant
Ochratoxin C	7.6 ± 0.4	1	No
Patulin	11.0 ± 0.2	0	No
Roquefortine C	4.7 ± 0.1	1	Slight
Sterigmatocystin	6.7 ± 0.2	2	No
T-2 toxin	13.2 ± 0.2	0	No
Tricarballylic acid	3.6 ± 0.2	0	Slight
α-Zearalenol	7.7 ± 0.6	1	No
Zearalenone	7.4 ± 0.2	1	No

3.2   Synthesis of ochratoxin A molecularly imprinted polymer

The challenge of designing and synthesizing a molecularly imprinted polymer (MIP) can be a daunting prospect to the uninitiated practitioner, not least because of the sheer number of experimental variables involved, e.g. the nature and levels of template, functional monomer(s), cross-linker(s), solvent(s) and initiator, the method of initiation and the duration of polymerization. Fortunately, a good number of "rules of thumb" have emerged in the literature that are helpful in this regard, however it is nevertheless useful to simultaneously bear in mind the basics of free radical polymerization processes. In bringing this information together in a useful fashion for synthetic purposes, it is constructive to highlight several of the more important considerations; whilst some of these synthetic considerations may be patently obvious, it is remarkable how often such things are often glossed over or indeed completely over-looked in the imprinting literature. In the following, attention is drawn to a number of factors pertaining to the template molecule and the selection of suitable functional monomers, cross-linkers, initiators and general polymerization procedure [82].

Of the imprinting strategies used, it has become evident that the use of non-covalent interactions between the print molecule and the functional monomers is the more versatile. The apparent weakness of these interaction types, when considered individually, may be overcome by allowing a multitude of interaction points simultaneously. Together with the fast association and dissociation kinetics of these bond types, so that in a short time many possible combinations can be checked before the correct partners associate, this protocol has proven advantageous. Furthermore, the use of non-covalent interactions in the imprinting step closely resembles the recognition pattern observed in nature [83].

3.2.1 Ochratoxin A as the target molecule

A wide variety of print molecules have been used in various imprinting protocols. In all molecular imprinting processes the template is of central important in that it directs the organization of the functional groups pendent to the functional monomers. Unfortunately, and for a variety of reasons, not all templates are directly amenable to templating. In terms of compatibility with free radical polymerization, templates should ideally be chemically inert under the polymerization conditions, thus alternative imprinting strategies may have to be sought if the template can participate in radical reactions or is for any other reason unstable under the polymerization conditions. The following are legitimate questions to ask of a template. Firstly, does the template bear any polymerisable groups? Secondly, does the template bear functionality that could potentially inhibit or retard a free radical polymerization, e.g. a thiol group or a hydroquinone moiety? Finally, will the template be stable at moderately elevated temperatures (e.g. at or around 60°C if AIBN is being used as the chemical initiator) or upon exposure to UV irradiation? [82].

In the study of ochratoxin A as the template, thermo-polymerization was employed by raising the temperature to 60°C instead of UV irradiation. The reason is that OTA decomposition occurs under exposure to UV light. OTA is heat resistant and it was reported that OTA solutions as ethanol or methanol can be stored in refrigerator more than a year without loss [41].

3.2.2. Choice of functional monomers for MIP preparation

Several polymer systems have been developed for use in molecular imprinting technology. By far the most readily used are either polyacrylate-based or polyacrylamide-based systems. Another approach is the polystyrene-based systems that are used to a lesser extent.

The choice of functional monomer was based on the findings ochratoxin A non-imprinted polymer [84]. N-phenylacrylamide (PAM) is a strong base (pK$_a$ = 13.6) with an amide functional group that can form a hydrogen bond with OTA, which is a weak acid (pK$_a$ = 3.5), as illustrated in Fig. 3. The PAM molecule also consists of a benzene ring that can offer π-π interaction with the aromatic moiety of OTA.

The rigid structure of PAM also enhances its molecular recognition potential for the ochratoxin A. It is expected to provide a means to tune the stability of template-functional monomer complexes. In an amide, not only the carbonyl carbon, but also the amide nitrogen has essentially trigonal planar bonding patterns (Figure 25).
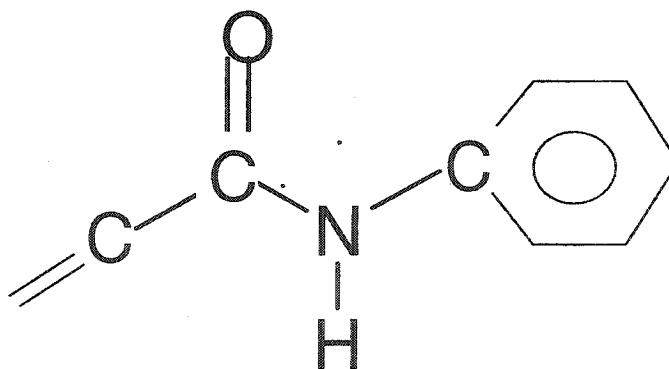
Figure 25. N-phenylacrylamide. The labeled atoms all lie in the same plane

The trigonal planar geometry at the amide nitrogen can be understood on the basis of the following resonance structures, which show that the bond between the nitrogen and the carbonyl carbon has considerable double-bond character.
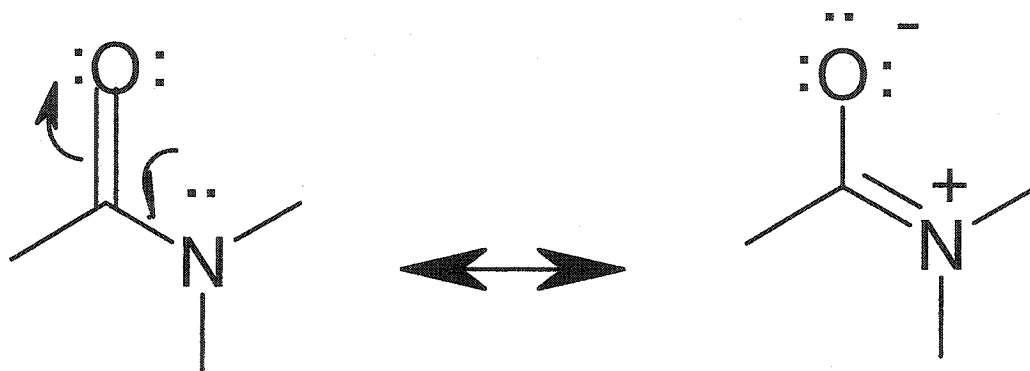
Figure 26. Amide resonance structure

Because of this trigonal planar geometry at nitrogen, secondary and tertiary amides can exist in both E and Z conformations about the carbonyl-nitrogen bond; the Z conformation predominates in most secondary amides because, in this form, van der Waals repulsions between the largest groups are avoided.

Z   E
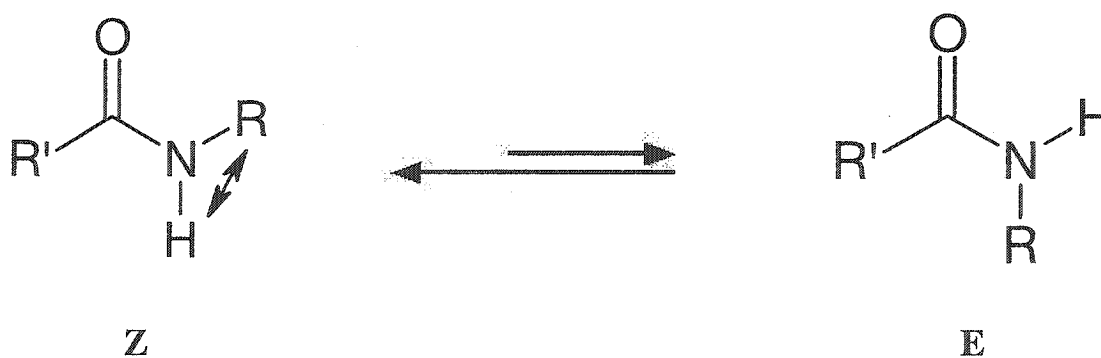
Figure 27. Z and E conformations of N-phenylacrylamide

The inter-conversion of E and Z forms of amides is too rapid at room temperature to permit their separate isolation, but is very slow compared with rotation about ordinary carbon-carbon single bonds. A typical energy barrier for rotation about the carbonyl-nitrogen bond of an amide is 71 kJ/mol (17 kcal/mol), which results in an internal rotation rate of about ten times per second. (In contrast, an internal rotation in butane occurs about 10^{11} times per second.) The significant double-bond character in the carbon-nitrogen bond causes the relatively low rate of internal rotation; recall that rotation about double bonds is much slower than rotation about single bonds [85].

3.2.3 Choice of a cross-linker

A very high degree of cross-linking (70-90%) is necessary for achieving specificity. Only a limited number of cross-linkers have been utilized (Figure 28). The solubility of the cross-linker itself in the pre-polymeric solution and the solubility of the template species reduce the number of possibility. Nevertheless, several different cross-linkers have been tried with different degrees of success. Originally, isomers of divinylbenzene were used for cross-linking of styrene and other functional monomers into polystyrenes. Later, it was found that acrylic or methacrylic acid based systems could be prepared with much higher specificity. Ethylene glycol dimethacrylate (EDMA) and 1,1,1-trimethylolpropane trimethacrylate (TRIM) are presently commonly employed. EDMA provides polymers with mechanical and thermal stability, good wettability in most rebinding media and rapid mass transfer [86]. Comparing with EDMA as a cross-linker, TRIM provides same recognition properties for a large variety of target templates. The MIPs prepared from TRIM possessed a higher load capacity than similar MIPs prepared using EDMA [87]. Therefore, TRIM was used as the cross-linker in this study.
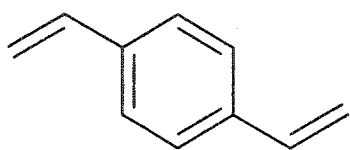
4-Divinylbenzene
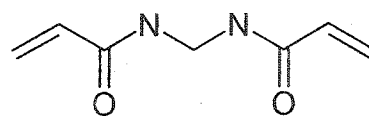
N, N'-Methylene-bisacrylamide
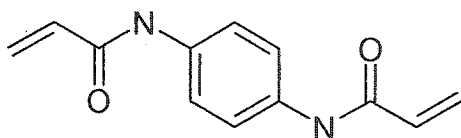
N, N'-Phenylene-bisacrylamide
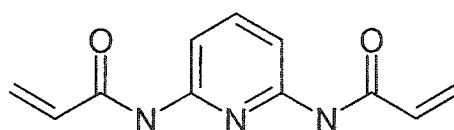
2,6-Bisacrylamideopyridine
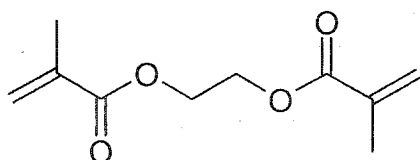
Ethylene glycol dimethacrylate
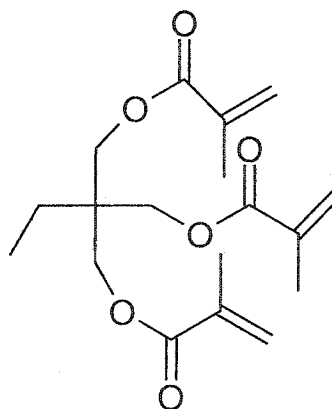
1,1,1-Trimethylolproprane trimethacrylate

Figure 28. Selection of cross-linkers

3.2.4 Stoichiometry of imprinting

An increasing number of interacting functional groups in a molecule will increase the selectivity, whereas the morphology is important for site accessibility and the mass transfer properties of the sorbent. With the present systems, the upper limit in the molecular weight of the template – analyte is approximately 1000 [1]. On one hand, these molecules must be able to diffuse into and out of the polymer; on the other hand, an increased number of interactions will slow dissociation from the binding sites. Molecularly imprinted polymers can recognize one or a group of analytes depending upon the template choice.

The ochratoxin A molecularly imprinted polymer was prepared following the well-established non-covalent imprinting protocol, with formulations comprising cross-linking agent, functional monomer and template in 20:4:1 molar amounts [88].

3.2.5 Choice of a porogen

The solvent plays an important role in the outcome of a molecular imprinting process. A role that is particularly pronounced self-assembly systems. As porogen in the polymerization, the solvent governs the strength of non-covalent interactions in addition to its influence on the polymer morphology. Generally, the more polar the porogen, the weaker the resulting recognition effect becomes, as a consequence of the influence of the solvent polarity on non-covalent interactions. The best imprinting porogens, for accentuating the binding strengths, are solvents of very low dielectric constant, such as toluene and dichloromethane. The use of more polar solvents will inevitably weaken the interaction forces formed between the print species and the functional monomers resulting in poorer recognition. On the other hand, the porogen influence on the structure of the prepared polymers may compensate for this apparent drawback

on the specific surface area and the mean pore diameter is dramatically dependent on the type of porogen used. To maximize the stability of the template-functional monomer complexes, chloroform as an ion-pair and hydrogen bond preserving porogenic solvent. Polymerization was initiated with AIBN and performed under thermal conditions.

3.3 Molecularly Imprinted Solid Phase Extraction and pulsed elution

Molecularly imprinted solid phase extraction and pulsed elution is a good example of solid phase extraction. Sorbents for MISPE-PE need to work only in "on" and "off" modes, strongly absorbing and easily releasing an analyte, while moderate strength of retention with good resolution is required for chromatographic stationary phases. Furthermore, the column efficiency, negatively affected by peak broadening and tailing, is much less significant in MISPE-PE applications.

3.3.1 Removal of template from OTA-MIP

After the MIP particles were packed into a micro-column, 5% $NaHCO_3$ in water was able to remove OTA at a slow flow rate of 0.1 mL/min. The micro-column was then ready for OTA determination by MISPE-PE.

The choice of a solution to remove OTA from MIP is based on the OTA solubility. OTA is soluble in organic solvents at pH less than 7.0, while OTA is present in the dissociated form (or ion) and dissolves in water under neutral and alkaline conditions. Under alkaline conditions, OTA can be deprotonated for desorption. The solution, 5% $NaHCO_3$ in water, was also used in liquid-liquid extraction for OTA sample preparation.

Removing the template molecule from the polymer is necessary not only to make the binding sites available for binding of the analyte but also limit possible interference of the template with the analyte's quantification. Some small amount of template molecule always remains behind in the polymer despite the best attempts to extract it. Residual template presence in the molecularly imprinted polymer and subsequent leaching (bleeding) in practical usage is considered to be a drawback of the present state of molecularly imprinted polymer technology by some. However, it is unrealistic to expect that molecularly imprinted polymers can be developed that show no leaching because that could rule out the analyte's use as the template molecule for the imprinting process in trace analytical methods. Neglecting this prerequisite has actually slowed down the implementation of the technology. The practical consequence of this occurrence is that a suitable structural analogue of the analyte should be available for molecularly imprinted polymer synthesis [89].

3.3.2 Choice of mobile phase for MISPE

In the recognition step similar questions about the choice of solvent arise. Since all non-covalent forces are influenced by the properties of the solvent, non-polar solvents normally lead to the best recognition. When applying the polymers to gradually more polar solvents, the recognition is diminished. Also, the morphology is affected since the swelling of the polymers is dependent on the surrounding medium. Thus, the swelling is most pronounced in chlorinated solvents, such as chloroform and dichloromethane, as compared to, e.g. acetonitrile and tetrahydrofuran. This swelling behaviour may lead to changes in the three-dimensional configuration of the functional groups taking part in the recognition in the sites resulting in poorer binding capability. As a rule of thumb, the best choice of recognition solvent should be

more or less identical to the imprinting porogen in order to avoid any swelling problems, although this is not necessarily a prerequisite. The polymer swelling taking place when polymers are prepared in organic porogens and subsequently used in aqueous phase, is however not necessarily a gross obstacle. The swelling in water is approximately the same as for many other solvents, such as acetonitrile.

An important unsolved aspect that still limits the practical application of MIPs is their poor performance in polar media. Although it is desirable to achieve affinity separation and sensing in water, MIPs usually do not work equally well in aqueous media due to the disruption of hydrogen bonds and competition process between solvent and template molecules for their binding to the polymer functional groups. A significant and possible contribution to loss of polymer affinity also originates from the difference in the structure of the polymer binding sites in organic solvent (traditionally used for polymer preparation) and in water due to differences in polymer swelling. It suggested that, in order to prevent this from happening, it is desirable to perform both, synthesis and separation or sensing in the same environment, e.g. in water [90].

However, in this study, the OTA-MIP exhibited a high affinity for OTA in water and many organic solvents. It was shown that the contribution of strong ionic interaction provided by the amide group and hydrophobic binding provided by the benzene ring in PAM was sufficient for OTA to reach high % binding at most solvents. DDW was chosen as an optimal mobile phase for MISPE-PE determination of OTA. First, DDW is readily available without the high cost and toxicity of organic solvents. Second, the ~100% binding in water was higher than the 95% binding in acetonitrile. Recondition became easier when water was used as the mobile phase. Thus, employing water as the mobile phase, an inexpensive and robust MISPE method was easily developed to facilitate the rapid screening of wheat extract samples for OTA.

3.3.3 Loading OTA samples for MISPE

Methanol/acetic acid (9:1 v/v) was found to be an optimal sample solvent for OTA loading in MISPE due to several advantages. First, OTA is stable in the acidic solution [41]. Second, since methanol is one of the most potent desorbents, adsorption of OTA on glassware walls would be minimal [91]. Third, it was easy for the sample solution to wet the hydrophobic MIP particles and achieve rapid OTA binding. Last, the acetic acid played an important role in sample loading and simultaneous MIP conditioning, which indicated that the mechanism was acid-base chemistry. Water as the mobile phase made the procedure of proton transfer easier than an organic solvent.

3.3.4 Desorption of OTA by PE

Pulsed elution is a method of using a strong solvent or solution to elute an analyte rapidly. OTA is soluble in organic solvents at pH less than 7.0, while OTA is present in dissociated form (or ion) and dissolves in water under neutral and alkaline conditions. Under alkaline conditions, OTA would be deprotonated for rapid desorption. Guided by this hypothesis, several basic solutions were tested for the PE of OTA from the MIP micro-column. Although 5% $NaHCO_3$ in water or 5% $NaHCO_3$ in methanol/water (2:8 v/v) could achieve PE, broad peaks were observed. When methanol/TEA (99:1 v/v) was employed for PE, a sharp peak was obtained. One plausible explanation is that this solution was able to wet the hydrophobic MIP particles easily. The PE mechanism is shown as acid-base chemistry as well. Note that OTA exhibited enhanced fluorescence detection sensitivity under the basic condition of PE with methanol/TEA (99:1 v/v), as compared to neutral or acidic condition.

3.3.5 Confirmation using mass spectrometer

Figure 29 shows that no OTA was detected during the loading step. It strongly suggested 100% binding. The first PE produced a large peak, which indicated that most the extracted OTA was eluted from the micro-column. Good agreement was observed between the results obtained from MS and fluorescence detection, as shown in Figure 30. Since a single injection of PE could not elute all the OTA from the micro-column, two injections of PE were necessary. The same operation (step) was essential when the real samples were tested using MISPE-PE-HPLC.
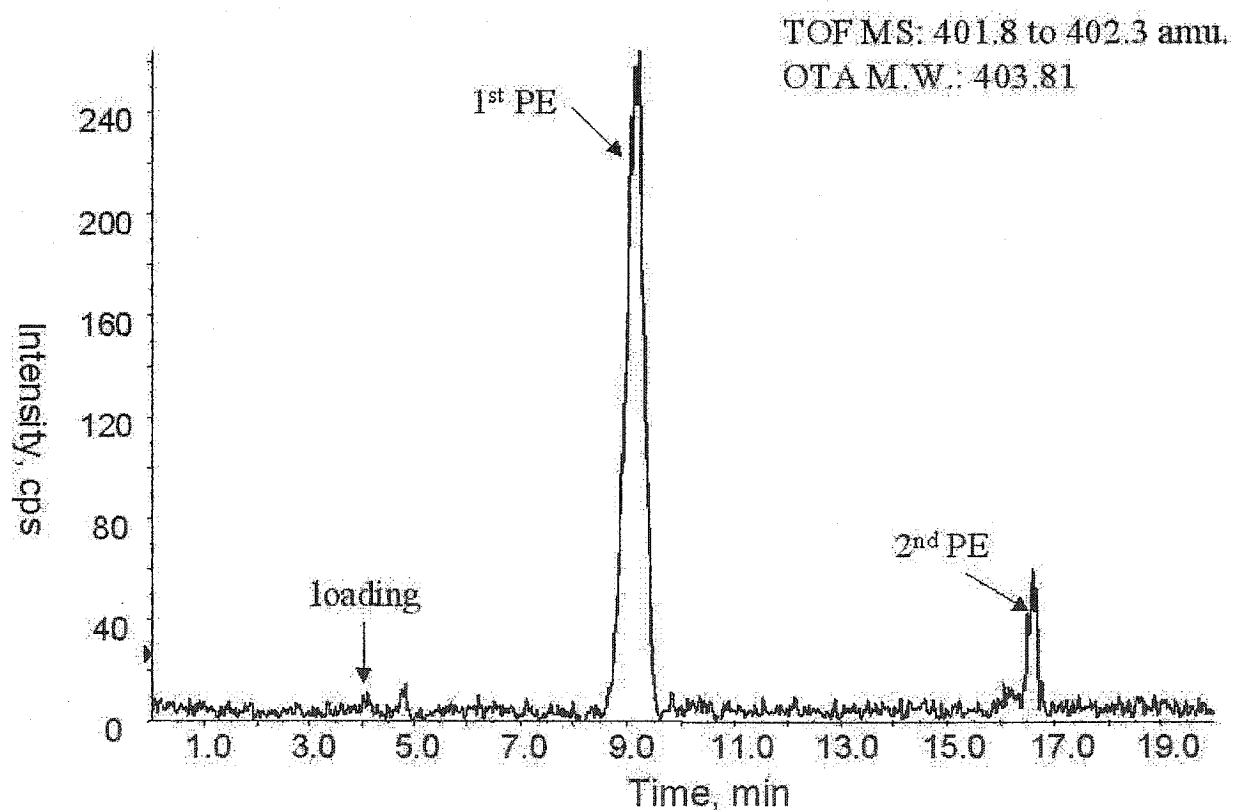
Figure 29. MISPE-PE-MS extracted ion chromatogram of the deprotonated molecule [M-H]⁻ at *m/z* 402 from an OTA standard solution (100 ng/mL). (Note: cps is counts per second)
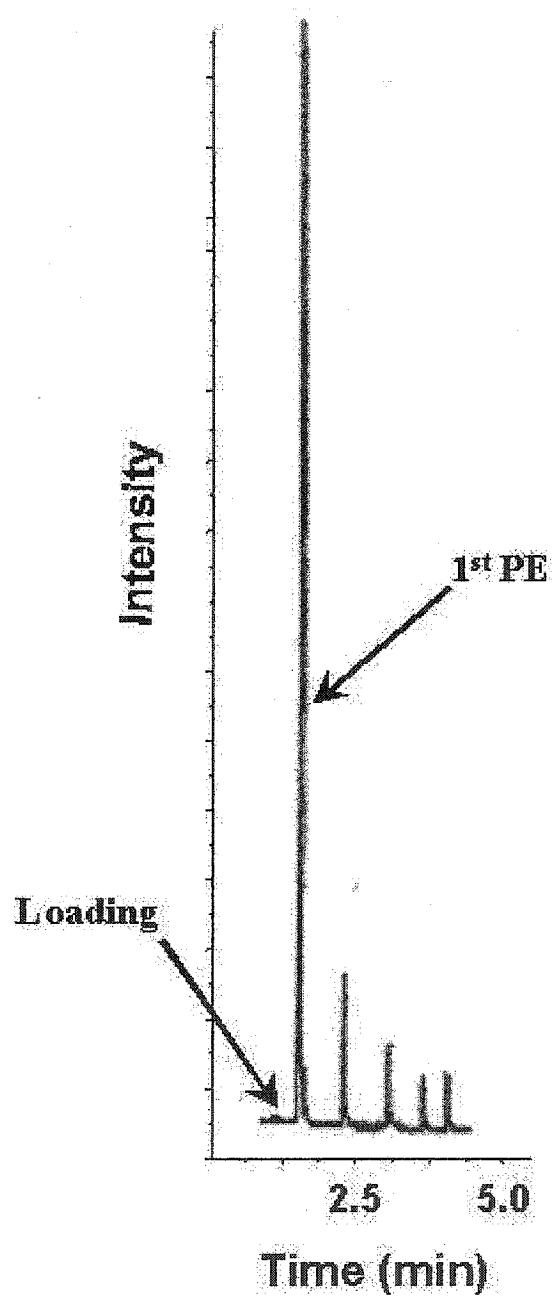
Figure 30. A chart from MISPE-PE using fluorescence detection

3.3.6 Standard calibration curve and recovery

A standard calibration curve for MISPE-PE of OTA is presented in Fig. 31. The MISPE – PE peak area was linearly related ($R^2 = 0.9973$) to the mass of OTA loaded on the MIP micro-column up to 30 ng. Deviation from linearity began near 40 ng, which was approaching the micro-column binding capacity for OTA. The detection limit was found to be 5 ng/mL (or 0.1 ng in 20 μL of sample injected) for OTA, based on three standard deviations of the blank signal.

The excellent clean-up obtained with molecularly imprinted polymer-based SPE columns can be explained by the fact that little discrimination will occur between individual components of the same polarity when using conventional SPE materials. Whereas matrix components with the same polarity will be washed away with the molecularly imprinted polymer phase because they lack the same spatial arrangement of functional groups present in the analyte that causes selective recognition [1].

The MIP acted like a plastic antibody that could specifically recognize OTA as the target molecule. Table 7 summarizes the results of OTA recovery for wheat extracts using MIP. Wheat extracts spiked with OTA offered a recovery of 103±3%, which demonstrated excellent affinity strength of the MIP to bind OTA during MISPE. Figure 32(a) shows that other matrix components in the wheat extract could not bind with the MIP and were eluted as a big breakthrough peak. On the contrary, the breakthrough peak of an OTA standard solution only arose from the background fluorescence signal of methanol/acetic acid (99:1 v/v) due to light scattering. Using the control polymer micro-column, a recovery of 125±5% was obtained. A small breakthrough peak could be observed in Fig. 32(b). Obviously, other matrix components in the wheat extract bound onto the control polymer particles due to poor selectivity. The potential interferences in wheat extracts are lactic acid and amino acids.

Table 7.   Recovery of OTA using MIP for wheat extracts

Sample (number)	Spiked OTA (ng)	Recovery (%)	STD
Canadian Western Red Spring Wheat (T50)	5.0	100.2	2.5
		103.6	
		105.1	
Canadian Western Amber Durum Wheat (T17)	10.0	101.2	2.5
		104.8	
		105.4	
Canadian Eastern White Winter Wheat (T23)	20.0	99.0	3.9
		104.5	
		105.4	
Average		103.0	3.0
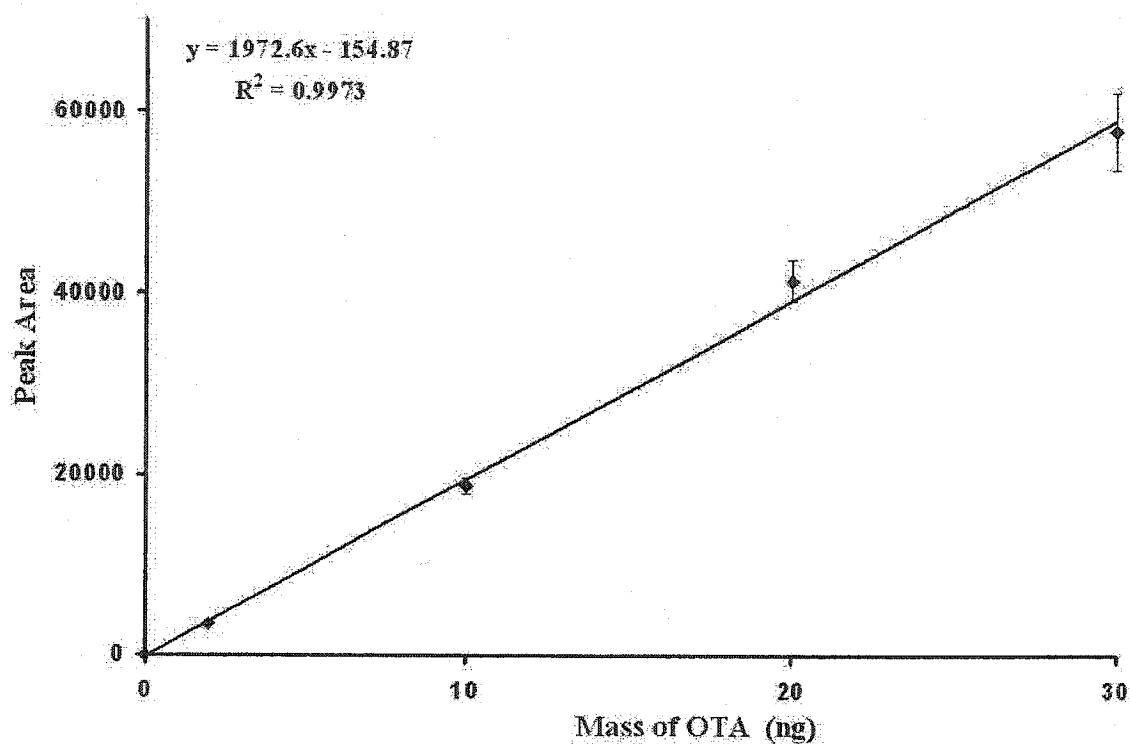
Figure 31. Standard calibration curve for MISPE-PE of OTA, using water as the mobile phase, OTA in MeOH/HOAc (99:1 v/v) as the injection solution, and MeOH/TEA (99:1 v/v) as the pulsed elution solution. Each data point represents the average result of triplicate analyses.
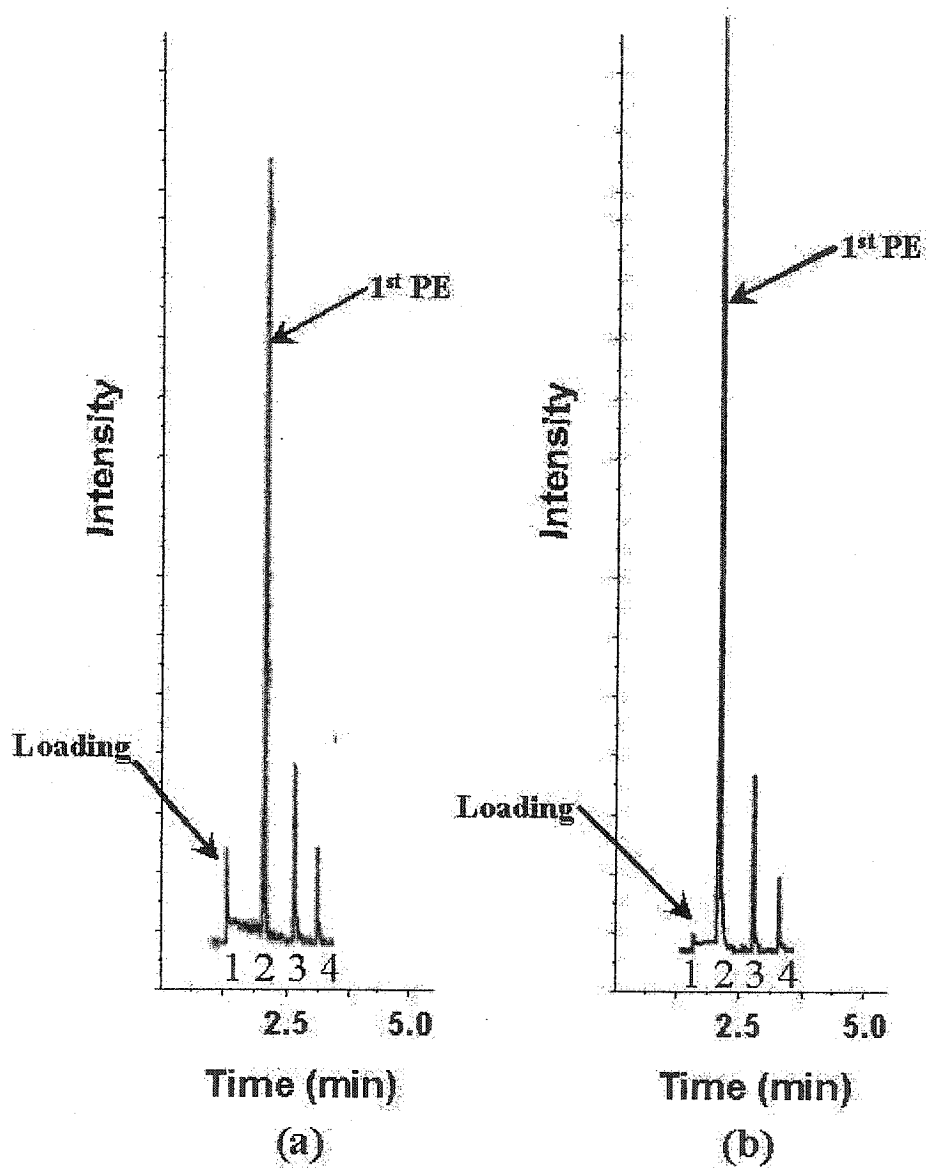
Figure 32. Analyses of wheat extract spiked with 2.0 ng/mL OTA by: (a) MISPE-PE, and (b) control polymer SPE-PE. From left to right in each diagram: breakthrough peak (wheat extract matrix components), 1st PE (OTA + matrix components), 2nd PE peak (OTA + matrix components), and 3rd PE (blank).

3.4 MISPE-PE-HPLC

3.4.1 MISPE for HPLC

In the development of an analytical method, such as a HPLC method, most researchers focus their efforts on separating the analyte from the nearest eluted components. When these components are already partially removed in the sample pretreatment step, HPLC method development becomes simpler. Using MIP-based SPE will limit the presence of new potentially interfering components in extracts and may avoid redevelopment of analytical methods such as drug development applications, matrix changes or samples with co-medications. The cross-reactivity observed for structurally related components is strongly dependent upon the structure and functional groups of the template molecule used for making the molecularly imprinted polymer. It is essential that the functional group types of the template and their 3-D orientation be very similar to those of the target analytes to obtain cross-reactivity. Furthermore, the non-interacting remaining functional groups in the analytes should be similar in size or smaller than the corresponding functional groups of the template molecule [1, 92].

3.4.2 Development of MISPE-PE-HPLC method

The principle of pulsed elution is to use a strong solvent of solution to elute the analytes rapidly. Its advantage has been previously reported in several applications [30, 31, 32, 33, 34, 35, 36, 37, 38]. However, there are always non-specific bindings on the surface of molecularly imprinted polymers due to hydrophobic interactions and broken cavities on the surface. One solution is to develop the method of differential pulsed elution (DPE) followed by final pulsed elution (FPE) [30, 31, 32, 33, 34, 35, 36, 37, 38]. However, it is tedious to develop a MISPE-DPE-FPE method because of the similar properties between the analyte and interference

compounds or structural analogues from sample matrix components. An alternative approach is to employ the well-developed high performance liquid chromatography technique to separate the analyte and interference compounds. An online molecularly imprinted solid phase extraction, pulsed elution and high performance liquid chromatography method was developed as a hyphenation setup that would shorten the total analysis time for rapid screening a large number of samples.

Besides the short time of analysis, this hyphenation design has also provided other advantages. The optimized MISPE-PE conditions can be satisfied to the requirement of OTA standards storage and the enhanced fluorescence, which has been mentioned in the previous sections. Fluorescence sensitivity was increased when MISPE-PE was hyphenated with ion-pair high performance liquid chromatography. Moreover, the optimal mobile phase provided with a good separation between OTA peak and the wine matrix peaks, and a short and reproducible retention time of 3.65 min for OTA. This retention time was far shorter than those of most reported methods, 10 or more minutes [93, 64].

Pulsed elution is a kinetic technique. After methanol/TEA (99:1 v/v) was injected to perform PE, there was a certain time of delay for transfer of the eluted OTA from the injector to the 100-µL sample loop of switch valve. A simple way to determine the delay time was to directly connect the switch valve to the fluorescence detector without any HPLC column. An optimal delay time was found to be 7 seconds through using one OTA standard solution for MISPE-PE. It was better to use a large sample loop (100 µL) that guaranteed the capture of all eluted OTA.

After ochratoxin A was eluted from the micro-column, it was in a dissociated form. A basic HPLC mobile phase was necessary to sustain this form in order to have an enhanced

fluorescence signal. A mobile phase with a pH of 9.8 was here employed for the highest possible fluorescence. It was interesting to find that a slight change in the mobile phase composition didn't significantly affect the retention time.

Figure 33 shows the standard calibration curve for MISPE-PE-HPLC with a fair linearity ($R^2 = 0.9966$). The detection limit was found to be 0.05 ng/mL for OTA, based on three standard deviations of the blank signal.
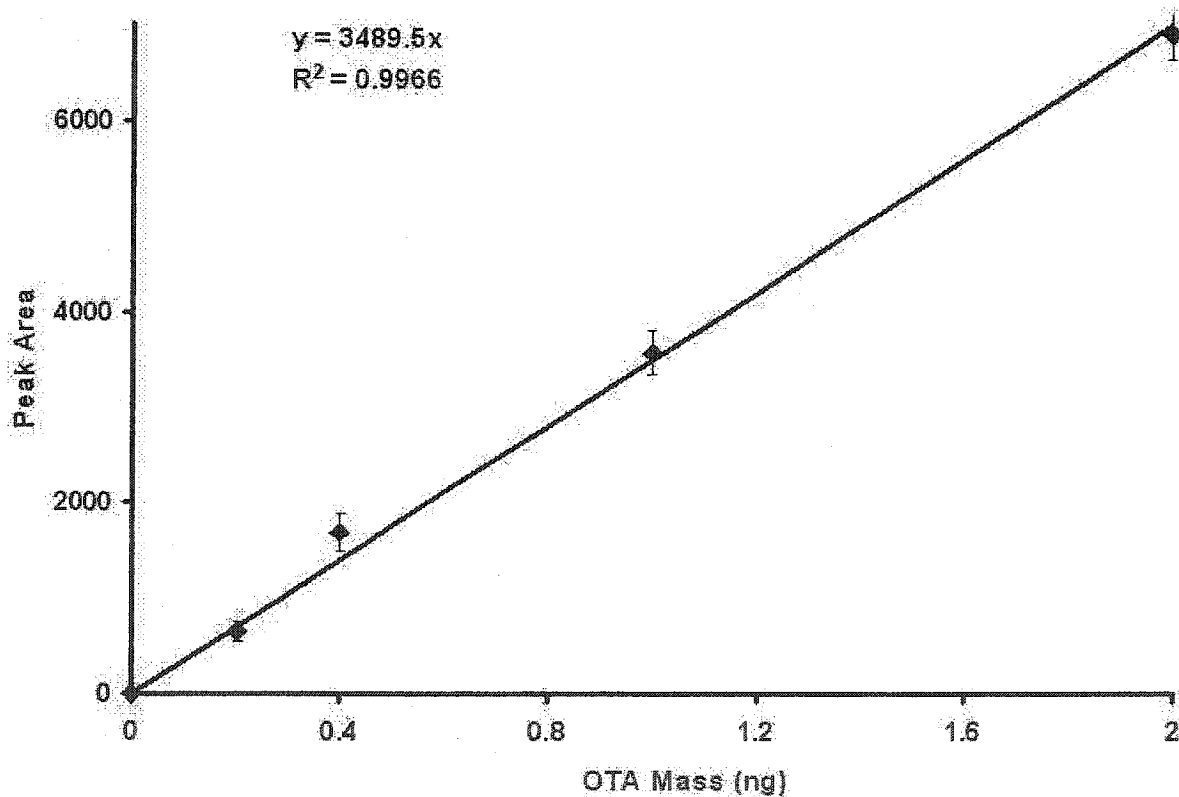
Figure 33. Calibration curve of OTA using MISPE-PE-HPLC

3.4.3 Determination of OTA in wines

Good recoveries were obtained when J. M. Saez *et al* used phenylsilane cartridges to clean up samples [94]. A similar procedure of solid phase extraction was employed in this study only with slight change. SPE was necessary to improve the recovery before the wine sample was injected into the MISPE-PE-HPLC system for OTA analysis. Without the SPE cleanup, the recovery was only about 25%. The potential interferences are tartaric acid and glucose. However, after the SPE cleanup, the recovery improved to 89±4%. It indicated that SPE removed some interfering matrix components so the cross-reaction was minimized. Good agreement was obtained between this result and that reported [64]. MIPSE played a key role in removing matrices from the wine sample further. A comparison of wine solutions, one passed only through phenyl solid phase extraction and another further cleaned-up by MIP, only the latter one provided a good separation between OTA and matrix peaks, as shown in Figure 34. Moreover, the total peak area for matrix components was decreased by a factor of 2. MISPE technique is therefore good for the HPLC analysis. Based on the calibration curve, the OTA concentrations of wine samples were listed in Table 8. Table 9 summarizes OTA recovery at 100% of the test concentration. The concentrations of OTA found the three wine samples were compared to some recent published values. The value obtained for the bottles of French and Italian red wines in the present study were within the range from recent surveys, albeit at the higher end of the concentration ranges [95, 96]. The value obtained for the Australian wine was higher than those reported in the largest available recent survey of Australian wines [97]. This suggests the possibility that the values may represent over-estimate in some cases. These measurements were not confirmed by an existing validated method.

The effect of different MISPE flow rates on %recovery was next investigated. When MISPE-PE-HPLC was employed to determine OTA in wine samples, two different MISPE flow rates, 0.1 mL/min and 0.5 mL/min, showed the similar recoveries (% deviation = 0.88%). The result further indicated that the PAM-based MIP has high affinity for OTA and water was the ideal mobile phase for MISPE.

After the micro-column was used for about one year to carry out different tests, the OTA-MIP still showed good usability and reproducibility. Since the MIP column is chemically robust to be reusable for numerous runs, MISPE-PE incurs a lower cost ($ 0.5 per sample analysis) as compared to immunoaffinity column.

Table 8.   Results of analysis for red wine samples

Sample	Sample origin (Brand name)	OTA (µg/L)	OTA (µg/L) Average	STD	RSD (%)
1	Italy (Dragani)	4.14	4.58	0.43	9.3
		4.60			
		4.99			
2	France (Chateau Timberlay)	0.79	0.82	0.03	3.7
		0.83			
		0.85			
3	Australia (Yalumba)	2.15	2.21	0.09	4.1
		2.16			
		2.31			

Table 9. Results of OTA recovery for red wine samples

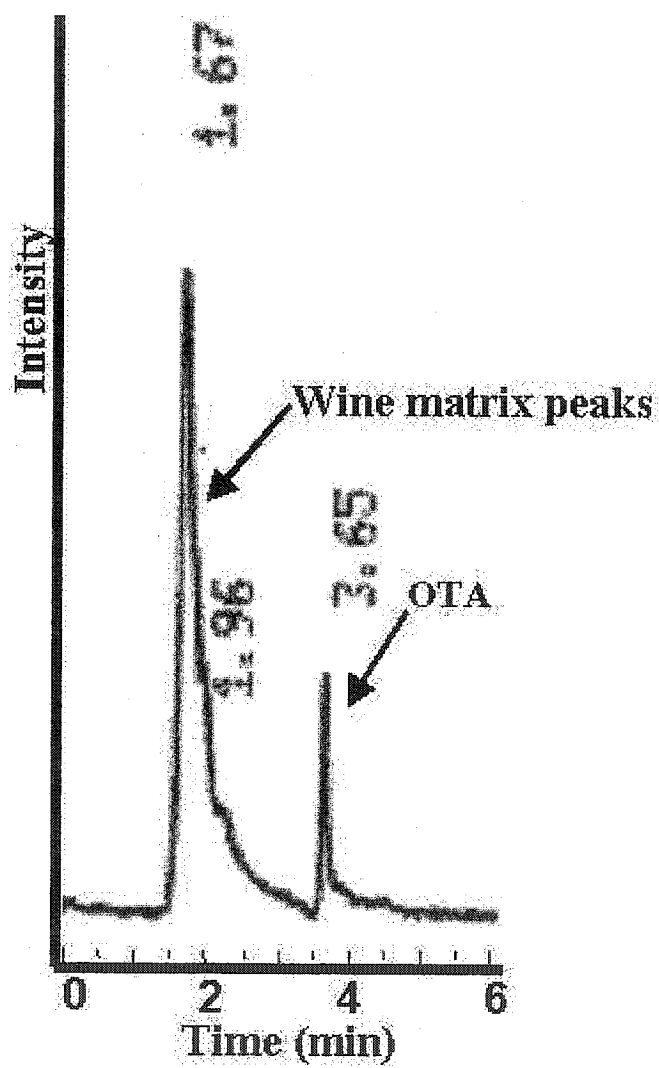| Sample	Sample origin (Brand name)	Spiked OTA (µg/L)	Recovery (%)	STD
1	Italy (Dragani)	4.00	85.6	5.3
			86.7	
			95.3	
2	France (Chateau Timberlay)	1.00	85.4	4.0
			88.7	
			93.4	
3	Australia (Yalumba)	2.00	86.9	2.9
			87.2	
			92.1	
Average			89.0	4.1

Figure 34. Ochratoxin A chromatogram using MISPE-PE-HPLC

3.5 Molecular modeling

An interesting new topic of MIP research is molecular modeling. The software package, SYBYL-6 (Tripos, St. Louis, MO) has recently been used to simulate monomer-template interactions [90, 98, 99]. All these structures were charged using the Gasteiger-Huckel approximation method, and refined using the molecular mechanics method applying an energy minimization with the MAXIMIN2 command. Then, the Leapfrog algorithm was applied to screen the library of functional monomers for their possible interactions with the template. The result from each run was examined by evaluating the binding scores. The monomers giving the highest binding scores and capable of forming the strongest complexes were used for preparation of MIPs. The software, Hyperchem, was also employed for MIP molecular modeling [100, 101, 102, 103]. F. Chapuis *et al* [103] used Hyperchem to perform molecular modeling. Molecular mechanic has permitted to find conformations of low energy; they were refined using semi-empirical mechanic. The conformation that possessed the lowest energy was refined with ab initio mechanic in order to obtain the electronic distribution. Molecular modeling provided a better understanding of the retention mechanism involved in the SPE procedure and was a useful guide for reducing non-specific interactions.

So far the software, Spartan, was not used for the purpose of MIP molecular modeling. Spartan was initially attempted to choose a best functional monomer with high affinity for OTA in this study. However, more research has to be done to predict molecular interactions using software such as Hyperchem, SYBYL, Spartan [104] and Gaussian 03 [105], for the future work.

3.5.1 Determination of atomic charges for the monomers and OTA

It would be more useful to be able to predict the interactions between OTA and the functional monomer(s). Molecular recognition is based on both steric criteria and interactions resulting from the electronic properties of the molecules, so molecular modeling can be helpful when it allows the calculation of atomic charges. The Spartan software was used to calculate the atomic charges. It only focused on those atoms that could potentially form hydrogen bonding.

Several polymer systems have been developed for use in molecular imprinting technology. By far the most readily used systems are either polyacrylate-based or polyacrylamide-based. Another is the polystyrene-based system, which has been used to a lesser extent. Spartan calculation can give the reason. Figure 35 shows the atomic charges and molecular structures. The nitrogen atoms in 4-vinylpyridine (4-VP) and 1-vinylimidazole (VI) have low positive partial charges, which cannot form strong H-bonding with OTA. Although aromatic properties exist in 4-VP and VI, the % bindings were low as shown in the previous results. Moreover, there is no hydrogen atom that can form H-bonding between molecules. It also explains the weak interaction between OTA and 4-VP or VI. Compared with monomers containing an/the acrylate group, the positive partial charges of nitrogen or oxygen in an/the acrylamide group are lower and the positive charges of hydrogen are higher. A conclusion can be drawn that polyacrylamide-based MIPs offer stronger H-bonding than polyacrylate-based MIPs. Interaction energies between monomers and OTA should be calculated in order to compare polyacrylamide-based MIPs with polyacrylate-based MIPs. Although the nitrogen, oxygen and hydrogen atoms in the chosen acrylamide monomers show close partial charges, PAM was observed to afford the highest affinity for OTA due to the phenyl group, which played a key role in strong interaction with OTA.
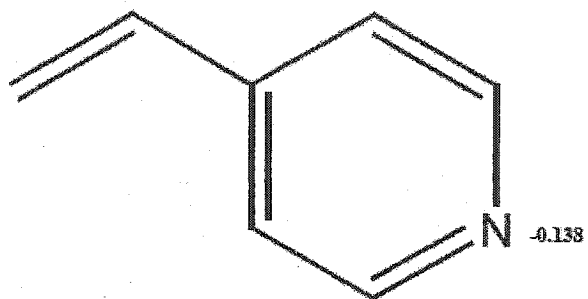
4-Vinylpyridine (4-VP)
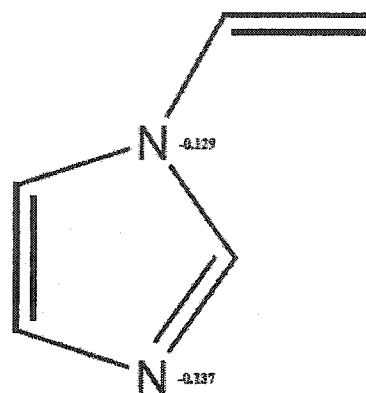
1-Vinylimidazole (VI)
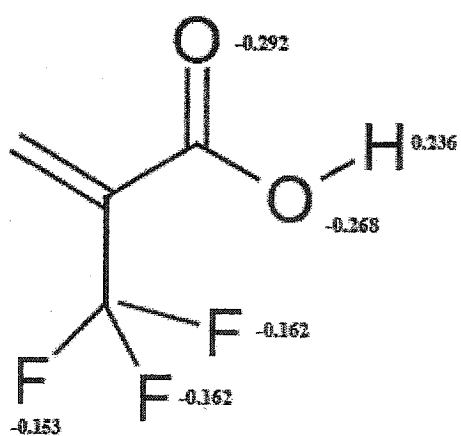
2-(Trifluoromethyl)acrylic acid (TFMAA)
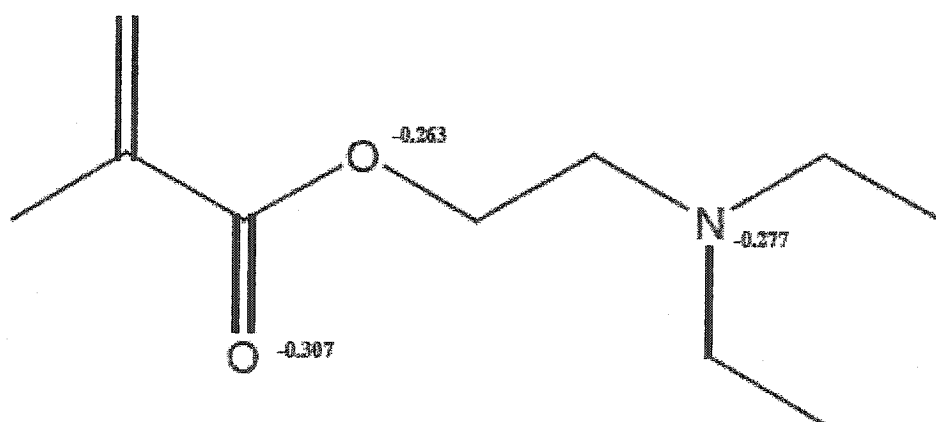
2-(Diethylamino)ethyl methacrylate (DE-MA)

88
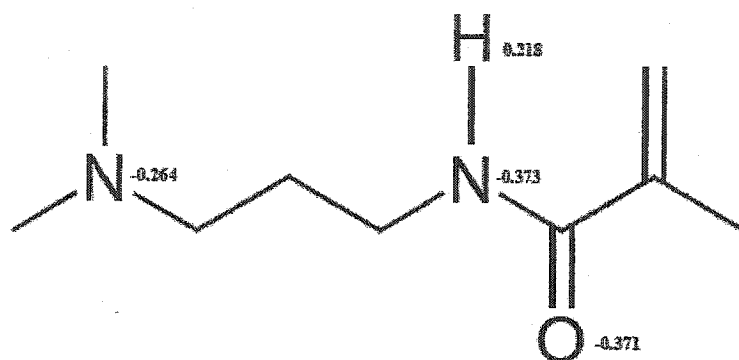
N-(3-(Dimethylamino)propyl) methacrylamide (DMP-MAAd)
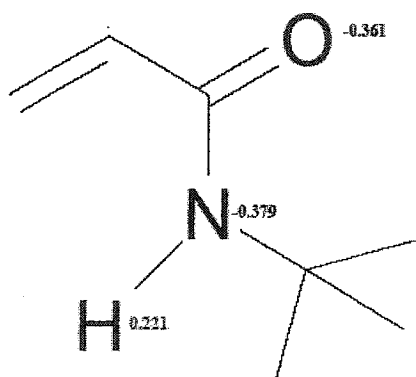
N-tert-Butylacrylamide (N-BAAd)
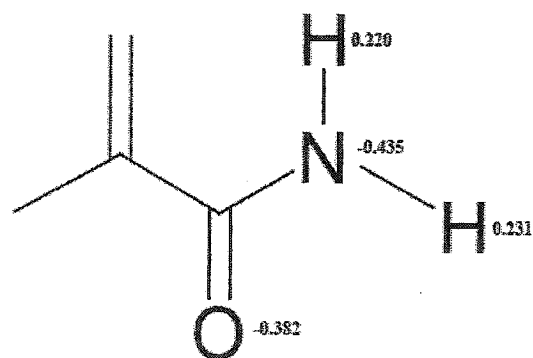
Methacrylamide (MAAd)
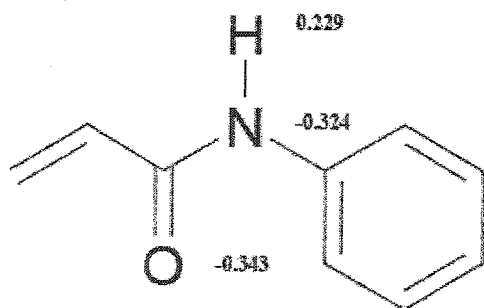
(E-) N-Phenylacrylamide (PAM)
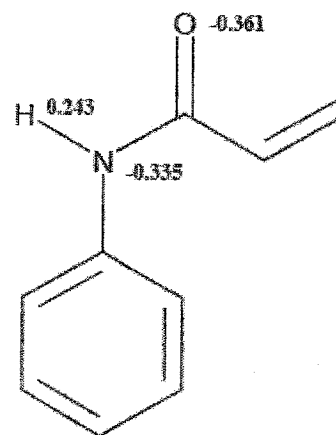
(Z-) N-Phenylacrylamide (PAM)

Figure 35. Molecular structures and atomic charges of monomers used to test affinity for OTA

Figure 36 shows the partial charges of atoms in OTA that can form H-bonding. It indicates that there are multiple points to form H-bonding. It is important to choose a monomer like PAM that can form two hydrogen bonds simultaneously, so as to strengthen the overall interaction with OTA.
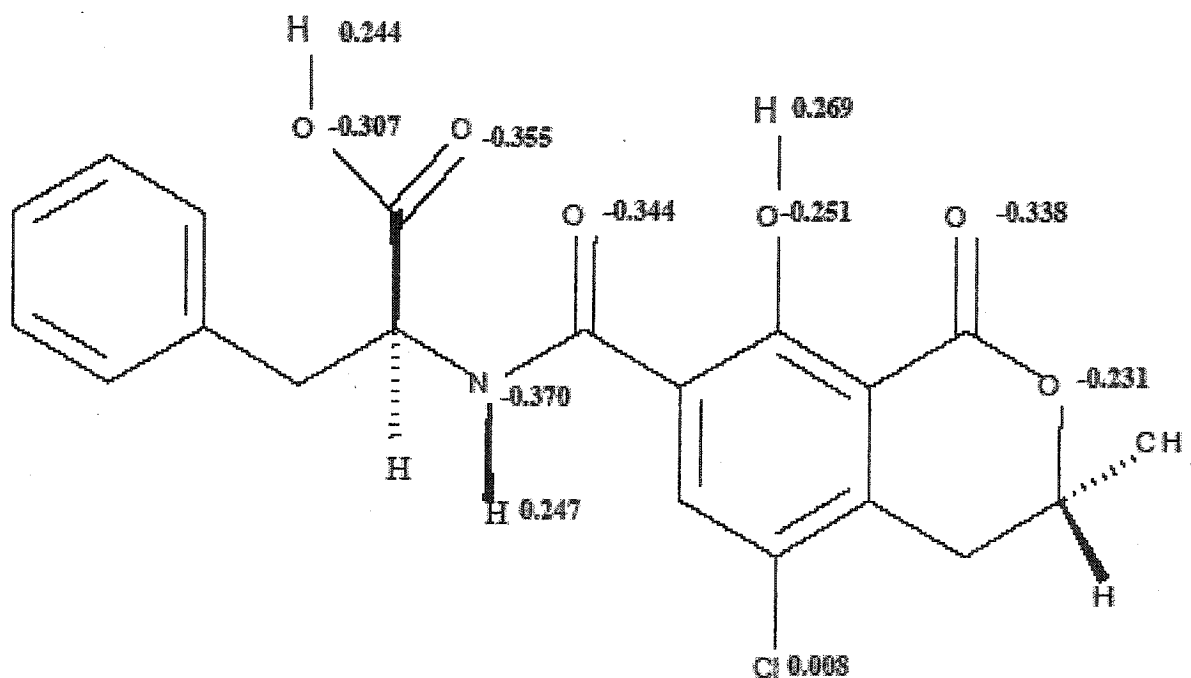
Figure 36. Partial charges of some atoms in OTA

3.5.2 Stability comparison of PAM isomers

Because of a trigonal planar geometry at nitrogen, secondary and tertiary amides can exist in both E and Z conformations about the carbonyl-nitrogen bond. The Z conformation predominates in most secondary amides because, in this form, van der Waals repulsions between the largest groups are avoided. Figure 37 shows the formation heat values calculated by Spartan. The heat of formation for E-PAM is 10.881 kcal/mol while that for Z-PAM is 11.445 kcal/mol.

Good agreement was obtained between the results of experimental data and Spartan calculations. It is interesting to find that there is no big difference in atomic charges between the two isomers shown in Figure 37.

Z   E
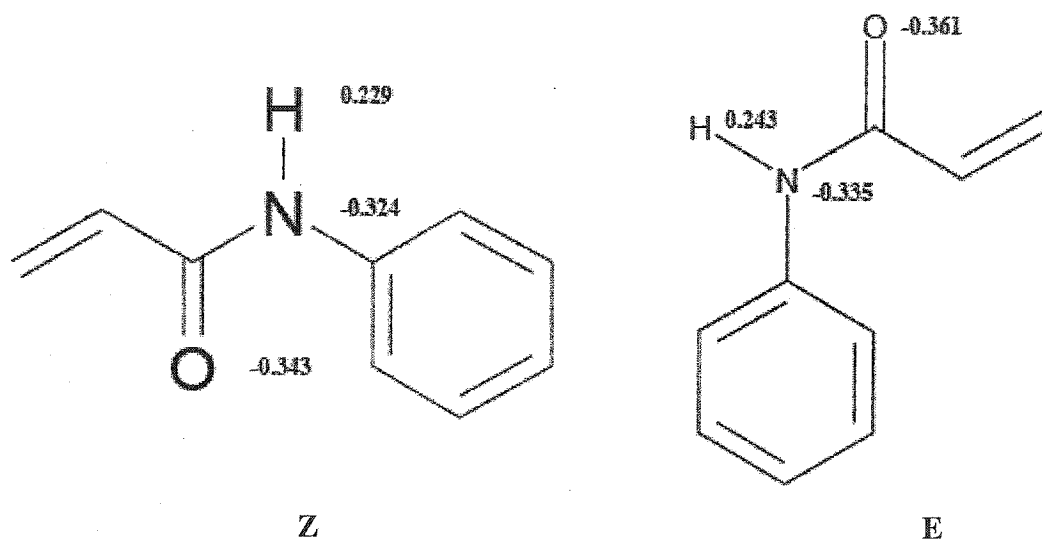
Heat of formation: 10.881 kcal/mol   Heat of formation: 11.445 kcal/mol

Figure 37. Comparison of Z-PAM with E-PAM in terms of atomic charges and heats of formation

3.6   Comments on molecularly imprinted polymers

Molecularly imprinted polymers can provide a strong contribution to the selectivity and sensitivity of analytical methods. Theoretically, they can solve a given selectivity problem, reduce method development time, reduce analysis time, and possibly allow the use of more conventional detectors. The last aspect can save money and facilitate method transfer to quality control laboratories and contract research.

From a practical point of view, developing a MIP sorbent is much quicker than developing biological antibodies (i.e., weeks versus one year). However, compared with conventional SPE sorbents, molecularly imprinted polymers must be synthesized for each new analyte or class of analytes. If specific molecularly imprinted polymer materials can be produced for specific analytes or classes of analytes, such as drugs of abuse or environmental pollutants, then the disadvantage becomes less significant. One issue that can limit the use of molecularly imprinted polymers is related to the cost and toxicity of template molecules.

The fast-growing research activities in molecular imprinting may contribute to the development of more rapid screening procedures for new molecularly imprinted polymers, reduced template consumption, and a higher success rate because of the availability of more highly developed polymerization protocols. Time will tell if a large range of imprints for biomarkers, toxins and drug residues will become commercially available. The upcoming development techniques for affinity phases based upon phage display [106] or recombinant-type antibodies might remove a principal advantage of molecularly imprinted polymers in time, though these biomolecules will not reach the same chemical stability as MIPs. The development of less expensive and very selective mass spectrometers could be seen as a threat to the use of molecularly imprinted polymers for several applications, but molecularly imprinted polymers can also remove matrix components that affect the ionization and identification of the target analytes in mass spectrometry.

3.7 Future work

Uniform molecularly imprinted polymer could be synthesized because it may decrease non-specific binding. Differential pulsed elution would be developed to remove the potential

non-specific binding from structural analogues. The technique should be applied to more real samples in order to further develop the technique of molecularly imprinted solid-phase extraction and pulsed elution. The next instrumental design would be online molecularly imprinted solid phase extraction capillary electrophoresis, which could minimize solvent consumption. Moreover, capillary electrochromatography for OTA analysis could also be developed using the ochratoxin A molecularly imprinted polymer. A laser induced fluorescence method should be introduced in order to improve detection limit. Lastly, molecular modeling could be continued.

CHAPTER IV

CONCLUSION

This research addressed the synthesis of a stable OTA-selective molecularly imprinted polymer for solid phase extraction, and the development of fast analytical methods for application to real-world samples, wheat extracts and red wines.

Since the price of OTA is pretty high, the approach of making non-imprinted polymers was first taken in this research. 1,1,1-Trimethylolpropane trimethacrylate (TRIM) was employed as a cross-linker and several monomers were chosen to synthesize non-imprinted polymers that were packed into micro-columns individually. Multiple loading injections were performed to determine the monomer's affinity for OTA. Comparing the first loading injection breakthrough peak area with the saturation breakthrough peak area, the percentage binding data for the first injection was obtained. The greater the difference between the first injection peak area and saturation peak area, the higher affinity the monomer has for OTA. The monomer with the highest affinity was found to be N-phenylacrylamide, which had never been used in the MIP field. PAM is a strong base that can pair up with OTA as a weak acid. The molecular structure of PAM consists of a benzene ring that can offer hydrophobic interaction with OTA. This molecule has an amide group that can form hydrogen bonds with OTA. The poly(PAM-*co*-TRIM) polymer exhibited high affinity for OTA in many organic solvents and water. Water was chosen as the optimal mobile phase for the development of a rapid screening method. Nearly 100% binding could be achieved for OTA in methanol/acetic acid (99:1 v/v), which also conditioned the MIP simultaneously. Pulsed elution was developed based on the property of OTA solubility. OTA is soluble in organic solvents at pH < 7.0 but dissolves in water as the dissociated form (or anion) under neutral and alkaline conditions. Under alkaline conditions, OTA was deprotonated for rapid desorption from the non-imprinted micro-column. After several basic solutions were tested for pulsed elution of OTA, methanol/TEA (99:1 v/v) proved

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to be the best because this solvent mixture was able to wet the hydrophobic particles easily. The mechanism was summarized as acid-base chemistry for the binding and pulsed elution. It was interesting to find that the mechanism was totally different comparing water with organic solvents (such as chloroform) as the mobile phase. The PE peak area was linearly related (R^2 = 0.9923) to the mass of OTA loaded on the non-imprinted micro-column up to 40 ng. The detection limit was found to be 7.8 ng/mL for OTA. The recovery of 125±5% was found when the method of NISPE-PE was applied to wheat extracts.

Based on the above results, molecularly imprinted polymer particles were synthesized from PAM and slurry-packed into a micro-column for selective solid-phase extraction of OTA. With water flowing at 0.5 mL/min, a total binding capacity of 30 ng OTA was determined for 20 mg of MIP particles. MISPE conditions were optimized using OTA in methanol/acetic acid (99:1 v/v). Nearly 100% binding could be achieved from one 20-μL injection of sample containing up to 30 ng of OTA. Pulsed elution (PE) using methanol/triethylamine (99:1 v/v) was good for the quantitative desorption of OTA. The results of MISPE-PE using fluorescent detection were confirmed by mass spectrometry. Good agreement was obtained between two results. The method of MISPE-PE was applied to wheat extracts. The MISPE-PE peak area was linearly related (R^2 = 0.9973) to the mass of OTA loaded on the MIP micro-column up to 30 ng, and the limit of detection was 5.0 ng/mL. Wheat extracts spiked with OTA offered a recovery of 103±3%. Each MISPE-PE analysis required less than 5 min to complete.

To improve the detection limit of the MISPE-PE method, a re-designed instrumentation was employed, online molecularly imprinted solid phase extraction, pulsed elution and ion-pair high performance liquid chromatography. An alkaline mobile phase (CH$_3$CN/NH$_4$OH, 15:85 v/v, pH = 9.8) for HPLC was employed to enhance OTA fluorescence, reproduce the short

retention time of 3.65 min, and achieve a good separation of OTA, OTB and wine matrix peaks. A standard calibration curve afforded a fair linearity ($R^2 = 0.9966$). The detection limit was found to be 0.05 ng/mL for OTA. For red wine analysis, after the interfering matrix compounds were removed by solid phase extraction using a phenyl cartridge, the extract in methanol/acetic acid (99:1 v/v) was directly injected into the MISPE-PE-HPLC system. The 89±4% recovery of this method was satisfactory for quantitative analysis.

After the OTA-MIP micro-column was used more than one year to carry out many different tests, the OTA-MIP still showed good usability and reproducibility. Since the MIP column is chemically robust to be reusable for numerous runs, MISPE-PE incurs a significantly lower cost for each sample analysis as compared to immunoaffinity columns.

Spartan software was used to predict the interactions between OTA and functional monomers using the atomic charges and the heats of formation.

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