

Cranfield University

Institute of BioScience and Technology

Academic Years 2000-2004

Nicholas W. Turner

**Development of a
Molecularly Imprinted Polymer
Specific for Ochratoxin A:
Theoretical and Sensor Applications.**

Supervisors:
Professor S.A. Piletsky
Professor N. Magan

Ph.D. Thesis

January 2004

This thesis is submitted for the degree of Doctor of Philosophy.

For
Daphne, Theo,
William and Thelma

Abstract

In this work the development of two molecularly imprinted polymers, specific for ochratoxin A, is presented. Ochratoxin A is produced by several *Aspergillus* and *Penicillium* species and is common in cereals and other starch rich foods and has also been found in coffee, dried fruits, wine, beer and meats. It demonstrates potent teratogenic, immunosuppressive, mutagenic and carcinogenic properties. The toxin is also linked to Balkan Endemic Nephropathy, a chronic kidney disease found in South-Eastern Europe. Due to this the European Union has set limits on foodstuffs ranging between 2-10 ng g⁻¹. Therefore the requirement of a simple and inexpensive biosensor to monitor this legislation is a necessity. Currently detection is performed by chromatographic methods such as HPLC, and by ELISA formats.

In this work two polymeric materials, rationally designed by computational modelling and synthesised using molecular imprinting, are studied. The modelling is complimented with a Nuclear Magnetic Resonance (NMR) study. The first polymer (Polymer A) consisted of 1 mol of acrylamide and 1 mol of methacrylic acid to 1 mol of template. This material demonstrated an unusual binding mechanism, working solely in aqueous solvents. A theoretical mechanism for this binding is presented and discussed.

The second polymer, again rationally designed, but under different conditions, consisted of 1 mol of N,N- diethylamino ethyl methacrylate (DEAEM) to 1 mol of template. This polymer demonstrated high affinity for the template in acetonitrile.

Polymer A is used in combination with an ion-exchange SPE protocol (developed for this purpose) for the extraction of OTA from maize.

Both polymer compositions are used in development of a MIP membrane optical sensor, with partial success seen in the detection of OTA in grape juice and white wine.

Acknowledgements:

I wish to offer my heartfelt thanks (and my heartfelt condolences for putting up with me) to my supervisors and colleagues.

Professor Sergey Piletsky, who has guided, cajoled, badgered, scared and supported me through these past four years, without his help I would not be where I am today. Thank you does not say it well enough.

Professor Naresh Magan for helping a stranger get by in the world of mycotoxins and EU projects. *Dr. Kal Karim*, one of the helpful people I have ever had the pleasure of meeting and working with (even with his dubious taste in movies). *Dr Elena Piletska*, *Dr Iva Chianella*, and the rest of the *MIPers* at Silsoe, past and present, for their help and making the lab a bearable place.

I wish to thank the European Union Framework 5 (PLQLRT-1999-01380) (OTA PREV) for funding this work and I send my regards to all who worked on this proposal.

I would also like to thank *Dr. Michael Whitcombe*, for work with the NMR analysis, *Dr. Michael Malecha* for work with the particle analysis and *Prof. Claudio Baggiani* for access to analogues of OTA.

My family for their support and my dearest *Sinéad*, soon to be my wife, I thank you for all that you are and more. I love you.

And the rest....

Lennard (RIP), *Quiche* (old school), *Blaid Ddu* (new school), *Class of Edgewood 1098*, *Dragons*, *Harts*, *Vipers* and even the odd *Gryphon*.

Bethor, *Chuffson*, *Quincy*, *Sir* and *Swervo*. And all those I do not have room for...

Game of D anyone? YCII.

Contents

Contents	1
List of Figures	7
List of Tables	12
List of Abbreviations	15
INTRODUCTION AND LITERATURE REVIEW	16
1.1 Introduction	17
1.2 Mycotoxins.....	18
1.2.1 Ochratoxins	19
1.3 Mycotoxin detection.....	25
1.3.1 Sample pre-treatment: extraction and clean-up.....	27
1.3.1.1 Liquid-liquid partition.....	28
1.3.1.2 Supercritical fluid extraction (SFE).....	29
1.3.2 Solid phase extraction (SPE)	29
1.3.2.1 Silica based materials.....	30
1.3.2.2 Ion Exchange Materials	31
1.3.2.3 Mycosep™ columns	31
1.3.2.4 Immunoaffinity columns.....	32
1.3.3 Chromatographic determination.....	33
1.3.3.1 Thin Layer Chromatography (TLC)	34
1.3.3.2 High Performance Liquid Chromatography (HPLC).....	35
1.3.3.3 Gas Chromatography (GC)	37
1.3.4 Mass Spectroscopy	39
1.3.5 Enzyme Linked Immunosorbent assay (ELISA)	40
1.3.6 Capillary Electrophoresis (CE).....	42
1.4 Molecularly Imprinted Polymers	44

1.4.1	History	44
1.4.2	Theory	46
1.4.2.1	Covalent or pre-organised imprinting	47
1.4.2.2	Non-covalent imprinting	49
1.4.3	Thermodynamics	50
1.4.4	Practical recommendations for polymer design	52
1.4.4.1	Monomer selection	53
1.4.4.2	Template requirements	56
1.4.4.3	Porogen	57
1.4.4.4	Cross linker	57
1.4.4.5	Initiator	58
1.4.4.6	Polymerisation conditions	59
1.4.5	MIPs for toxins	59
1.5	Objectives	62

MATERIALS AND METHODS..... 66

2.1	Introduction	67
2.2	Materials	67
2.3	Methods	70
2.3.1	Computational design of an imprinted polymer specific for OTA	70
2.3.1.1	Design of molecular model of template	70
2.3.1.2	Design of functional monomer database	71
2.3.1.3	Screening of the template against the database using the Leapfrog™ algorithm	74
2.3.1.4	Refining step (simulated annealing)	76
2.3.1.5	Analysis of monomer template interaction in organic solvent	77
2.3.2	Polymer monolith synthesis	77
2.3.2.1	Polymer A	77
2.3.2.2	Polymer B	79
2.3.3	Polymer washing	80

2.3.3.1	Electrochemical elution of OTA from polymer B.....	81
2.3.4	Imprinted membrane production	82
2.3.4.1	Polymer A	83
2.3.4.2	Polymer B	84
2.3.5	Washing of polymer membranes	85
2.3.5.1	“Burning” of residual OTA in membrane by UV irradiation.....	85
2.3.6	Polymer characterisation.....	85
2.3.6.1	A note on fluorescence	85
2.3.6.2	Measuring OTA fluorescent signal	86
2.3.6.3	Measurement of OTA in solution for SPE experimental phase.....	86
2.3.6.4	Fluorescent analysis of OTA in membrane	87
2.3.6.5	Membrane specificity and affinity	88
2.3.7	Nuclear Magnetic Resonance (NMR)	89
2.3.8	Particle Size Analysis.....	89
2.3.9	Solid Phase Extraction (SPE) Analysis.....	90
2.3.9.1	MIP-based SPE	91
2.3.9.2	Binding of OTA in different pH.....	92
2.3.9.3	Binding of OTA in different ionic concentrations	92
2.3.9.4	Binding of different OTA concentrations.....	93
2.3.9.5	Binding of OTA in varied concentration of acetonitrile (MeCN).....	93
2.3.10	Cross reactivity studies.....	94
2.3.11	Sensor design and analysis.....	95
2.3.11.1	Preparation of grain extract	95
2.3.11.2	Membrane study with model solutions	96
2.3.11.3	Membrane studies with wine and grape juice	96
2.3.11.4	Membrane study with grain extract	97
2.3.12	SPE experiments in grain samples.....	97
2.3.12.1	Identification of suitable materials for extraction of OTA from grain.....	97
2.3.12.2	IEX extraction combined with MIP extraction	98
2.3.12.3	Study on the effect of the extract on binding of OTA to polymer A	98

EXPERIMENTAL RESULTS.....	100
3.1 Introduction	101
3.2 Polymer Characterisation	101
3.2.1 Molecular Modelling	101
3.2.1.1 Design of molecular model of OTA	101
3.2.1.2 Rational design of polymer using molecular modelling	103
3.2.2 Nuclear magnetic resonance	110
3.2.3 Measurement of OTA by fluorescence.....	116
3.2.4 Polymer synthesis	116
3.2.5 Washing of polymer particles	116
3.2.5.1 Polymer A	116
3.2.5.2 Polymer B	119
3.2.6 Imprinted membrane production	120
3.2.7 Fluorescent analysis of OTA in membrane	122
3.2.8 Particle size analysis	124
3.2.9 SPE analysis	126
3.2.9.1 Polymer behaviour.....	126
3.2.9.2 Cross reactivity studies.....	131
3.3 Sensor design and analysis.....	134
3.3.1 MIP membranes in optical sensing	134
3.3.1.1 Membrane study in model solutions	134
3.3.1.2 Membrane studies with grape juice and wine	136
3.3.1.3 Membrane studies with grain extract.....	139
3.3.2 SPE experiments in grain samples	139
3.3.2.1 Identification of suitable materials for extraction of OTA from grain.....	139
3.3.2.2 Celite 545.....	140
3.3.2.3 Activated Carbon/ AlO_3	141
3.3.2.4 Polymer B	142
3.3.2.5 IEX cartridges	143
3.3.2.6 Combination of IEX and Polymer A MIP extraction.....	149

DISCUSSION.....	152
4.1 Introduction	153
4.2 Polymer design	153
4.2.1 Computer modelling	153
4.2.2 Design of the molecular model.....	155
4.3 Nuclear magnetic resonance.....	159
4.4 Polymer synthesis.....	162
4.4.1 Synthesis.....	162
4.4.2 Washing	163
4.4.2.1 Polymer A	163
4.4.2.2 Polymer B	164
4.4.2.3 Membranes.....	165
4.5 Measurement of OTA by fluorescence.....	166
4.6 Characterisation of polymer A.....	168
4.6.1 SPE analysis of polymer behaviour	168
4.6.2 Cross reactivity.....	171
4.6.3 Polymer conformation	172
4.7 Membrane analysis	176
4.7.1 Polymer A.....	178
4.7.2 Polymer B.....	179
4.8 SPE experiments for maize analysis.....	181
CONCLUSIONS AND FURTHER WORK.....	186
4.1 Introduction	187
4.2 Conclusions	187
5.3 Further work.....	189
REFERENCES.....	192

List of Figures

Figure 1.1: Examples of common mycotoxins demonstrating structural diversity of these compounds.....	20
Figure 1.2. Structural representations of ochratoxin A and B.	22
Figure 1.3: Structural representations of the dihydroisocoumarins, ochratoxin α , ochratoxin β and mellein.	23
Figure 1.4: Schematic of molecular imprinting theory.....	47
The template shown in blue and monomers (red and yellow) interact to form a pre-polymerisation complex. This complex keeps its steric and chemical properties throughout the polymerisation process and then the template can be removed, leaving an “imprint” behind.	47
Figure 1.5: Schematic representation of a covalent imprinted binding cavity (From Wulff, 1995).	48
Figure 1.6: Schematic representation of non-covalent imprinting.....	51
Figure 1.7: Ethylene-glycol dimethacrylate (EDGMA)	58
Figure 1.8: 1,1'-azobis(cyclohexane-carbonitrile) (ACHN)	58
Figure 2.1: A 3D representation of ochratoxin A, as simulated by the SYBYL 6.9 molecular modelling software.....	71
Figure 2.2: Electrophoresis set-up with SPE cartridge.	82
Figure 2.3: Polymerisation set-up for membrane production.....	83
Figure 2.4: Instrumentation set-up for membrane polymer study.	87
Figure 2.5: Schematic of the SPE process.....	91
Figure 2.6: Analogues used for cross reactivity studies.	95
Figure 3.1: A minimised 3D representation of ochratoxin A, as simulated by the SYBYL 6.9 molecular modelling software.	102

Figure 3.2a: Predicted interaction between OTA and acrylamide from Leapfrog screening performed in vacuum. The yellow dotted lines signify hydrogen bonding between templates.	106
Figure 3.2b: Predicted interaction between OTA and methacrylic acid from Leapfrog in vacuum. The yellow dotted lines signify hydrogen bonding between templates.	106
Figure 3.3: Predicted interaction between OTA and DEAEM from Leapfrog in acetonitrile.	107
Figure 3.4: Example of the refining step solvation box. OTA (green) surrounded by methacrylic acid and acrylamide molecules in solvated box. This system has been minimised.	108
Figure 3.5: Final result of refining step. This figure shows the predicted interactions between OTA, acrylamide and methacrylic acid.	109
Figure 3.6: Final result of refining step. This figure shows the predicted interactions between OTA and DEAEM.	110
Figure 3.7: OTA, numbered for NMR identification.	111
Figure 3.8a: NMR spectra at 270MHz of OTA in acetonitrile (MeCN).	113
Figure 3.8b: NMR spectra of OTA and acrylamide in MeCN (1:5).	113
Figure 3.9a: NMR spectra at 270MHz of OTA in acetonitrile (MeCN).	114
Figure 3.9b: NMR spectra of OTA and methacrylic acid in MeCN (1.5).	114
Figure 3.10a: NMR spectra of OTA (close up showing groups 2 and 8).	115
Figure 3.10b: NMR spectra of OTA in the presence of DEAEM in the ratio 1:4 (close up showing groups 2 and 8).	115
Figure 3.11a: Fluorescent emission spectra for OTA in 50 mM sodium phosphate buffer (pH 7.5).	117

Figure 3.11b: Fluorescent excitation spectra for OTA in 50 mM sodium phosphate buffer (pH 7.5).	118
Figure 3.11c: Linear properties of OTA fluorescence in sodium phosphate buffer solution (100 mM, pH 7).....	118
Figure 3.12: The presence of OTA in successive eluted solutions during electrophoresis, measured by fluorescence. Bars indicate standard error.	119
Figure 3.13: Degradation of OTA fluorescent signal with UV exposure, performed on polymer B membrane. Bars indicate standard error.....	121
Figure 3.14: Examples of the background fluorescence for both MIP and NIP of polymer A. $\lambda^{Ex} = 379$ nm. The line at 424 nm is used for comparative measurements.	122
Figure 3.15: Increase in fluorescent signal relating to bound OTA to polymer A MIP and NIP respectively, dependent of concentration. Bars indicate standard error.....	123
Figure 3.16: Example of image taken during particle size analysis. This is a picture of polymer A in the presence of water at 10x magnification.	125
Figure 3.17: Relative changes (%) in average size of polymer particles (polymer A) in different solvents as compared with the size of the particles in water. The bars indicate standard error of particle size distribution.....	125
Figure 3.18: Effect of pH on binding of OTA to MIP of polymer A. Bars indicate standard error of measurement.....	126

Figure 3.19: Effect of concentration of sodium phosphate buffer on binding of Ochratoxin A to the MIP of polymer A at pH 7.5. Bars indicate standard error of measurement..... 127

Figure 3.20: Binding of different concentrations of OTA to polymer A MIP. Solutions prepared with 100 mM sodium phosphate buffer pH 7.5. Bars indicate standard error of measurement. 129

Figure 3.21: Effect on binding of OTA to MIP of polymer A in the presence of acetonitrile (MeCN). Solutions prepared with 100 mM sodium phosphate buffer pH 7.5. Bars indicate standard error of measurement..... 130

Figure 3.22: Release of OTA from polymer A MIP in successive washes. 100 mM sodium phosphate buffer pH 7.5 and de-ionised water were used as eluents. Bars indicate standard error of measurement..... 130

Figure 3.23a: Chromatogram of L-Phe-CHNA standard 100 ng/ml in 100 mM sodium phosphate buffer. The peak at 3.79 indicates the fragment used for concentration comparison..... 132

Figure 3.23b: Chromatogram of L-Phe-CHNA after elution in water from MIP polymer A. The peak at 3.65 indicates the fragment used for concentration comparison. 133

Figure 3.24: Fluorescent measurement of polymer A MIP membrane demonstrating the effect of the binding of OTA in acetonitrile. The bars indicate standard error of measurement. 134

Figure 3.25: Fluorescent measurement of polymer B MIP membrane demonstrating the effect of the binding of OTA in acetonitrile. The bars indicate standard error of measurement. 135

Figure 3.26: Fluorescent measurement of polymer A blank membrane demonstrating the effect of the binding of OTA from grape juice and white wine. The bars indicate standard error of measurement.	136
Figure 3.27: Fluorescent measurement of polymer A MIP membrane demonstrating the effect of the binding of OTA in grape juice and white wine. The bars indicate standard error of measurement.	137
Figure 3.28: Calibration curve of detection of OTA in grape juice using a fibre-optic set-up integrated with MIP membrane. Bars indicate standard error in measurement.	138
Figure 4.1: Representation of the switch between conformations caused by presence of buffer or water on the polymer.....	175

List of Tables

Table 1.1: Classes of major mycotoxins and their physiological effects.	21
Table 1.2: Examples of SPE phases used in the extraction and clean up of mycotoxin samples.....	32
Table 1.3. Examples of TLC protocols used for the detection of common mycotoxins.	35
Table 1.4: Examples of protocols using HPLC for common mycotoxins.	38
Table 1.5. Examples of GC protocols for common mycotoxins.	39
Table 1.6. Examples of protocols and detection limit using ELISA for the most common mycotoxins.	41
Table 1.7: Maximum levels of Ochratoxin A in foodstuffs, as set by the European Union legislation (FAO, 1997).....	43
Table 2.1: Virtual library containing the most commonly used functional monomers.	73
Table 2.2: Move frequencies selection with the explanation of each.....	75
Table 2.3: Composition of Polymer A.....	78
Table 2.4: Composition of Polymer B.....	79
Table 2.5: Composition of polymer A membrane.	84
Table 2.6: Composition of Polymer B.....	85
Table 3.1: The binding scores between OTA and monomers in a vacuum (dielectric constant = 1).	104
Table 3.2: The binding scores between OTA and monomers in acetonitrile (dielectric constant = 37.5).	105

Table 3.3: Peak designation of OTA (270 MHz). Based on the numeration in Figure 3.7.....	112
Table 3.4: Recovery of template and its analogues by polymer A MIP in water.	131
Table 3.5: Results of spiked extract binding to MIP and blank membrane..	139
Table 3.6: Fluorescence of maize extract components (measured at 434 nm) filtered through Celite 545 column.....	140
Table 3.7: Fluorescence of 100 ppb OTA solution (measured at 434 nm) filtered through Celite 545	141
Table 3.8: Fluorescence of maize extract components (measured at 434 nm) filtered through C/AlO ₃ column	141
Table 3.9: Fluorescence of 100 ppb OTA solution (measured at 434 nm) filtered through C/AlO ₃ column.	142
Table 3.10: Fluorescence of 100 ppb OTA solution filtered through blank polymer B.....	143
Table 3.11: Fluorescence of 100 ppb OTA solution filtered through IEX cartridges.	144
Table 3.12: Release of model OTA sample from IEX cartridges.....	144
Table 3.13: Fluorescence of 100 ppb OTA solution filtered through NH ₂ cartridge.	145
Table 3.14: Release of OTA in 0.2 M sodium phosphate buffer (pH 7).	146
Table 3.15: Extract cleaning using NH ₂ IEX cartridge.	147
Table 3.16: Binding and recovery of OTA from real samples using NH ₂ IEX cartridge.	148

Table 3.17: Recover of OTA using NH ₂ IEX cartridge and modified protocol (MeCN washing stage is excluded).....	148
Table 3.18: Binding and release of OTA and extract in the presence of 1% SDS.....	149
Table 3.19: Binding of OTA from IEX adjusted buffer solution to MIP polymer A cartridge.....	150
Table 3.20: Binding of OTA from IEX adjusted buffer solution containing maize extract to MIP polymer A cartridge.....	151
Table 3.21: Binding of OTA from buffer solution containing 1% lecithin to MIP polymer A cartridge.....	151

List of Abbreviations

This thesis uses standard chemical and mathematical nomenclature with the following added abbreviations.

4 – VP	4- vinylpyridine
ACCN	1,1-azobis cyclohexane(carbonitrile)
CE	Capillary Electrophoresis
CHNA	4-chloro-1-hydroxy-2-naphthoic acid (CHNA)
DEAEM	N,N- diethylamino ethyl methacrylate
DON	Deoxynivalenol
DMF	Di-methyl formamide
EGDMA	Ethylene glycol dimethacrylate
ELISA	Enzyme-linked immunosorbent assay
EU	European Union
GC	Gas Chromatography
HPLC	High Performance Liquid Chromatography
IEX	Ion Exchange
LLP	Liquid-Liquid Partition
L-Phe-CHNA	N-(4-chloro-1-hydroxy-naphthoylamido)-(L)-phenylalanine
OTA	Ochratoxin A
OUA	Oligourethane acrylate
MeCN	Acetonitrile
MeOH	Methanol
MIP	Molecularly Imprinted Polymer
MS	Mass Spectroscopy
NIP	Non Imprinted Polymer
NMR	Nuclear Magnetic Resonance
ppb	Parts per billion
TLC	Thin Layer Chromatography

Chapter 1

Introduction and Literature Review

1.1 Introduction

This introduction explains the reasoning behind this project and describes the structure of the thesis.

This project presents the development of a molecularly imprinted polymer specific for ochratoxin A and its use in solid phase extraction and in sensing of this compound. The project was part of the European Union Framework 5 proposal (PLQLRT-1999-01380) (OTA PREV). Several universities and companies situated across Europe were involved in this work, with the efforts co-ordinated from Uppsala, Sweden. The overall aim of the EC OTA PREV project was to reduce the intake of OTA from cereals by 50%+, and provide tools for corrective and preventive action. The work presented here is a part of Workpackage 7 entitled "Development of rapid biosensors for ochratoxin detection using molecular imprinted polymers". Further information can be found at the end of this chapter.

Each chapter will have a short introduction and conclusion, allowing the reader to follow the progress of the project. Overall conclusions, recommendations and a plan for further work will be presented in the final chapter.

The following literature review discusses the importance of mycotoxins as a whole, how they are produced, and the general techniques used in their detection and quantification. Particular attention is devoted to ochratoxins, their production, toxicity and importance in the ecosystem, and an explanation

of the analytical interest in this family of mycotoxins. A short history of molecular imprinted polymers is presented together with a demonstration of their importance and the rationale behind using them in this project.

1.2 Mycotoxins

The name mycotoxin is combination of the Greek word for fungus 'mykes' and the Latin word 'toxicum' meaning poison. The term 'mycotoxin' is usually reserved for the relatively small (MW ~700), toxic chemical products formed as secondary metabolites by a few fungal species that readily colonise crops in the field or after harvest. These compounds pose a potential threat to human and animal health through the ingestion of food products prepared from these commodities.

Contamination can occur pre- or post harvest (e.g. deoxynivalenol and T-2 toxin produced by *Fusarium* pre-harvest and ochratoxins (*Aspergillus* and *Penicillium*) and aflatoxins (*Aspergillus*) post harvest, although aflatoxin contamination can also be a field event.) (Lacey *et al.*, 1991)

Any crops that are stored for more than a few days become a potential target for mould growth and mycotoxin formation. Mycotoxins can occur both in temperate and tropical regions of the world, depending on the species of fungi. Major food commodities affected are cereals, nuts, dried fruit, coffee, cocoa, spices, oil seeds, dried peas, beans and fruit, particularly apples. Mycotoxins may also be found in beer and wine resulting from the use of contaminated barley, other cereals and grapes in their production. Mycotoxins

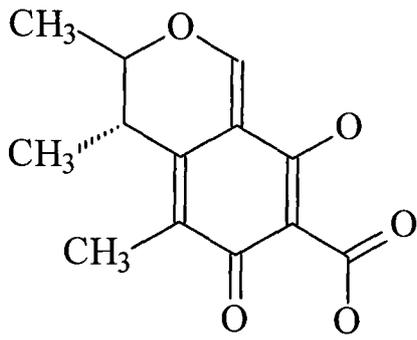
can also enter the human food chain via meat or other animal products such as eggs, milk and cheese as the result of livestock eating contaminated feed. They are often genotypically specific, but can be produced by one or more fungal species. For example ochratoxin A (OTA) is produced by some species of *Aspergillus*, such as *A. ochraceus*, mainly in tropical regions and by *Penicillium verrucosum*, a common storage fungus in temperate areas. In some cases one species can form more than one mycotoxin (Thrane, 1989; Frisvad, 1994).

Mycotoxins are chemically and structurally diverse (Figure 1.1). Since the majority of secondary metabolites are synthesised by simple biosynthetic reactions from small molecules (e.g. acetates and pyruvates). This is surprising, however this leads to the compounds having a diverse range of toxic effects, both acute and chronic (Table 1.1).

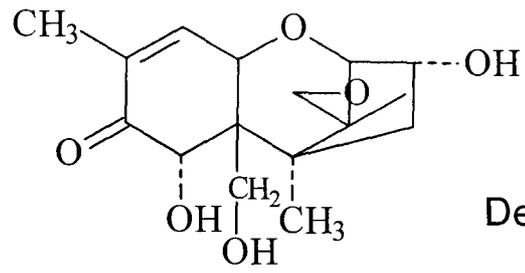
1.2.1 Ochratoxins

Ochratoxin A (OTA) was first discovered in 1965 as one of the first fungal metabolites that showed toxic behaviour towards animals (van der Merve, Steyn & Fourie, 1965).

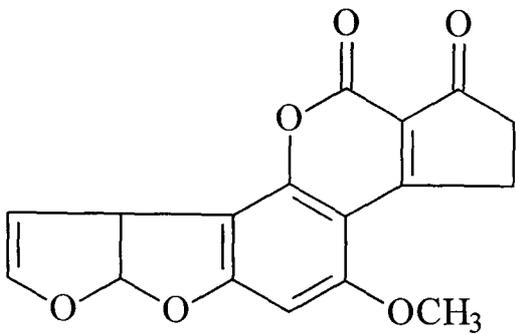
Structurally, it contains a 7-carboxy-5-chloro-8-hydroxy-3,4-dihydro-3R-methylisocoumarin, that is linked through the 7-carboxy group to L- β -phenylalanine by an amide bond. Its structure and structures of analogues are shown in Figure 1.2.



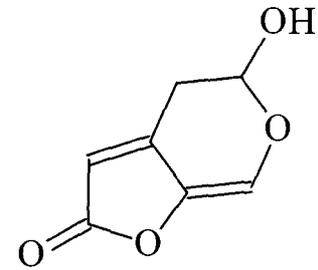
Citrinin



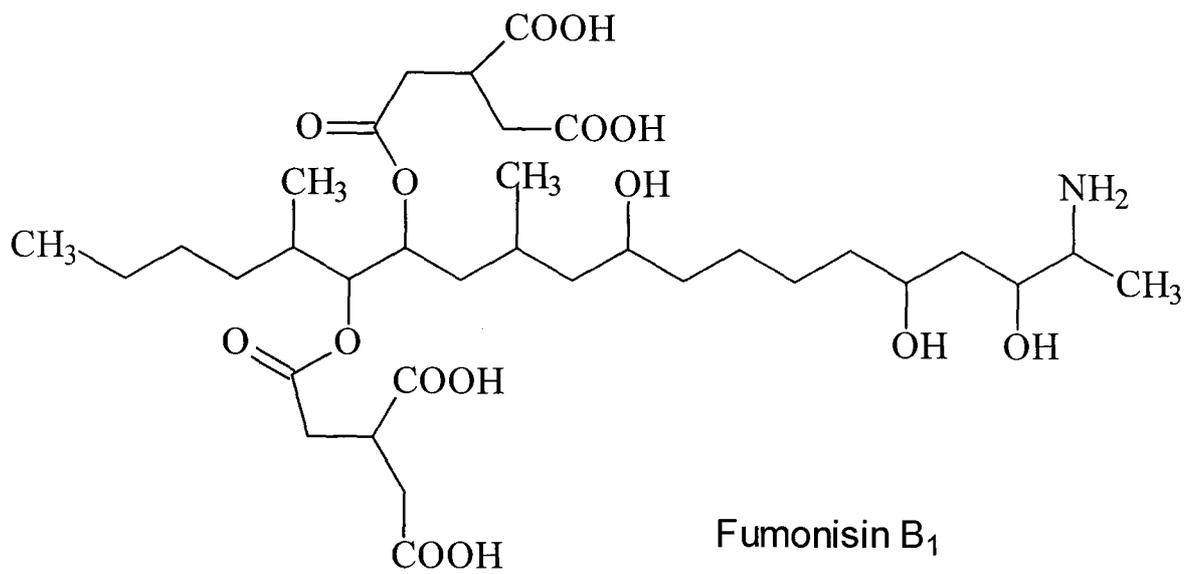
Deoxynivalenol (DC)



Aflatoxin B₁



Patulin



Fumonisin B₁

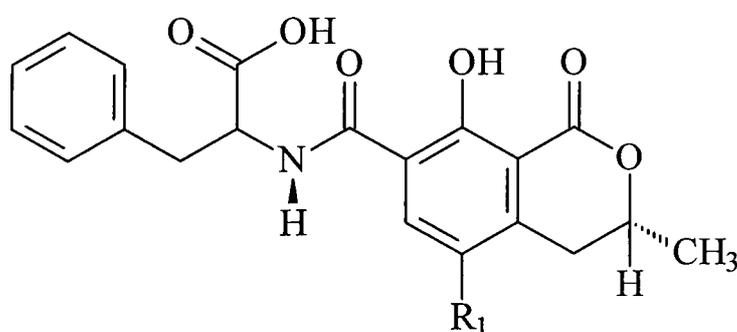
Figure 1.1: Examples of common mycotoxins demonstrating structural diversity of these compounds.

Table 1.1: Classes of major mycotoxins and their physiological effects.

Toxin	Major effects on mammalian systems.	References
Fumonisin	Carcinogenic, hepatotoxic, causative agent in leukoencephalomalacia in horses	Dutton, 1996; Vainio, Heseltine & Wilbourn, 1993; Rheeder <i>et al.</i> , 1992; Gelderbolm <i>et al.</i> , 1991; Kellerman <i>et al.</i> , 1990; D'Mello, Placinta & Macdonald, 1999
Aflatoxins	Carcinogenic	IARC, 1993; Ellis, Smith & Simpson, 1991.
Ochratoxins	Carcinogenic, nephrotoxic, hepatotoxic, teratogenic.	IARC, 1993; MAFF, 1993
Patulin	Lung and brain haemorrhaging.	McKinley & Carlton, 1991
Trichothecenes	Immuno-depressants, gastrointestinal haemorrhaging	Frisvad, 1994; D'Mello, Placinta & Macdonald, 1999
Zearalenone	Estrogenic activity	D'Mello, Placinta & Macdonald, 1999
Citrinin	Nephrotoxic	Reddy & Berndt, 1991

Ochratoxin α , ochratoxin β and mellein (Figure 1.3) are dihydroisocoumarins produced by the same group of fungi. These have similarities to the main ochratoxins and are linked to the biosynthesis of OTA.

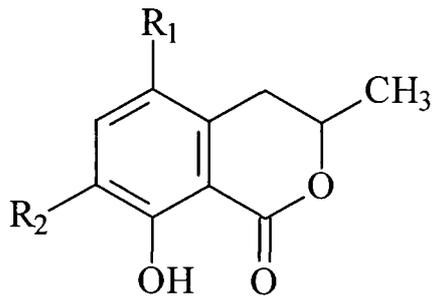
The biosynthesis of these compounds has been studied using fermentation methods and radio-labelling of precursor groups (Ferriera & Pitout, 1969; Searcy, Davis & Diener, 1969; Steyn, Holzapfel & Ferreira 1970). A full pathway has not been elucidated, although a version has been proposed (Huff & Hamilton, 1979). From this scheme it appears that the pentaketide intermediates are formed from acetate units (Ferriera & Pitt, 1969), and that of these, ochratoxin β was more likely to be transformed into OTA than ochratoxin α , suggesting that chlorination is a penultimate step (Harris & Mantle, 2001). This step protects the compound from degradation by carboxypeptidases in animal digestion and also lends itself to the high toxicity seen with OTA, as compared with OTB. There is also evidence that suggests that OTB is formed as a degradation of OTA.



Ochratoxin A: R₁ = Cl

Ochratoxin B: R₁ = H

Figure 1.2. Structural representations of ochratoxin A and B.



Ochratoxin α : R1 = Cl; R2 = COOH

Ochratoxin β : R1 = H; R2 = COOH

Mellein R1 = H; R2 = H

Figure 1.3: Structural representations of the dihydroisocoumarins, ochratoxin α , ochratoxin β and mellein.

OTA has an empirical formula of $C_{20}H_{18}ClNO_6$ and a molecular weight of 403.8. It is a white odourless, crystalline solid, which produces a light brown solution in methanol. It has a melting range of 441 –446 K. Further physico-chemical data can be found in Pohland *et al.* (1982).

OTA is produced by several *Aspergillus* and *Penicillium* species, which are natural opportunistic biodeterioration agents. The natural occurrence of these fungi is widespread, since both these species grow over a wide range of conditions (substrate, pH, moisture and temperature) (Frivad & Lund, 1993; Harwig, Kuiper-Goodman & Scott; 1983; Lee & Magan, 2000; Ramos *et al.*, 1998). The impact of these fungi is evident in countries such as those in South-Eastern Europe, with lower standards of food storage and ideal environments for fungal growth.

OTA is most common in cereals and other starch rich foods and has also been found in coffee, spices, and dried fruits (IARC, 1993; Studer-Rohr *et al.*, 1995). Generally the concentration of OTA does not exceed a few ppb. More recently OTA has been also discovered in human and animal fluids, meats, beers and wines (Brera *et al.*, 2002; Gharbi *et al.*, 1993; Jorgensen, 1998).

OTA is a heat stable compound, which leads to the intake of OTA leads to its build-up in the circulatory system, liver and other tissues such as adipose and muscle, from intake in food. Its presence in animal feeds and tissue can lead to OTA intake in humans, as it passes through the food chain. OTA exhibits unusual toxicokinetics with a half-life of 35 days in man after oral ingestion (the longest known for living mammals) (Schlatter, Studer-Rohr & Rasonyi, 1996).

The biological effects of OTA are well documented. Reported immunosuppressive (Stormer & Lea, 1995), teratogenic (Marquardt & Frolich, 1992), fertility inhibition (Biró *et al.*, 2003), mutagenic and carcinogenic (IARC, 1993; de Groene *et al.*, 1996) effects have been described in the literature and several reviews have covered the subject (Kuiper-Goodman, 1996; Creppy, 2002; Mantle, 2002; Petzinger & Weidenbach, 2002).

It is also linked with Balkan Endemic Nephropathy (BEN), a chronic kidney disease found in South-Eastern Europe (Petkova-Bocharova, Chernozemsky & Castegnaro, 1988; Mantle, 2002).

The IARC have classified OTA as a possible carcinogen (group 2B) (IARC, 1993). OTA toxicity appears to be related to its ability to inhibit protein synthesis by competing with phenylalanine in the reaction catalysed by phenylalanyl-tRNA synthetase and other systems requiring this amino acid.

Due to these findings many countries have set limits on OTA levels in food, typically between 1-10 ppb depending on the type and quality of the foodstuff. These regulations require accurate and suitable methods of detection and quantification.

1.3 Mycotoxin detection

Most mycotoxins are chemically stable so they tend to survive storage and processing even when cooked at quite high temperatures such as those reached during baking bread or breakfast cereal production. This makes it important to avoid the conditions that lead to mycotoxin formation, which is not always possible and not always achieved in practice. Mycotoxins are notoriously difficult to remove and the best method of control is prevention (Bullerman, Schroeder & Park, 1984).

The presence of a recognised toxin-producing fungus does not, in fact, mean that the associated toxin will also be present, as many factors are involved in its formation. Equally, the absence of any visible mould will not guarantee freedom from toxins as the mould may have already died out while leaving the toxin intact.

Fungi generally tend to develop in isolated pockets and are not evenly distributed in stored commodities. Therefore, it is important to develop a

protocol to ensure that if a sample is taken for analysis it is representative of the whole consignment. Grab samples generally give very low estimates of mycotoxin content.

The fact that most mycotoxins are toxic in very low concentrations requires sensitive and reliable methods for their detection. Sampling and analysis is of critical importance since failure to achieve a satisfactory verified analysis can lead to unacceptable consignments being accepted or satisfactory loads being unnecessarily rejected.

Due to the varied structures of these compounds it is not possible to use one standard technique to detect all mycotoxins, as each will require a different method. What works well for some molecules is inappropriate for others of similar properties, or for the same molecule in a different environment/ matrix. Likewise, practical requirements for high-sensitivity detection and the need for a specialist laboratory setting create difficulties for routine analysis. Therefore, depending on the physical and chemical properties, procedures have been developed around existing analytical techniques, which offer flexible and broad-based methods of detecting compounds.

It would be desirable to have simple detection methods to be used by non-scientific personnel and which are fast and inexpensive. The application of simpler, cheaper and effective solutions for the detection of mycotoxins is increasingly being required, due to their perceived importance, based around their toxicity and requirements of legislation for limits on amounts in foods.

A successful detection method should be robust, sensitive and have a high degree of flexibility, over a wide range of compounds, but which can be very specific when required. All techniques should be reproducible to a high level, and the results gained must be relevant and easy to analyse. For fieldwork, the system should also be rapid and portable. There are many methods used, of which many are lab-based but there is no single technique that stands out above the rest, although analytical liquid chromatography, commonly linked with mass spectroscopy is gaining popularity. Many of the following techniques have been combined to form protocols, which are used in laboratories today.

1.3.1 Sample pre-treatment: extraction and clean-up

Any method used for determination of a mycotoxin must rely on the correct extraction and clean-up methods. These steps are vital to a successful protocol, as they are time consuming (sample preparation is the main time factor in an analysis and takes approximately 2/3rd of the total) and will affect the final choice of detection procedure. The extraction method used to remove the mycotoxin from the biological matrix is dependent on the structure of the toxin. Polar metabolites, such as the fumonisins, require the presence of water, aided by the presence of organic solvents (Shephard, 1998). Hydrophobic toxins such as aflatoxins rely on use of organic solvents (Holcomb, 1992; AOAC, 1997). These can be direct extractions, or may be partitioned with other solvents, such as n-hexane as partial clean up, to remove excess components of the biological matrix. The choice of extraction solvent is also dependent on the matrix from which the extraction is required,

as the differing chemical mixtures can affect it (Wilkes & Sutherland, 1998). The use of chlorinated chemicals for extraction is being gradually removed from use as they are proven ecological hazards (Montreal Protocol, 2003).

The clean up procedure used in a protocol is the most important step, as the purity of the sample affects the sensitivity of the results. Trace amounts of a target molecule may be masked by interfering compounds, found not only in the matrix but in the chemicals, materials and solvents used in the technique. Glassware should also be free of material, such as alkaline detergents, which can form salts with the compounds and lower detection rates (AOAC, 1997). Several methods exist, and have all been recorded for use with clean up of mycotoxin samples (Scott, 1995).

1.3.1.1 Liquid-liquid partition

Liquid-liquid partitioning (LLP) involves using the different solubility of the toxin in aqueous phase and in immiscible organic phase, to bring the compound into one solvent leaving the rest of the matrix in the other. Thus, solvents such as hexane and cyclohexane are used to remove non-polar contaminants, e.g. lipids and cholesterol. The procedure is effective for several toxins and works well in small-scale preparations (Bauer & Gareis, 1987). However, it is time consuming, and is dependent on which matrix is being used, and which compounds are being determined. Disadvantages lie with contamination from within the sample, and possible loss of sample by absorption onto the glassware.

1.3.1.2 *Supercritical fluid extraction (SFE)*

SFE uses a supercritical fluid, such as CO₂ to extract the required compound from the matrix. This works well due to the high solvating power, and density of the solvating liquid. However it has not been included in routine analysis due to high costs and the need for specialised equipment (Holcomb, Thompson-Jr. & Cooper, 1996).

1.3.2 **Solid phase extraction (SPE)**

The basic principle of SPE technology is a variation of chromatographic techniques based around small disposable cartridges packed with silica gel, or bonded phases which are in the stationary phase. The sample is loaded in one solvent, generally under reduced pressure, rinsed, where most of the contaminants are removed, and eluted in another solvent. This system can be used “on” and “off” line. These cartridges have a high capacity for binding of small molecules.

These cartridges contain different bonding phases, ranging from silica gel, C₁₈ (octadecylsilane), ion exchange materials, both anionic and cationic, to affinity materials such as immunoabsorbents and molecular imprinted polymers (MIP) (Hurst W & Martin Jr, 1998; Scudamore & MacDonald, 1998; Solfrizzo, Avantiaggiato & Visconti, 1998; Supelco, 1998; Visconti & Pascale, 1998; Jornet, Busto & Guasch, 2000; Visconti, Pascale & Centonze, 2000; Sharma & Mårquez, 2001; Zambonin, Monachi & Aresta, 2001; Mateo *et al.*, 2002).

The SPE systems have many advantages when compared to LLP, e.g. they use considerably less solvent and they are faster in operation. In addition to cleaning sample they can also be used to effectively pre-concentrate the sample giving better detection results. SPE has found widespread use and is an integral part of many extraction and detection protocols.

The disadvantage of SPE is that it is impossible to find a single universal type of cartridge useful for extraction of all toxins. Each type can operate in certain conditions and its performance can be affected by pH, solvent and ion concentration in the sample. Nowadays solid phase extraction is by far the most popular technique used in routine analysis of mycotoxins.

1.3.2.1 *Silica based materials*

Silica gel is a very popular material, frequently used for solid phase extraction. The surface of silica particles is heterogeneous, with a variety of silanol groups present which can bind target compounds through multiple electrostatic interactions. The addition of various functional groups can widen the application of this material. Normally this is done by reacting an organosilane with a long chain aliphatic derivative (e.g. C-18 phase) (Supelco, 1998). Silica gel can be used directly or after modification for mycotoxin detection (see Table 1.2). More frequently, however, it is a hydrophobic phase which is used in environmental and food analysis of these toxins, performed in both polar and non-polar solvents.

Alexander Leitner *et al.* (2002) demonstrated the use of C-18 reverse phase materials in the extraction of OTA from wine. They have shown that the use of

cartridges made of this material combined with mass spectroscopy offers good results (detection at sub-ppb level) which are comparable to clean up with OTA specific immunosorbents.

1.3.2.2 *Ion Exchange Materials*

Ion exchange materials are often used in SPE to isolate and pre-concentrate toxins found in aqueous solutions, (e.g. fumonisins). They utilise electrostatic interactions formed between the target molecule and charged groups bonded to the silica material (Supelco, 1998). The samples are set to a pH value where both groups are charged, and filtered through the SPE cartridge. The bound material is removed by addition of a strong ionic solution or by altering pH. Several types exist, in both anionic and cationic phases. SAX (strong anion exchange) is the favoured material for mycotoxin extraction. Some examples of the application of SPE cartridges in extraction of mycotoxins are given in Table 1.2. Pelegri *et al.* (1997) offered a sensitive protocol for OTA detection and quantification using strong anionic columns in clean up. This protocol gave a detection limit of 0.02 ng/ml using HPLC with fluorescence detection.

1.3.2.3 *Mycosep™ columns*

These columns are made up of several absorbents specifically selected for recovery of individual mycotoxins, packed into a plastic tube, and used to remove the entire matrix leaving the desired compound in solution on top of the column (Akiyama, 2001; Mateo *et al.*, 2002). This method is practical, portable and quick and no additional rinsing steps are required, however

Table 1.2: Examples of SPE phases used in the extraction and clean up of mycotoxin samples.

Toxin	SPE phase	Matrix	Reference
Fumonisin	C18-RP, SAX, Immunoaffinity	Various (Review)	Shephard, 1998
Alternariol	C18-RP	Apple juice	Delgado <i>et al</i> , 1996
Ochratoxin A	Immunoaffinity, C18-RP	Wine	Leitner <i>et al</i> , 2002
Aflatoxin B1	C18-RP	Meat	Toribo <i>et al</i> , 1999
Trichothecenes	Mycosep, C18-RP	Grain	Radová, Holadová, & Hajiová, 1998

columns are designed per analyte and therefore not useful in multi toxin analysis. A Mycosep™ column specific for OTA has been developed, which works in tandem with other columns to ensure clean up from a variety of matrices like grain, wine and coffee.

1.3.2.4 Immunoaffinity columns

Antibody based immunoaffinity materials have been intensively investigated over the past few years, and have been shown to be of great value in mycotoxin analysis protocols (Zimmerli & Dick, 1995; Scudamore & MacDonald, 1998; Solfrizzo, Avantaggiato & Visconti, 1998; Visconti & Pascale, 1998; Visconti, Pascale & Centonze, 2000; Sharma & Márquez,

2001; Mateo *et al.*, 2002). They offer excellent recovery of analyte, because of the specificity gained by using monoclonal or polyclonal antibodies. The main disadvantage in using these materials is high costs involved as each column can only be used once (due to denaturation of antibodies). This has been taken further with the development of sequential injection immunoassay (SIIA) (Garden & Strachan, 2001). This is a colorimetric system, which utilises a jet ring flow cell packed with beads coated with an immuno-affinity material. These beads bind the toxin and an absorbance across the beads is measured, to give a highly accurate quantification of toxin concentration, comparable to ELISA.

Clean up of OTA samples has been reported in many different matrices, with a high degree of success. The use of IAC in wine samples (Visconti, Pascale & Centonze, 2000) demonstrated the importance of such materials. Commercially available columns were used in combination with HPLC and fluorometric detection to produce a protocol for quantification in red, rose and white wines. The results were an improvement on previous work (Zimmerli & Dick, 1995) and showed excellent detection limits (0.01 ng g^{-1}) depending on the matrix. Thus red wine proved to be more difficult to analyse.

1.3.3 Chromatographic determination

There are several types of chromatographic methods available for mycotoxin analysis (Shephard, 1998; Valenta, 1998). The review by Betina (1989) which covers the period prior to 1990 offers a detailed study on the use of these methods. As in most other examples referred to in the present review, sample

pre-treatment plays a major part in the analysis. This is normally done by LLC or by SPE depending on the matrix (Roch et al., 1995; Zimmerli & Dick, 1995; Holcomb, Thompson-Jr & Cooper, 1996).

1.3.3.1 *Thin Layer Chromatography (TLC)*

Traditionally the most popular method used for mycotoxins analysis is thin-layer chromatography (TLC), which offers the ability to screen large numbers of samples economically. The use of TLC analysis for mycotoxins has been well documented and is still popular for both quantitative and semi-quantitative purposes. This is due to its high throughput of samples, low operating cost and ease of identification of target compounds, using UV-Vis spectrum analysis. As with HPLC several methods have been developed to obtain the best results with each separate class of mycotoxin and both one-dimensional and two-dimensional analyses are used frequently (Lin *et al.*, 1998). Table 1.3 shows examples of TLC protocols used for detection of common mycotoxins.

For greater accuracy TLC is replaced with the more versatile method high-performance liquid chromatography (HPLC) (Frisvad & Thrane, 1987).

For OTA detection TLC is not commonly used, with very few examples in the literature, as HPLC has superseded this technique. The best example is that of Dawlatana *et al.* (1996) where a series of solvent steps were used to separate OTA from rice, and quantify it by fluorescence. This method required large quantities of solvent, intensive laboratory procedures and was

characterised by a lack of automation. However it was possible to use it to screen rapidly a variety of samples.

Table 1.3. Examples of TLC protocols used for the detection of common mycotoxins.

Toxin	Protocol	Ref
Aflatoxin	Silica gel with fluorescence densitometry, from rice wine.	Nawaz, Coker & Haswell, 1992
Ochratoxin A	Silica gel with fluorescence densitometry, from rice.	Dawlatana <i>et al.</i> , 1996
Trichothecenes	Comparison with HPLC for detection in food	Yagen, Sintov & Bialer, 1986
Fumonisin B ₁	Fluorescent determination from maize	Rottinghaus, Coatney & Minor, 1992

1.3.3.2 High Performance Liquid Chromatography (HPLC)

The application of liquid chromatography (LC) and HPLC is split into three main areas of interest concerning the mycotoxins. Firstly they are used for qualitative and quantitative analysis; secondly, they are used for “clean up” of samples and thirdly, they are used for large scale preparations of toxins for laboratory use (Shephard *et al.*, 1990; Reinhard & Zimmerli, 1999).

For each of these requirements different protocols and equipment are used.

For example, small mini columns are used for sample pre-treatment in

comparison to large scale preparative column chromatography methods used for preparation of mycotoxin standards.

Modern analysis of mycotoxins relies heavily on HPLC with various adsorbents depending on the physical and chemical structure of the mycotoxin. Reversed-phase columns are predominately used for separation and purification of polar toxins (Shephard *et al.*, 1990; Reinhard & Zimmerli, 1999), and normal phases used for more hydrophobic molecules (Giacomelli *et al.*, 1998).

In essence most of the protocols used for HPLC detection of mycotoxins are very similar. The most common found detection methods are UV or fluorescence detectors, which use the presence of a chromophore in the molecules. A number of toxins already have natural fluorescence (e.g. ochratoxin A, aflatoxins, citrinin) and can be detected directly in HPLC by fluorescent detection (Valenta, 1998), but many do not. These mycotoxins, like fumonisins produced by *Fusarium* species, lack a suitable chromophore, and their determination requires derivatisation (Shephard, 1998). Examples of derivatising agents are o-phthaldialdehyde (OPA) and 9-(fluorenylmethyl)chloroformate (Holcomb, Thompson & Hankins, 1993). Derivatisation can be performed either pre- or post- extraction (Neely & Emerson, 1990; Kussak *et al.*, 1995; Jimenez, Mateo & Mateo, 2000; Chiavaro *et al.*, 2001).

The main benefit of using HPLC, along with the high quality of separation, and low limits of detection, is the possibility to combine multiple detection systems

(fluorescent, UV, diode array) with this technology, allowing for multiple detection of compounds from one sample. It can also be automated (Hurst & Martin Jr., 1998), which offers a major advantage over other techniques, such as TLC and enzyme linked immunoassay (ELISA). Table 1.4 shows some examples of use of HPLC for detection of common mycotoxins.

HPLC is the industry standard for mycotoxin detection and numerous protocols exist for OTA analysis (Valenta, 1998). Most rely on sample clean up using SPE, and HPLC separation and detection by either fluorescence or mass spectroscopy. For example Visconti, Pascale & Centonze (1999) used commercially available immunoaffinity columns for clean up and separation on a reversed phase HPLC C18 column with fluorometric detection to determine the presence of OTA in wine. This system allowed the authors to detect OTA down to 0.01 ng/ml. This detection limit is also typical for other protocols.

1.3.3.3 *Gas Chromatography (GC)*

GC is regularly used to identify and quantify the presence of mycotoxins in food samples and many protocols have been developed for these materials. Normally the system is linked to mass spectroscopy (MS), flame ionisation (FI) or Fourier transform infrared spectroscopy (FTIR) detection techniques in order to detect the volatile products (Young & Games, 1994; Onji et al., 1998). Most mycotoxins are not volatile and therefore have to be derivatised for analysis using GC (Scott, 1995). Several techniques have been developed for the derivatisation of mycotoxins. Chemical reactions such as silylation or polyfluoroacylation are employed in order to obtain a volatile material (Scott,

1993). Table 1.5 gives examples of detection of common mycotoxins using GC.

Table 1.4: Examples of protocols using HPLC for common mycotoxins.

Toxin	Protocol	Ref
Ochratoxin A	LC- Electrospray ionisation- tandem MS in human food (wheat, coffee, beer)	Becker <i>et al.</i> , 1998
Ochratoxin A	SPE extraction with HPLC- fluorometric detection in wine	Jornet, Busto & Guasch, 2000
Citrinin	HPLC- time resolved luminescence (TRL) in soft cheese	Vasquez <i>et al.</i> , 1996
Aflatoxins	HPLC-amperometric detection in various media	Elizalde-Gonzalez, Mattusch & Wennrich, 1998
Zearalenone, Trichothecenes, Fumonisin	Study of clean up methods and analysis by HPLC-Fluorometric or photodiode array	Mateo <i>et al.</i> , 2002

As with other mycotoxins, OTA cannot be directly analysed by GC as it is not volatile. Several examples of OTA detection utilising derivatisation have been published (Jiao *et al.*, 1992). This approach gave a detection limit of 0.1 µg/kg. However, the use of GC detection is not expected for commercial protocols due to existence of cheaper and faster alternatives such as HPLC.

Table 1.5. Examples of GC protocols for common mycotoxins.

Toxin	Protocol	Ref
Fumonisin B ₁	GC-MS method using B ₁ as internal standard before derivatisation	Plattner & Branham, 1994
Deoxynivalenol, Ochratoxin A	GC-MS method compared to electronic nose for quantification and detection	Olsson <i>et al.</i> , 2002
Alternariol, Altenuene	GC-MS detection with derivatisation by silylation	Scott, Weber & Kanhere, 1997
Deoxynivalenol, T-2 (+ 6 others)	Direct analysis without derivatisation	Onji <i>et al.</i> , 1998
Trichothecenes	Fast screening using GC-MS	Nielsen & Thrane, 2001
Trichothecenes	GC with flame ionisation detection from wheat	Schothorst & Jekel, 2001

1.3.4 Mass Spectroscopy

Mass spectroscopy (MS) became the industry standard detection method allowing highly accurate and specific detection of toxins. These detectors can be linked to any other separation techniques, as described in sections (e.g. HPLC and GC systems) which will increase the separation and identification power of these systems (Becker *et al.*, 1998; Olsson *et al.*, 2002). The limiting factors in the use of MS as an analytical tool, are the high cost of equipment; complex laboratory requirements and limitations in the type of the solvents used in extraction and separation.

Leitner *et al.* (2002) presented an excellent comparison of mass spectroscopy and fluorescent detection for OTA analysis. The results for both appeared to be comparable offering sensitive tools with sub-ppb detection levels.

1.3.5 Enzyme Linked Immunosorbent assay (ELISA)

Many other separation techniques exist which are capable of running alongside or in place of chromatographic methods. Among these are enzyme linked assays (ELISA) which became very popular recently due to their relatively low cost and easy application.

Commercially available ELISA kits for detection of mycotoxins are normally based on a competitive assay format that uses either a primary antibody specific for the target molecule or a conjugate of an enzyme and the required target (Morgan, 1989; Pestka *et al.*, 1995). The complex formed will then interact with a chromogenic substrate to give a measurable result. They can be portable, rapid and are highly specific as well as simple to use. The disadvantage of these kits lies in the fact that they are single use, which can increase costs for bulk screening. Additionally, competitive ELISA suffers from having a limited detection range due to the narrow sensitivity of the antibodies, be they mono- or polyclonal. The development of antibodies for most mycotoxins, due to their small size, require development of a carrier molecule usually, a protein (e.g. bovine serum albumin), to achieve immunogenicity. The conjugation process can also be responsible for decreases in assay selectivity. In general however these problems can be resolved and the application of ELISA in mycotoxin detection is increasing.

Kits have been developed in several formats such as microtitre plates and bead-based assays, or membranes, and can also be linked with other techniques such as electrochemical sensors and Surface Plasmon Resonance (SPR) (Mullet, Lai & Yeung, 1998; Porter *et al.*, 2001). The examples of protocols used for mycotoxin detection are shown in Table 1.6.

The detection of OTA using ELISA based systems has become important in recent years, with several commercial products becoming available. These test kits offer rapid, portable analysis of OTA, however they suffer from both high cost and relatively high cross reactivity.

Table 1.6. Examples of protocols and detection limit using ELISA for the most common mycotoxins.

Toxin	Protocol	Ref
Aflatoxins	Sequential injection immunoassay in spiked food	Betina, 1989
Aflatoxins	Detection of producing moulds and target molecule from peanuts and maize	Yong & Cousin, 2001
Ochratoxin A, T-2	Membrane based flow through system	De Saeger <i>et al.</i> , 2002
Fumonisin	ELISA in Spanish beers	Torres, Sanchis & Ramos, 1998

1.3.6 Capillary Electrophoresis (CE)

The individual detection of closely related toxins requires sophisticated separation technique in addition to highly sensitive detection. The effective separation of components can be based upon charge and mass dependent migration in an electrical field. The fast separations can be accomplished by capillary electrophoresis in aqueous buffer solutions, excluding the need for organic solvents. Sensitive fluorescence-based detection methods have been described for aflatoxins (Pena *et al.*, 2002) and fumonisins (Wilkes & Sutherland, 1998).

There does not appear to be any information in the literature, which shows the application of capillary electrophoresis for the detection or quantification of OTA; however this does not mean that the technique would not be valuable as OTA, been a natural fluorophore would favour the techniques described by Wilkes and Pena (Wilkes & Sutherland, 1998, Pena *et al.*, 2002).

In conclusion, a broad range of detection techniques used for practical analysis and detection of a wide spectrum of mycotoxins are available. Ochratoxin A in particular has numerous protocols developed for quantification and detection in food matrices. However, most of these products are not cost effective or lack the analytic ability (especially in the sense of being not portable or fast enough) to support the legislation placed by the European Union on foodstuff quality, with regards to levels of OTA (Table 1.7). The development of an accurate, rapid, robust, reusable analytic

method is required. One solution may be the development of Molecularly Imprinted Polymers (MIP), specific for OTA.

Table 1.7: Maximum levels of Ochratoxin A in foodstuffs, as set by the European Union legislation (FAO, 1997).

Product	Maximum level ($\mu\text{g kg}^{-1}$)
Cereals (including rice and buckwheat) and derived cereal products	No limit as yet.
Raw cereal grains (including raw rice and buckwheat)	5
All products derived from cereals (including processed cereal products and cereal grains intended for direct human consumption)	3
Dried vine fruit (currants, raisins and sultanas)	10
Green and roasted coffee and coffee products, wine, beer, grape juice, cocoa and cocoa products and spices	Under consideration.

1.4 Molecularly Imprinted Polymers

1.4.1 History

In the simplest terms, the principle of molecular recognition can be described as proposed by Fischer, who in late 19th century explained the enzyme-substrate recognition as a lock and key mechanism. This occurs by complementary fitting of a substrate molecule (key) to a depression or active site in the larger enzyme molecule (lock). The molecules “recognise” each other through the formation of hydrogen bonds, ionic interactions, hydrophobic binding, formation of reversible covalent bonds etc.

Throughout the 1930's a Ukrainian group in Kiev led by Polyakov, working with silica based adsorbents, discovered that silica gels prepared in the presence of organic compounds developed affinity towards template molecules (Polyakov; 1931; Polyakov 1933; Polyakov, Kuleshina & Neimark; 1937). This was, to our best knowledge, the first example of synthetic materials with biomimetic recognition properties. This work remained largely unnoticed by other researchers until 1947 when Francis Dickey, a student of Linus Pauling, essentially repeated this work developing silica gels specific for methyl orange and its derivatives (Dickey, 1949). The “footprint” theory proposed by these authors was eagerly accepted and actively pursued by other researchers who in next twenty years developed whole range of substrate-specific inorganic materials (Beckett and Youssef, 1963; Bartels, 1967).

In 1972 Wulff and co-workers published the first formal methodology for the molecular imprinting in organic polymers (Wulff & Sarhan, 1972). They have polymerised sugar template esterified with polymerisable boronic acid, and shown that resulting material was capable of selective recognition and enantioseparation of template and its structural analogues. Shortly after, Takagishi & Klotz (1972) published similar work, utilising a slightly different method. In this work they cross-linked linear polymer in the presence of template and showed that this material has slightly higher affinity to the template as compared with blank polymer, made in the absence of template.

Arshady & Mosbach (1981) demonstrated the first example of non-covalent imprinting (polymerisation of functional monomers with template that do not form covalent bonds with each other). The template recognition by MIPs in this system was based mainly on electrostatic interactions between template and functional monomers. Practically all early work in molecular imprinting has been made developing adsorbents for batch binding or chromatography (Wulff & Vesper, 1978; Norrlöw, Glad & Mosbach, 1984; Ekberg & Mosbach, 1989; Ansell, Kriz & Mosbach, 1996). This technology is still the most popular in modern imprinting, with cross-linkers, such as ethylene glycol dimethacrylate (EGDMA), monomers, such as methacrylic acid and organic solvents, such as toluene and chloroform remaining in active use nowadays for the design of MIPs.

Further expansion of work in 1990's involved development of new recognition systems such as metal chelation and expansion of MIPs application towards development of MIP sensors and membranes.

The discoveries made by these few groups led to the almost exponential growth in the study of MIP technologies, a trend that is continuing to this day (Wulff, 1994 ; Piletsky, Alcock & Turner, 2001). In 2000 the First International Workshop on Molecularly Imprinted Polymers was held in Cardiff, UK (Selligren, 2003), and a formal society dedicated to the future of this area of science was started in 1999 (SMI, 2003)

1.4.2 Theory

The theory behind the molecular imprinting can be likened to modelling clay. Any imprint left in a piece of clay, such as a child's hand, when baked, will leave a defined impression in its surface. This impression will be specific to that child, and another person would not be able fit his hand, nor any other part of his anatomy into it.

Figure 1.4 demonstrates the basic scheme, which forms the basis of molecular imprinting theory. There are three distinct stages to the process. Firstly the template and monomers are mixed together in solution which acts also as a porogen. This allows for interactions between the template and functional groups in the monomers to form. These complexes are then incorporated into a three-dimensional matrix by polymerisation of the monomers with cross-linker. The template is then removed from the resulting

polymer, normally by solvent extraction. This leaves a polymeric matrix with specific cavities formed within it, which are chemically and sterically compatible for the template, allowing selective rebinding.

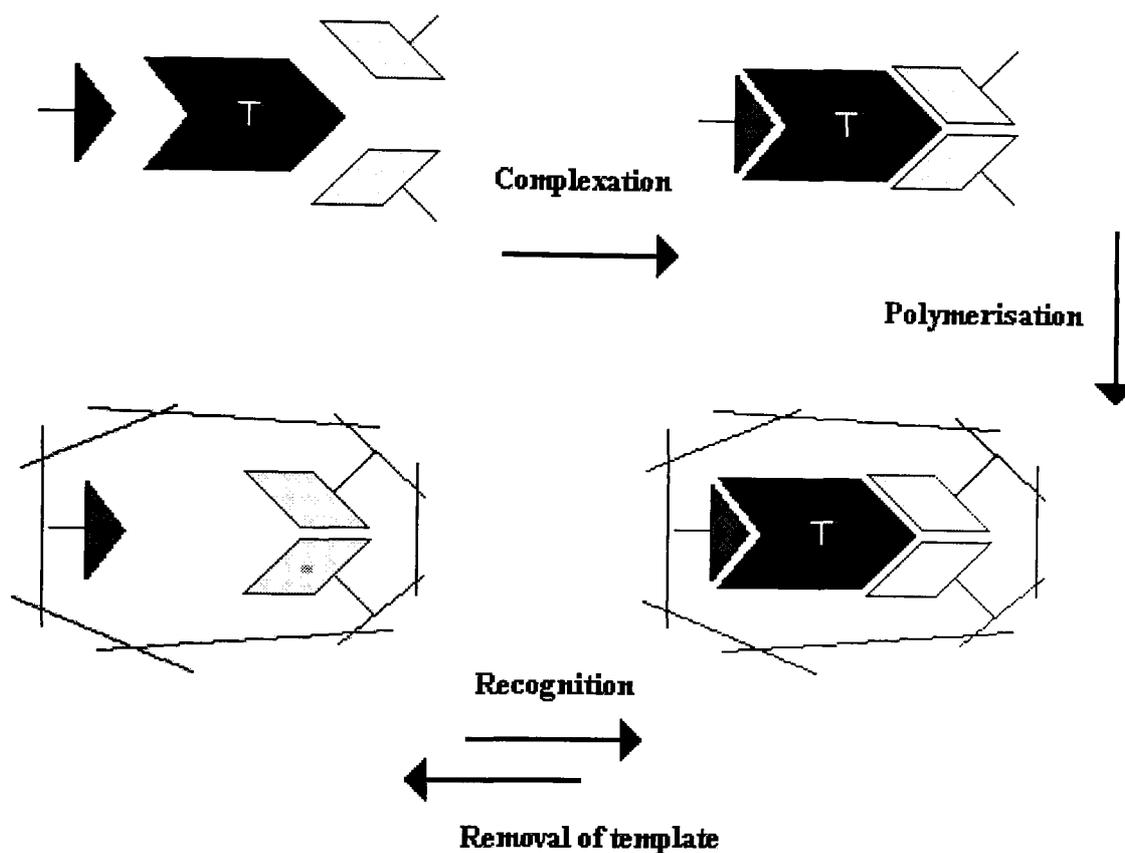


Figure 1.4: Schematic of molecular imprinting theory.

The template shown in blue and monomers (red and yellow) interact to form a pre-polymerisation complex. This complex keeps its steric and chemical properties throughout the polymerisation process and then the template can be removed, leaving an “imprint” behind.

1.4.2.1 Covalent or pre-organised imprinting

In this type of imprinting the functional monomers and template are bonded to each other, prior to polymerisation, via covalent interactions. This conjugate is

then polymerised, under conditions that will not interfere with the pre-organised complexation. Once the polymerisation stage is over, the template is removed from the matrix by cleaving the linkages in the initial complex (Figure 1.5).

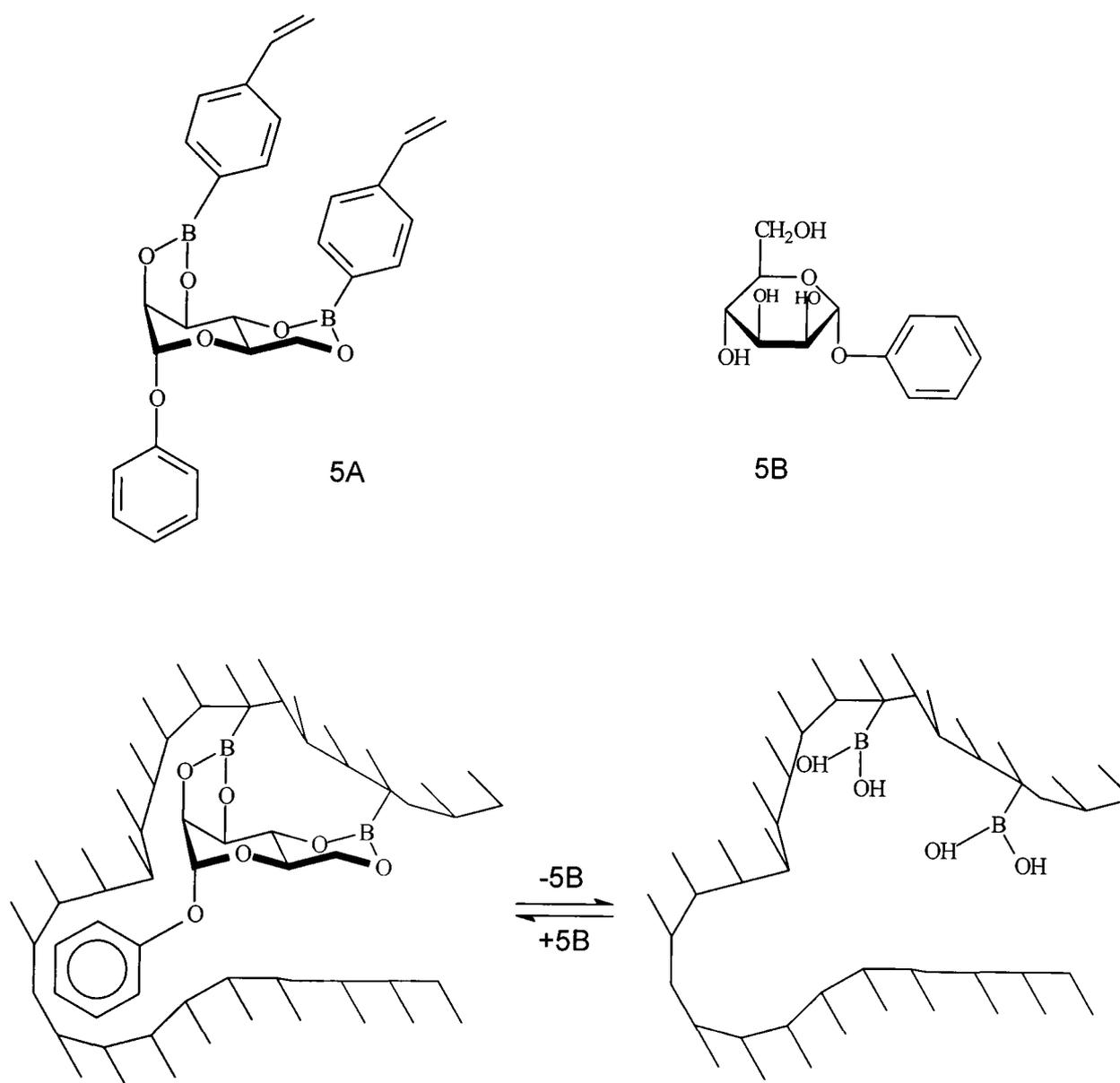


Figure 1.5: Schematic representation of a covalent imprinted binding cavity (From Wulff, 1995).

The template 5B (phenyl- α -D-mannopyranoside) is bound by esterification to two molecules of 4-vinylphenylboronic acid, (chosen as they undergo rapid and reversible reactions with the diols). The monomer template complex (5A) undergoes polymerisation and then the template is removed by treatment with water or methanol. The binding is reversible as shown.

This technique has been demonstrated with several different bond types including ketals (Shea & Dougherty, 1986), phosphonic esters (Sellergren & Shea; 1994), imides (schiff base) (Wulff, Best & Akelah, 1984) and carboxylic amides (Sellergren & Andersson, 1990). The advantages to this method are obvious. The pre-arrangement of the template/monomer complex is stable and stoichiometric, which allows the structure of the binding sites to be easily predicted. The stability of the initial complex is high enough to allow a wide range of polymerisation conditions to be used.

However, the required cleavage of covalent bonds for template removal causes problems as there are a limited number of reversible systems and that the cleavage of these available requires reasonably harsh conditions, which in some instances, can affect the polymer structure. Also, the use of the polymer once formed, can cause problems as the binding and release of the template is never complete.

1.4.2.2 Non-covalent imprinting

Non-covalent interactions include electrostatic (e.g. ionic, dipole-dipole interactions and hydrogen bonding) and hydrophobic interactions, coordination bond formation and Van der Waals forces.

These charge dependent interactions allow for a complex to be formed prior to polymerisation between the template and the functional monomers (Figure 1.6). After polymerisation the template can be removed by using suitable solvents.

This technique is more generic and can be tailored to a much wider range of templates, as the synthesis of a defined pre-polymerisation complex is unnecessary. However, in comparison to covalent imprinting, it lacks the strict stoichiometry of molecular complexes which is responsible for the generation of populations of binding sites with different affinities. The large excess of monomers used can lead to a high number of non-specific binding sites, which lowers polymer specificity.

The binding and release of template is efficient due to the weak interactions between polymer and template and can be affected using mild changes in environment. Since the interactions between monomers and template are weak, the polymerisation conditions need to be chosen carefully to maximise them and preserve the formed complexes during the polymerisation stage.

This project has been designed and performed using more generic non-covalent imprinting approach, therefore this review covers to larger degree this technique rather than that of covalent imprinting.

1.4.3 Thermodynamics

The accepted theory behind imprinted polymerisation as shown in Figure 1.4, demonstrates the importance of the physical and chemical conformation of the binding 'pocket'. The complex between the target molecule (template) and functional monomers forms these pockets, which are then bound into place with a three dimensional polymer structure made of a suitable cross linker.

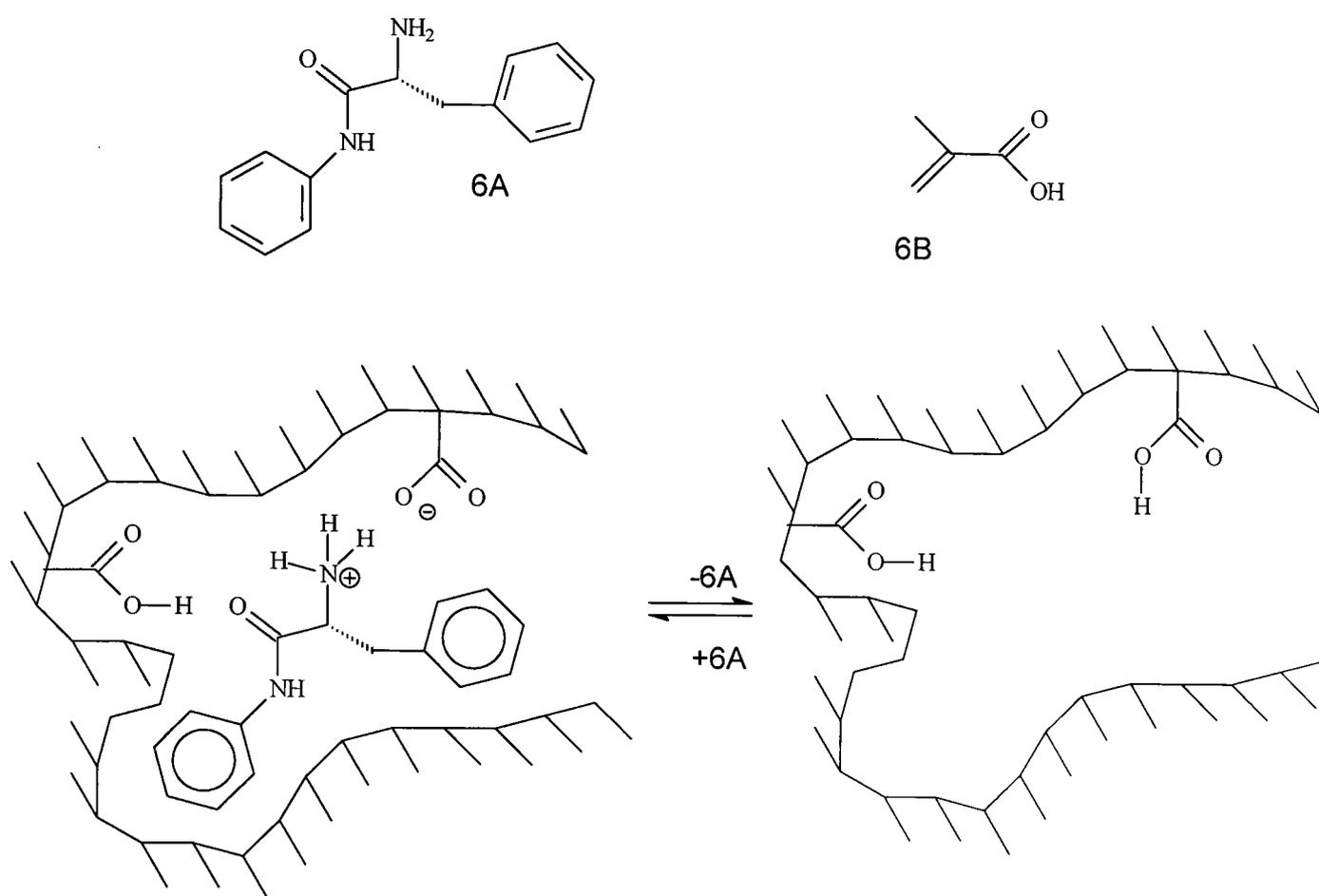


Figure 1.6: Schematic representation of non-covalent imprinting.

The template L-phenylalanine anilide (**6A**) was polymerised in the presence of methacrylic acid (**6B**). One methacrylic acid unit forms a hydrogen bond and another forms an electrostatic bond (Sellergren, 1988)

The polymer is produced by a thermal or photo-initiated reaction in a suitable solvent, which acts as a porogen. Once polymerised, the template can be removed leaving a cavity or pocket, complimentary to the original template.

The complex formation of these sites is under thermodynamic control and obeys the equation:

$$\Delta G_{\text{bind}} = \Delta G_{\text{t+r}} + \Delta G_{\text{r}} + \Delta G_{\text{h}} + \Delta G_{\text{vib}} + \Sigma \Delta G_{\text{p}} + \Delta G_{\text{conf}} + \Delta G_{\text{vdW}}$$

Equation 1.1: where the Gibbs free energy changes are ΔG_{bind} the complex formation, $\Delta G_{\text{t+r}}$ the translational and rotational, ΔG_{r} the restriction of rotor upon complexation, ΔG_{h} the hydrophobic interactions, ΔG_{vib} the residual soft vibrational modes, $\Sigma \Delta G_{\text{p}}$ the sum of the interacting polar group contributions, ΔG_{conf} the adverse conformational changes and ΔG_{vdW} the unfavourable van der Waals interactions (Holroyd *et al.*, 1993).

1.4.4 Practical recommendations for polymer design

It is clear from attempts made to predict these complexes that several factors are important for a successful imprint: the choice of cross-linker (Wulff, Vietmeier & Poll, 1987), solvent (porogen) (Selligren & Shea, 1993), initiator (Selligren & Shea, 1993; O'Shannessy, Ekberg & Mosbach, 1989) and the polymerisation conditions (Selligren, Dauwe & Schneider, 1997; Piletsky *et al.*, 2002).

It is, however, the choice of monomers, which has proven to be the determining factor in successful imprinting. Monomers are selected on the basis of their ability to interact with the template, which contribute to $\Sigma \Delta G_{\text{p}}$, ΔG_{h} and $\Delta G_{\text{t+r}}$ in Equation 1.1. This choice can be performed either by rationally by screening a library of suitable candidates against the template,

selecting those which strongly interact with the template at a suitable point or by empirically testing a series of polymers in order to identify a suitable candidate. The important point in selection of monomers and identification of polymerisation conditions is to be able to preserve the structure of pre-formed complex during the polymerisation step. We now know, in general, a lot more about complexation stage than about polymerisation process itself. It has proved difficult to obtain an exact model of the polymerisation process, due to lack of information on the processes taking place in real systems (e.g. chain propagation, phase separation, changes in complex structure).

Following the template extraction the surrounding polymer becomes subject to environmental changes that alter the physical conformation of the material. Changes in the pH, concentration of ions, presence of surfactants, solvent and changes in temperature have been shown to alter the conformation of polymer chains within the structure, in a number of studies. (McCormick and Brent Johnson, 1989; Shay, 1989; Qiu *et al.*, 2002; Holappa *et al.*, 2002; Piletsky *et al.* 2002). These changes mainly affect the complex formation by altering the ΔG_{conf} in Equation 1.1; however, all the described energies can be affected, depending on the changes made. These changes within an imprinted polymer will affect the sensitivity and affinity of the material towards the corresponding template.

1.4.4.1 *Monomer selection*

As explained in section 1.4.4 the monomer selection is important, as the interactions produced between the template and the monomers are what, in

essence, produce a successful imprint. Traditionally the selection of these monomers is made by subjective choice based on previously published experimental results, chemical logic (matching a negative monomer with a positive template), behaviour of similar systems and the individual experience of the chemist. This approach requires considerable time and knowledge in order to produce a quality imprint, and in today's fast moving world of science it is a luxury that we cannot afford.

Several different approaches have been designed to meet this requirement for speed and accuracy based on combinatorial or computational protocols. The first real attempt to develop a rational generic protocol was made using a combinatorial screening approach (Takeuchi, Fukuma & Matsui, 1999; Lanza & Sellergren, 1999). MIP libraries containing tens of polymers were synthesised and tested in batch binding experiments. These experiments were performed in situ using small amounts of materials (~55 mg). Measuring template leaching and polymer ability to re-bind template, the so-called MINI-MIPs libraries were used to select monomers to produce a final effective polymer.

This method, despite demonstrating effective results on small sample spreads, has limitations in its applicability for the testing of large libraries of co-polymers made of two or more monomers. This is due to the number of polymers that can be produced using a combination of two monomers (Equation 1.2). A library containing 100 monomers would require more than five thousand polymers to be synthesised and tested. A combination of three

or more monomers per polymer would amount to huge numbers of materials to be screened, which severely limits the use of the method. This also assumes that the monomers are used in set ratios (i.e. 1:1). Any change to this, to study the effect of different monomer ratios would elicit an increase to the total numbers of polymers required for screening.

$$A=(n^2+n)/2$$

Equation 1.2: where A is the number of polymers and n is the number of monomers in the library.

Automation of the process is possible (Takeuchi, Fukuma & Matsui, 1999) but in practice the synthesis and screening of large libraries of polymers remains difficult, time consuming and expensive to be used in routine applications.

Thermodynamic calculations have been used also to suggest the rules which should be followed to generate a successful polymer (Nicholls, 1995). However, they were never used to predict the optimal composition due the complexity of performing these calculations on real systems, and the amount of resources and time required. Whitcombe *et al.* (1998) utilises mathematical modelling of the pre-polymerisation complexes and their relative binding, in order to predict the selectivity of the polymers prepared under different conditions, a method that works, even though it requires complex calculations and intensive polymer production and testing.

To avoid these complications, at Cranfield we have developed a computational approach suitable for the selection of monomers and

optimisation of monomer composition. The protocol is based on Sybyl 6.9 (Tripos, USA, 2003), a commercially available software, running on a Silicon Graphics Octane Workstation equipped with IRIX 6.5 operating system. A virtual library of polymerizable compounds can be used to identify the high affinity monomers and predict a stoichiometric ratio of template to monomer, by simulating the process of complex formation. This system can be tailored to include the effects of temperature, solvent, and cross-linker (Karim *et al.*, 2000; Piletsky *et al.*, 2001; Subrahmanyam *et al.*, 2001; Chianella *et al.*, 2002; Piletsky *et al.*, 2002). A full description of this method may also be found in the materials and methods section of this paper.

1.4.4.2 *Template requirements*

To select an ideal template several factors should be considered. The stability of the template under the polymerisation conditions is paramount. It should be preferably inert, without any double bonds that can be affected by free radical initiation. The template should have a low internal flexibility and high rigidity as these effect the steric formation of a polymer cavity, and lower the potential of flexible rebinding, both of which will offer greater homogeneity to the resultant polymer. The functionality of the template will decide the nature of the interactions. The nature and number of the functional groups in a template will directly affect the selectivity and affinity of the polymer (Sellergren, 1989).

It should be soluble in the porogen and this should complement the choice of monomers and cross linker. Also the required use of organic solvents (for

non-covalent interactions) affects the choice of template, as many multiple charged and zwitterionic molecules are unsuitable due to solubility.

1.4.4.3 *Porogen*

The role of the porogen is two fold. Firstly it acts as a solvent allowing the constituents of the polymer solution to mix and reach homogeneity before polymerisation occurs, and secondly it allows the modulation of the porosity of the polymer structure. With hydrophilic monomers a non-polar solvent is favoured as it allows cross linker and template to mix with the monomers, and vice versa. The choice of porogen is important, as it must be inert, as any interactions with the monomer mixture would lower the recognition properties of the resultant polymer. Therefore, in non-covalent imprinting a non-polar solvent is preferred over a polar one, which would interfere with the electrostatic interactions needed for a successful imprint.

1.4.4.4 *Cross linker*

The cross linker is the stable, inert building block of the bulk polymeric matrix, within which the monomer/template pockets are held. A suitable cross linker must possess the ability to polymerise under free radical initiation however it should not be sensitive to any interactions with the template. A selection of suitable compounds is commercially available with varying degrees of functionality.

The most popular cross linker is ethylene-glycol dimethacrylate (EDGMA) (Figure 1.7), a bi-functional compound, as it offers high mechanical and

thermal stability (Wulff, Vietmeier & Poll, 1987; Svenson & Nicholls, 2001). However it has been shown that with a higher branched cross linker, greater the load capacity and enantiomeric separation can be achieved (Kempe, 1996).

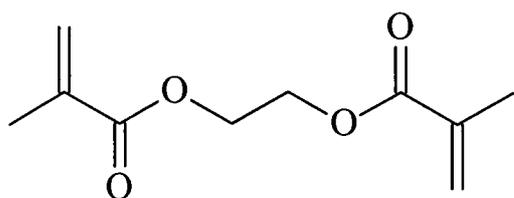


Figure 1.7: Ethylene-glycol dimethacrylate (EDGMA)

1.4.4.5 Initiator

The choice of initiator depends on the mode of polymerisation, be it by thermo- or photo- polymerisation, the required temperature of the reaction and in which solvent the polymerisation is to take place. The concentration of initiator is important in the polymerisation process, as it acts as a rate-determining factor. Usually the initiator is about 0.5 – 1% of the total pre-polymerisation solution.

Several are commercially available with the azobisnitriles the most commonly used in organic solvents (Figure 1.8).

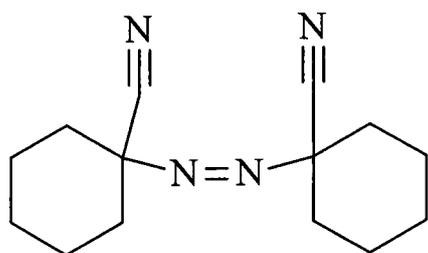


Figure 1.8: 1,1'-azobis(cyclohexane-carbonitrile) (ACHN)

1.4.4.6 *Polymerisation conditions*

From the past experiments a general observation for imprinted polymers is that optimum binding occurs when polymer is exposed to the same conditions as those used for polymerisation (Wulff *et al.*, 1986; Allender, Brain & Heard, 1997; Haginaka & Sanbe, 2001), therefore the physical properties of the system must be taken into consideration as well as the components.

Piletsky *et al.* (2002) demonstrate the importance of the temperature on the specificity and affinity of the polymer, with lower polymerisation temperatures offering better recognition. This study shows the importance of thermodynamic analysis and how it can aid recognition choice.

The duration of the polymerisation reaction is also critical. Traditionally when making monolithic polymers the reaction runs for 24 hrs to ensure all components have polymerised, however depending on the format these times vary.

1.4.5 **MIPs for toxins**

Toxic compounds offer attractive targets for the design and implementation of imprinted polymeric phases, for several reasons. With increased awareness in the environment and pollution control, improved foodstuff standards, excellent medical technologies and in general a better standard of living, the requirement to be able to detect such compounds efficiently and rapidly is needed. This, in turn, has increased the financial interest in these compounds, as products suitable for detection, quantification and removal of toxins are sought after to obtain a slice of the ever-growing market. This means more projects, aimed at producing reliable and robust materials for targeted

compounds are been funded, as they are deemed viable for product development.

Many toxins offer strong chemical functionality (from which they gain their toxicity), which increases their potential as targets, as this is a requirement for strong non-covalent imprinting. The raising of antibodies for ELISA (the obvious alternative) is affected by the inherent toxic nature of these compounds. The resultant materials have short shelf life and are subject to environmental conditions that can affect the binding and can degrade the material, therefore the use of MIPs is a viable alternative. Several examples appear in the literature from a wide range of materials, including nerve agents, pollutants and natural toxins.

The determination of nerve agents in human serum has been demonstrated using MIPs (Zi-Hui & Qin, 2001). They showed that a MIP produced for a derivative product of nerve agent could also be used to screen for other degradation products as well. Jenkins *et al.* (1999) use imprinting technology alongside laser luminescence technology to produce a sensor capable of detecting hydrolysis of chemical warfare agent Soman with a detection limit of ppt (parts per trillion).

In terms of environmental analysis the detection of herbicides has been the focus of several groups and several examples in the literature exist of MIPs produced for these compounds (Sergeyeva *et al.*, 1999a; Panasyuk-Delaney *et al.*, 2001; Kochkodan, Weigel & Ulbricht, 2002; Zhu *et al.*, 2002).

A typical example is that of a MIP synthesised for the highly toxic herbicide 2,4,5-trichlorophenoxyacetic acid. The binding properties and the selectivity of the polymer tested by HPLC. The polymer, used as a solid-phase extraction material for the clean-up of the template molecule and related herbicides from river water samples demonstrated quantitative recoveries comparable with those obtained with a traditional C₁₈ reversed-phase column when analysed by capillary electrophoresis, but with better sample clean-up (Baggiani *et al.*, 2001).

A MIP specific for Microcystin L-R, a potent algal toxin has been synthesised using the computational method (Chianella *et al.*, 2002). This polymer was found to have affinity and sensitivity comparable to polyclonal antibodies, while gaining superior cross reactivity. This work was furthered in the development of a SPE extraction/ piezoelectric sensor system. With a pre-concentration step of 1000 fold a limit of 0.35 nM was reached (Chianella *et al.*, 2003).

Two groups have published work based on MIP synthesis specific for OTA. The group of Lindner (Jodlbauer, Maier & Lindner, 2002) demonstrated the use of analogues, to mimic OTA, during polymerisation, seeking to prevent template leaching and produce inexpensive materials, however it requires complex synthetic chemistry. Likewise, their choice of monomers is specifically designed for the task. The results obtained show only moderate recognition of OTA in pre-determined experimental conditions which are not suitable to real sample extraction, With poor cross reactivity and without affinity/capacity results, this paper stands only as the first step in producing

useful materials. Baggiani demonstrates the use of mimics in polymerisation to develop a MIP for OTA. He demonstrates the importance of steric factors on recognition (Baggiani, Giraudi & Vanni, 2002).

1.5 Objectives

This work, and backbone of the thesis, is part of a project is sponsored by the European Union under the proposal No. PL QLRT-1999-01380 (OTA PREV)

The overall aim of the proposal is to reduce the intake of OTA from cereals by 50% +, and provide tools for corrective and preventive action.

Cranfield University at Silsoe (Partner 3) is involved in two parts of the project.

The section that concerns this work, is work package 7, part of Task 3 (Establishing a monitoring system).

This project was aimed at producing a sensor system for OTA to work within the requirements of new European legislation to limit the intake of mycotoxins, namely OTA in the food chain. The primary application was the design of a system capable of specifically binding OTA, from a grain extract, providing a measurable signal, within the range of 2-5 ppb. The secondary benefits were that this system would be usable for the detection of OTA in other foodstuffs. The system had to be robust, cost effective and simple to use. Its main use would be in food matrices where it would be used to confirm the presence of OTA before its acceptance. This would be *in situ*, at the production site or at the site of collection (grain storage).

This package is entitled “ Development of rapid biosensors of Ochratoxin detection using molecular imprinted polymers.”

Within this package work will be focused on five phases of development.

- A: Synthesis of MIP, specific for OTA.
- B: Analysis of specificity and cross reactivity of imprinted polymers.
- C: Development of assay based on MIPs.
- D: Evaluation of system performance using “real” samples.
- E: Comparison to other validated analytical methods.

The ideal detection limit of OTA is \approx 1-5 ppb.

The project requires a series of yearly deliverables.

First Year: Imprint polymer material with high stability, long operational life, low cost, good sensitivity, and specificity with respect to OTA.

Second Year: The development of imprinting polymers, which will specifically bind with OTA.

Third Year: Biosensor system meeting legislative requirements of the European Union on limits.

This thesis is aimed at the design of an imprinted polymer system for Ochratoxin A and development of a sensor for this toxin in natural products.

Figure 1.9 shows the order of research taken to achieve this. Two polymers

were computationally designed, synthesised and used in SPE and an optical sensor based on MIP membranes.

In the following chapters you will find the methodology and materials used to fulfil this task, the results of the experimentation and discussion on them.

Concluding remarks and further suggested work will also be covered.

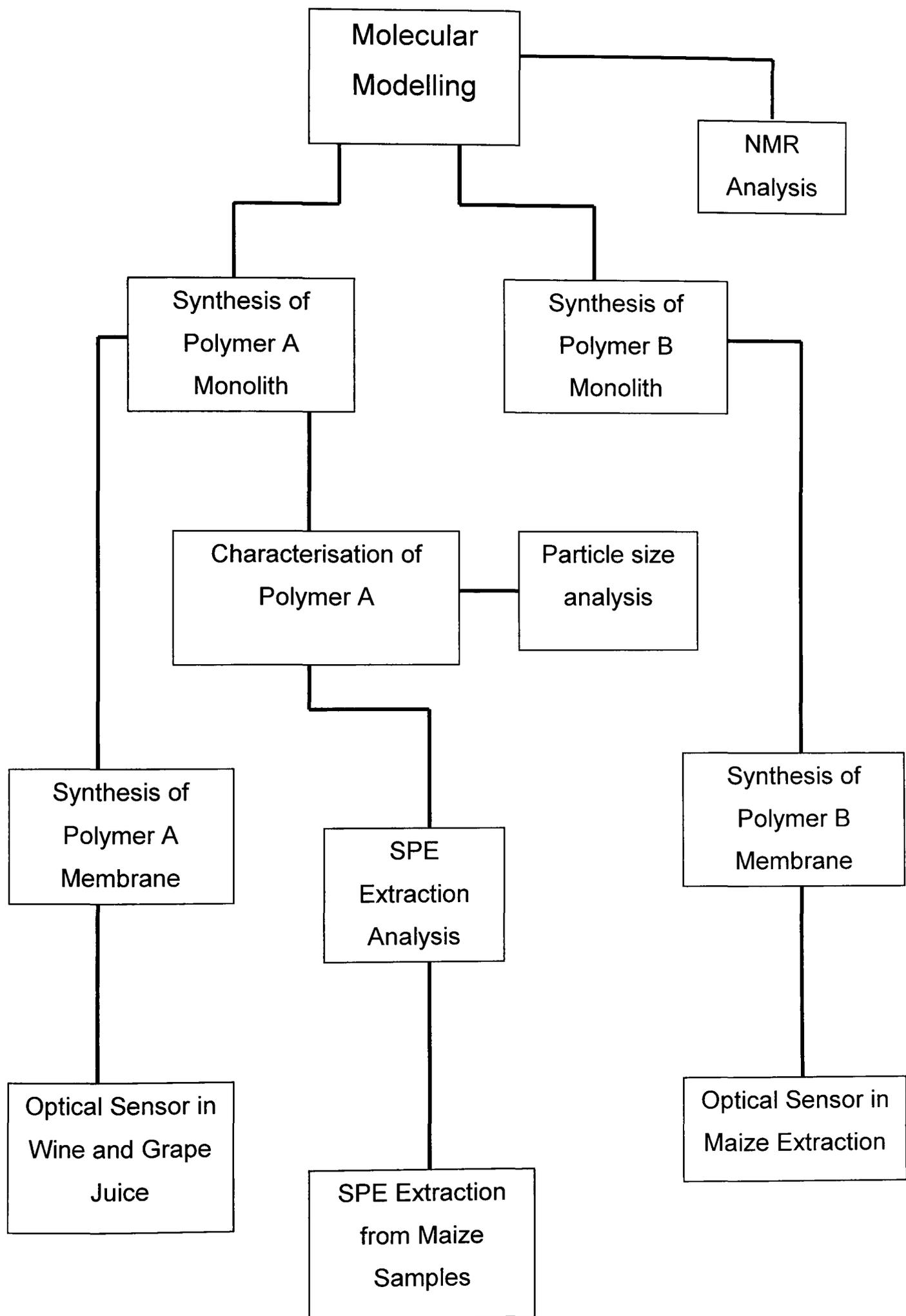


Figure 1.9: Flowchart of experimental research presented in this thesis

Chapter 2

Materials and Methods

2.1 Introduction

This chapter is divided in four sections. Firstly, a full description of materials and equipment used in this thesis is reported. The second section describes design and synthesis of the polymers. The third describes the methods used to characterise and analyse the polymer behaviours and the fourth is a description of methods used for the development of MIP-based sensor and MIP-based solid-phase extraction (SPE).

2.2 Materials

Acetonitrile, chloroform, dimethyl formamide (DMF), hexane, methanol, HPLC-grade water, ethylene glycol dimethacrylate (EGDMA), tri-ethylene glycol dimethacrylate (TEGDMA), keiselghur (Celite 545), carbon graphite powder, aluminium oxide, acrylamide, N,N-methylene bis acrylamide, methacrylic acid, N,N- diethylamino ethyl methacrylate (DEAEM), lecithin, phenylalanine, sodium perchlorate, sodium phosphate (mono and dibasic), Tween 20, and vinylimidazole were purchased from Sigma Chemicals (Poole, Dorset, UK).

Oligo-urethane acrylate (OUA) was kindly donated by the Institute of Macromolecular Chemistry, National Academy of Sciences of Ukraine (Kiev, Ukraine). 4-chloro-1-hydroxy-2-naphthoic acid (CHNA) and N-(4-chloro-1-hydroxy-naphthoylamido)-(L)-phenylalanine (L-Phe-CHNA) were kindly donated by Dipartimento di Chimica Analitica, Università di Torino, (Turin, Italy).

Deuterated acetonitrile (CD_3CN) and tetramethylsilane (TMS) were obtained from Cambridge Isotope Laboratories (Massachusetts, USA).

Ochratoxin A was purchased from ACROS organics (Fisher, Loughborough, Leicestershire, UK).

1,1-azobis cyclohexane(carbonitrile) (ACCN) was purchased from Aldrich Chemicals (Poole, Dorset, UK).

All the solvents were of analytical or HPLC grade and were used as received. All the buffers and solutions were prepared using reverse osmotic water (RO water). The pH of the RO water was measured consistently at 5.82. Spanish maize was used for grain sample studies. The wine and white grape juice was purchased from Tesco (Cheshunt, Hertfordshire, UK).

The IEX cartridges, (types CBA, NH_2 , SAX and SCX) were purchased from Varian (Harbor City, CA, USA) and the Strata NH_2 were purchased from Phenomenex (Macclesfield, Cheshire, UK).

1 ml and 4 ml vials, pipette tips and cuvettes were obtained from Supelco (Sigma-Aldrich, Poole, Dorset, UK). The sieves (with aperture size of 106, 63, 45 and 38 micron), utilised for polymer particle separation, were obtained from Endecotts Ltd. (London, UK).

A Shimadzu RF 5001PC Spectrofluorometer (Shimadzu Deutschland GmbH, Duisburg, Germany), a Buchi R-114 rotovapor (BÜCHI Labortechnik

AG, Switzerland) a Supelco Preppy vacuum manifold (Sigma-Aldrich, Poole, Dorset, UK), powered by a vacuum pump and a Hanna Instruments 8159 pH meter (Hanna Instruments Ltd, Leighton Buzzard, Bedfordshire, UK), were used in SPE analysis. A Fluoromax-2 (Instruments SA, UK) fluorimeter, with fibre-optic attachment was used for membrane analysis.

A "Labram 1B" Raman Microprobe (Instruments SA, UK) containing an integrated microscope, equipped with a high stability BX40 frame (Olympus, UK) with objectives up to 100x, was used for particle size analysis. Two UV sources were used for polymerisation; UVAprint 100 CVI was purchased from Dr. Hönle, Germany. The fibre-optic focused source (Cermax) was obtained from ILC Technologies, UK.

The workstation used to simulate monomers-template interactions was a Silicon Graphics Octane running IRIX 6.6 operating system. The workstation was configured with two 195 MHz reduced instruction set processors, 712 MB memory and a 12 GB fixed drive. This system was used to execute the software packages SYBYL 6.9 Tripos Inc. (St. Louis, MI, USA). NMR spectra were obtained on a Jeol JNM- EX270 FT-NMR (Japan) fitted with a Jeol NM-EVTS3 liquid nitrogen-based variable temperature system.

2.3 Methods

2.3.1 Computational design of an imprinted polymer specific for OTA

Utilising the Sybyl software, a rational design protocol has been developed (Piletsky et al, 2001).

This protocol contains four steps:

- Design of the molecular model of the template for screening.
- The design of molecular models of functional monomers and the creation of a virtual library (monomers database).
- Screening of the template against the database (library) using the Leapfrog™ algorithm.
- Refining step (simulated annealing).

From the first three steps we can predict the most suitable monomers which interact with the template molecule. The addition of the fourth step allows the prediction of the optimum stoichiometric ratio of monomers to template.

2.3.1.1 *Design of molecular model of template*

The template used in this study was ochratoxin A (OTA). A 2D structure of the template is drawn using the sketch molecule command in the Sybyl software and was translated into a 3D representation of the molecule (Figure 2.1). The chemistry of the template was checked to ensure that the program had correctly identified the type of chemical groups and chemical bonds present in the molecule and that the chirality is correct.

The molecule is then charged using the Gasteiger-Huckel method, placed under the rules of the Tripos force field, within a dielectric constant of 1

(default) and then minimised utilising the Powell method, for 2000 iterations until the molecule had reached an energy state of $0.001 \text{ kcal mol}^{-1}$. This takes approximately 2 minutes.

Once this has been performed, the template is saved and is ready for screening, and the next step of the protocol can be undertaken.

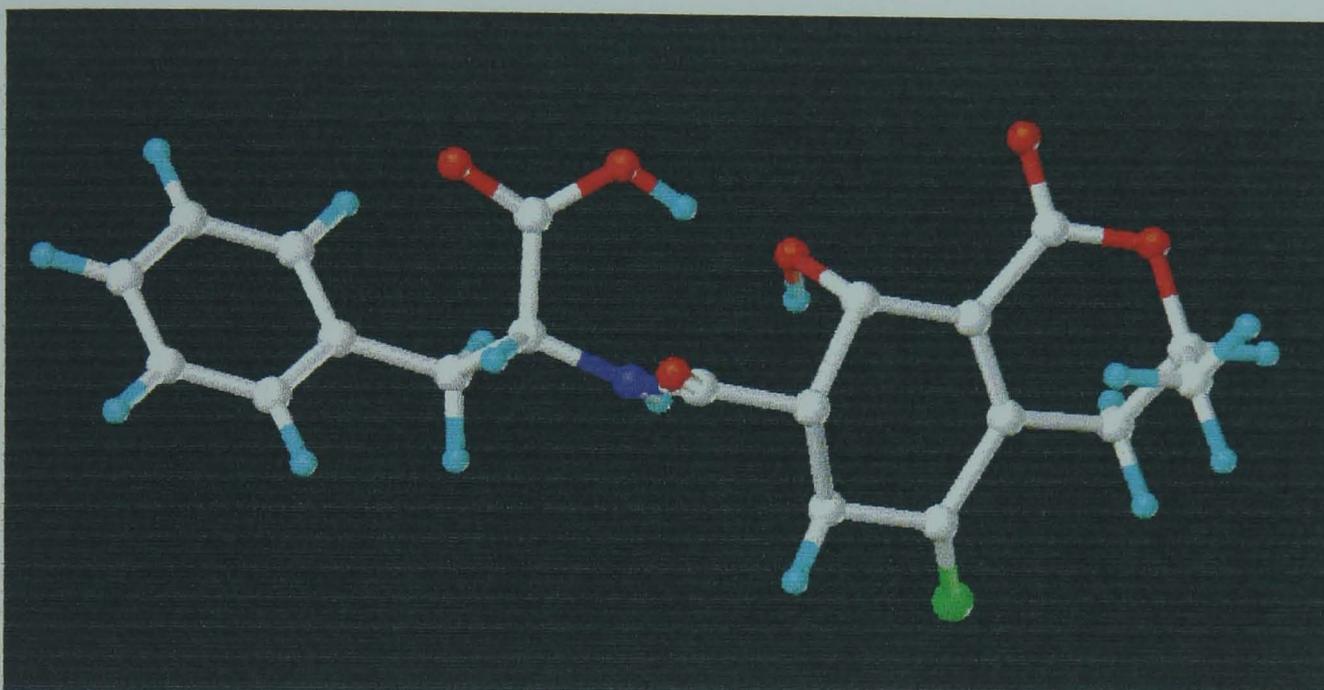


Figure 2.1: A 3D representation of ochratoxin A, as simulated by the SYBYL 6.9 molecular modelling software.

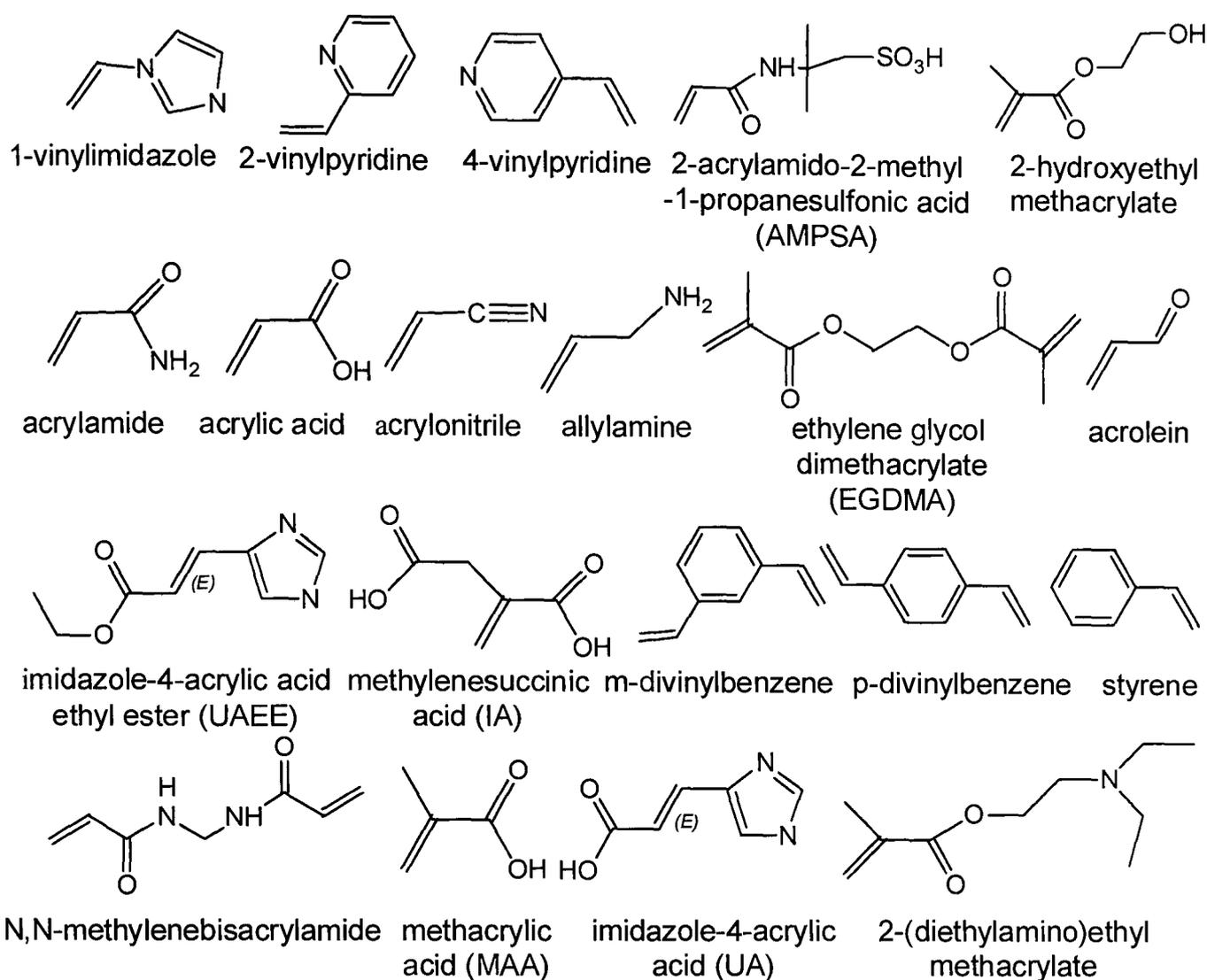
2.3.1.2 *Design of functional monomer database*

As we are dealing with a non-covalent binding system the monomers chosen to form the library are able to interact with the template through non-covalent means, such as electrostatic, hydrophobic van der Waals forces, dipole-dipole interactions. They also contain functional groups (such as a double bond) to be able to be polymerised through a radical mechanism. Molecular modelling software was used to screen all possible monomers included in the database for a suitable match. A database acts as a spreadsheet containing the structural information on the 20 most common functional monomers (Table

2.1). This small library contained acidic, neutral and basic monomers, capable of a diverse range of interactions with practically any type of template.

In this experiment the molecular structures of the monomers of the common library (Table 2.1) were refined (ensuring correct chemical structure), charged and minimised (by determining their lowest energy conformation using molecular dynamics), as described in section 2.3.3.1. Once this had been performed the library was prepared for screening against the template using the Leapfrog™ algorithm.

Table 2.1: Virtual library containing the most commonly used functional monomers.



Abbreviations: AMPSA: 2-acrylamido-2-methyl-1-propane sulfonic acid; EGDMA: ethylene glycol dimethacrylate; UAEE: urocanic acid ethyl ester, imidazole-4-acrylic acid ethyl ester; IA: itaconic acid, methylenesuccinic acid; UA: urocanic acid, imidazole-4-acrylic acid: DEAEM (2-(diethylamino)ethyl methacrylate).

2.3.1.3 *Screening of the template against the database using the Leapfrog™ algorithm*

The Leapfrog™ algorithm, a component of the SYBYL software package was used to be probed each of the monomers in the database against the template for any possible interaction. This is performed mainly on the basis of their binding score, calculated for electrostatic, dipole-dipole and Van der Waals interactions, which could be formed in the existing sterical environment. Mechanically this was performed by repeatedly trying to position different ligands (one each time) around of the template and then either keeping or discarding the results depending on calculated binding score.

The software is used to identify possible binding points on the minimised template model. The program was applied in DREAM Mode for 20,000 iterations, performed in one run. The database spreadsheet containing the structural information of monomers was inserted as data. The parameters used for Leapfrog applications are then selected. Most of the categories were left as default with the exception of energy startup, in which the inclusion of H-bonding energy was added, and relative move frequencies, which were tailored to the features of the monomers and the characteristics of desired result. In this experiment these parameters were set as described in Table 2.2.

Table 2.2: Move frequencies selection with the explanation of each.

Join: Move which tries to join different fragments	<table border="1"> <thead> <tr> <th colspan="2">= Move Frequencies</th> </tr> </thead> <tbody> <tr> <td>JOIN:</td> <td>0</td> </tr> <tr> <td>FUSE:</td> <td>0</td> </tr> <tr> <td>NEW:</td> <td>10</td> </tr> <tr> <td>FLY:</td> <td>1</td> </tr> <tr> <td>TWIST:</td> <td>1</td> </tr> <tr> <td>REFINE:</td> <td>4</td> </tr> <tr> <td>BRIDGE:</td> <td>0</td> </tr> <tr> <td>COMPLEMENT:</td> <td>0</td> </tr> <tr> <td>SAVE:</td> <td>2</td> </tr> <tr> <td>WEED:</td> <td>1</td> </tr> <tr> <td>CROSSOVER:</td> <td>0</td> </tr> <tr> <td>PRUNE:</td> <td>0</td> </tr> <tr> <td>OK</td> <td>Cancel</td> <td>Help</td> </tr> </tbody> </table>	= Move Frequencies		JOIN:	0	FUSE:	0	NEW:	10	FLY:	1	TWIST:	1	REFINE:	4	BRIDGE:	0	COMPLEMENT:	0	SAVE:	2	WEED:	1	CROSSOVER:	0	PRUNE:	0	OK	Cancel	Help
= Move Frequencies																														
JOIN:		0																												
FUSE:		0																												
NEW:		10																												
FLY:		1																												
TWIST:		1																												
REFINE:		4																												
BRIDGE:		0																												
COMPLEMENT:		0																												
SAVE:		2																												
WEED:		1																												
CROSSOVER:		0																												
PRUNE:		0																												
OK	Cancel	Help																												
Fuse: Move which try to fuse different fragments when a ring bond flanked by hydrogens is present in both fragments																														
New: A new ligand is started by aligning one of the fragments with respect of any of sitepoints																														
Fly: Move which tries to seek alternative minimum energy orientations for a ligand																														
Twist: Move very similar to Fly, it resembles more a conventional minimiser																														
Refine: One of the most important move, which can improve the newly found ligands																														
Bridge: Move which considers all available fragments as bridges and not just a single randomly chosen fragment																														
Complement: Move which chooses moiety complementary to a select cavity group as a ligand																														
Save: Move, which saves ligands that match the desired requirements																														
Weed: Move discards the worst ligands except the top 10, when the total number of ligands is greater than 100.																														
Crossover: Genetic move for generating the best hybridisations among the generally similar molecules generated by Leapfrog																														
Prune: Move which can delete moieties of a known ligand on the basis of their energy of the binding with the receptor																														

Complexes formed are saved in a SYBYL database and these were referenced via an internal spreadsheet, which serves to list the binding score of each monomer, and also indicates the binding position of each monomer around of template.

The results of a Leapfrog™ protocol allowed identifying complementary monomers for the template based on the strength and type of their interaction with the template. This also allowed for single and multiple point interactions (multiple interactions ensure superior imprinting) between different monomers

and template molecule to be identified and possible conflicts (monomers binding to same position on the template) to be identified.

Although, this is useful in predicting the monomers it does not predict any stoichiometric ratios or demonstrate any interactions that might exist between chosen monomers. Therefore a further step (simulated annealing) was applied.

2.3.1.4 *Refining step (simulated annealing)*

After monomer selection using Leapfrog™ a molecular dynamics program (simulated annealing) was used to predict and analyse the organisation of the monomers around the template molecule. This process is used to optimise the polymer monomer/template composition. In order to simulate the pre-arrangement of functional monomers with the template in the monomer mixture prior to polymerisation, multiple copies of the best monomers (methacrylic acid and acrylamide for polymer A, according to Leapfrog results) were placed around a molecule of OTA creating a solvation box.

The solvated box was then charged with the Gasteiger-Hückel method and its energy was minimised using Powell method, termination gradient, default parameters for dielectric constant and function and convergence at 0.05 Kcal mol⁻¹ in 2000 steps. Annealing conditions were fixed as 1000K-300K sweeping in 32,000 consequent steps. Equilibrium length was determined in 2,000 fs. The energy minimisation, performed among each of the molecular dynamics steps, was carried out under the default conditions (dielectric constant etc.). Periodic boundary conditions were applied both during energy

minimisation and molecular dynamics, to ensure that the monomers were kept close to the template.

At the end of the program, the number and the position of the functional monomers were examined giving information about the optimum MIP composition. The type and quantity of the monomers participating in the complex with template indicate the type and ratio of the template and monomers in optimised MIP composition.

2.3.1.5 Analysis of monomer template interaction in organic solvent

The same protocol as described in section 2.3.4 was used to analyse interactions between DEAEM and OTA. The dielectric constant was changed from default for that of acetonitrile (37.5) to mimic interactions in organic solvent. This was used instead of the solvent used for polymer synthesis (DMF) which has a dielectric constant of 36.7. This is because acetonitrile was intended for the solvent used for extraction from maize and the aim of the modelling was to maximise the interactions between the polymer and OTA in that solvent.

2.3.2 Polymer monolith synthesis

In this sub-chapter we describe synthesis of the polymers used in present study.

2.3.2.1 Polymer A

The composition of the first polymer (Polymer A), based on the molecular modelling performed as described in section 2.3.1 is shown in Table 2.3.

10.6 mg of methacrylic acid, 8.8 mg of acrylamide was added to 930.6 mg of EGDMA. This solution was degassed and 95 mg was taken and added to 100 mg of DMF, and mixed thoroughly, in a 1 ml vial. To this solution 5 mg of OTA was added, and sonicated until dissolved. To the prepared mixture 2 mg of initiator (ACCN) was added and dissolved. The entire solution was degassed and the vial was sealed, under a nitrogen atmosphere. The solution was then placed within an oil bath at 80°C for 24 hours.

Table 2.3: Composition of Polymer A

Component	MIP (mg)	MIP (mM)	NIP (mg)	NIP (mM)
OTA (Template)	5	0.0123	/	/
Methacrylic acid (Monomer)	1.06	0.0123	1.06	0.0123
Acrylamide (Monomer)	0.88	0.0123	0.88	0.0123
EGDMA (Cross linker)	93.06	0.47	93.06	0.47
DMF (Solvent)	100	1.36	100	1.36
ACCN (Initiator)	2	0.008	2	0.008

The molar ratio between OTA:Methacrylic acid:Acrylamide is 1:1:1

After this period the vial was removed and allowed to cool. The vial was then broken carefully and the resultant monolith was removed.

The monolith was then ground by hand, using a pestle and mortar in the presence of methanol. This procedure is performed slowly and carefully, due to small amounts of material. The polymer was sieved and fraction with particles size 38-106 microns collected. The fine particles (>38 micron) were also collected.

On average, with careful grinding ~60% of the total polymer was collected. The same procedure was performed, without the addition of the template (OTA) to produce a NIP (Non Imprinted Polymer).

2.3.2.2 Polymer B

The composition of the Polymer B is shown in Table 2.4.

Table 2.4: Composition of Polymer B.

Component	MIP (mg)	MIP (mM)	NIP (mg)	NIP (mM)
OTA (Template)	5	0.0123	/	/
DEAEM (Monomer)	2.29	0.0123	2.29	0.0123
EGDMA (Cross linker)	92.71	0.47	92.71	0.47
DMF (Solvent)	100	1.36	100	1.36
ACCN (Initiator)	2	0.008	2	0.008

The molar ratio of DEAEM:OTA is 1:1.

22.9 mg of DEAEM was added to 927 mg of EGDMA. This solution was degassed and 95 mg was taken and added to 100 mg of DMF, and mixed thoroughly, in a 1 ml vial. To this solution 5 mg of OTA was added, and sonicated until dissolved. To the prepared mixture 2 mg of initiator (ACCN)

was added and dissolved. The entire solution was degassed and the vial was sealed, under a nitrogen atmosphere. The solution was then placed within an oil bath at 80°C for 24 hours.

After this period the vial was removed and allowed to cool. The vial was then broken carefully and the resultant monolith was removed.

The monolith was then ground by hand, using a pestle and mortar in the presence of methanol. This procedure is performed slowly and carefully, due to small amounts of material. The suspension was sieved and fraction with size 38-106 microns collected. The fine particles (>38 micron) were also collected. On average, with careful grinding ~60% of the total was collected between 38-106 microns. The same procedure was performed, without the addition of the template (OTA) to produce a NIP.

2.3.3 Polymer washing

30 mg of polymer samples were weighed and packed into 1.5 ml SPE cartridges. The cartridges were then washed with methanol to condition material.

Each polymer was then washed thoroughly to ensure the removal of the entire template used in preparation, and any excess of unreacted components.

This is required to ensure that any “leaching” of template is kept to a minimum as it can affect the sensitivity of any following experimental studies.

In the case of polymer A each cartridge was subjected to a series of solvent washes designed to remove the presence of any bound template. 50 ml of methanol, 50 ml of acetone, 50 ml of methanol and 50 ml of water were slowly

filtered through the cartridge under reduced pressure. The eluent was collected and washed OTA detected fluorimetrically (Section 2.3.6). This washing step was repeated until no OTA could be detected after which the polymer was considered clean. The NIP polymer was subjected to the same washing step.

In the case of polymer B, this technique was repeated, however it proved difficult to remove OTA from the polymer using due to high affinity of the material. Further washing steps were used with excess (10 ml) of 50 mM HCl in 80% methanol and with 50 mM NaOH in 80% methanol.

2.3.3.1 Electrochemical elution of OTA from polymer B

A new set-up was designed for electrophoresis of OTA from polymer B (Figure 2.2). Electrochemical cells (1 ml top and 10 ml bottom as shown in Figure 2.2.) were filled with 100 mM solution of sodium perchlorate in acetonitrile and current (10 V, 100 mA) applied for 1 hour. The eluted OTA was measured fluorimetrically (Section 2.3.6).

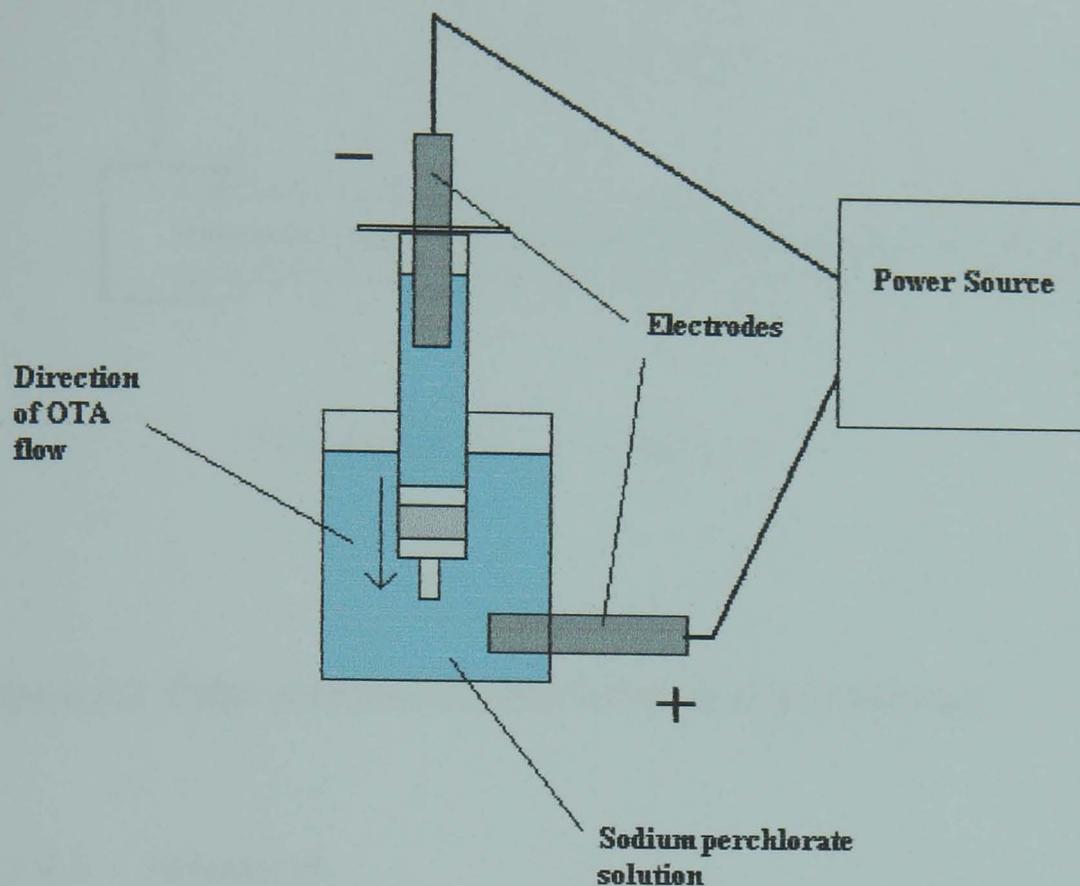


Figure 2.2: Electrophoresis set-up with SPE cartridge.

2.3.4 Imprinted membrane production

Two glass plates approximately 6cm^2 were taken and cleaned using water, detergent and methanol.

The plates were then coated with dichlorodimethyl silane to ensure that any polymer formed on its surface will not stick to the glass plates.

Two spacer sheets (~ 60 micron thick) of TeflonTM were cut and used to ensure exact thickness of membrane.

Tri-ethylene glycol dimethacrylate (TEDGMA) was mixed with oligourethane acrylate (OUA) in the ratio of 85/15%.

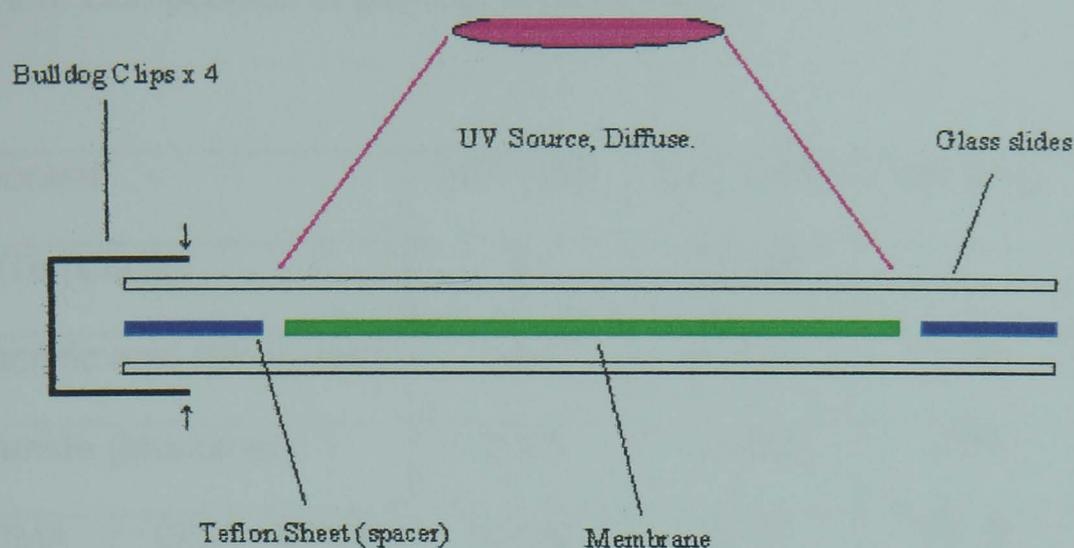


Figure 2.3: Polymerisation set-up for membrane production.

2.3.4.1 Polymer A

200 μ l of polymer solution, as described in Table 2.5 was placed between the glass plates held together with bulldog clips to ensure an even spread of solution. This was then exposed to a high intensity UV source (Dr Hönle, Germany) for 4 minutes (Figure 2.3). The addition of more solvent allows the polymer to spread evenly and keep its shape whilst the polymerisation process occurs. A NIP membrane was produced in the same manner. The polymer and glass plates were allowed to cool and then gently split apart. The polymer was then carefully peeled off the slide and dried between two sheets of filter paper.

Table 2.5: Composition of polymer A membrane.

Component	MIP (mg)	MIP (mM)	NIP (mg)	NIP (mM)
OTA (Template)	5	0.0123	/	/
Methacrylic acid (Monomer)	1.06	0.0123	1.06	0.0123
Acrylamide (Monomer)	0.88	0.0123	0.88	0.0123
TEGDMA / OUA (Cross linker)	93.06	0.47	93.06	0.47
DMF (Solvent)	200	2.72	200	2.72
ACCN (Initiator)	2	0.008	2	0.008

2.3.4.2 Polymer B

Using the protocol in section 2.3.4.1 MIP and NIP membranes of polymer B were produced, with the composition as described in Table 2.6.

The membranes were pinned between filter paper and suspended in stirred solutions of methanol, acetonitrile, acetone and water, to remove any excess OTA, and unreacted components. These solutions were changed every twenty minutes to ensure a steady diffusion of OTA from high concentration in membrane to low in solution. A series of 4 methanol, 4 acetonitrile, 4 acetone and 4 water washes were performed per membrane.

Table 2.6: Composition of Polymer B

Component	MIP (mg)	MIP (mM)	NIP (mg)	NIP (mM)
OTA (Template)	5	0.0123	/	/
DEAEM (Monomer)	5	0.0123	5	0.0123
TEGDMA / OUA (Cross linker)	90	0.47	90	0.47
DMF (Solvent)	200	2.72	200	2.72
ACCN (Initiator)	2	0.008	2	0.008

2.3.5 Washing of polymer membranes

2.3.5.1 "Burning" of residual OTA in membrane by UV irradiation

Each membrane was exposed to the UV source for fifteen minutes after washing to break down the OTA, and decrease the fluorescent signal. The kinetics of OTA decomposition was monitored fluorimetrically every minute for 10 minutes as described in section 2.3.6.4.

After UV treatment each membrane was washed again in methanol and water.

2.3.6 Polymer characterisation

2.3.6.1 A note on fluorescence

Due to significant influence of experimental conditions on OTA fluorescence, for some experiments (e.g. testing of grain samples) control samples were prepared by treating corresponding solutions in exactly the same way (e.g.

filtering through the polymer cartridge) and spiked with the expected maximum concentration of OTA. These samples were defined as positive standards. Non-spiked samples were defined as negative standards. Therefore to analyse change in OTA concentration the fluorescence of each sample was compared to both, positive and negative standards.

2.3.6.2 *Measuring OTA fluorescent signal*

A 100 ppb solution of OTA in 50 mmol phosphate buffer (pH 7.5) was produced and utilised to identify the optimum excitation wavelength. 3 ml of this solution was placed in a 4 ml uPVC cuvette. An emission scan was performed using the Shimadzu RF 5001PC Spectrofluorophotometer between 250 nm and 650 nm with an emission wavelength set at 400 nm. Using the peak identified by this and the same chemical solution, an excitation scan was performed to identify the optimum emission peak. Once this had been performed this first experiment was repeated to confirm the initial excitation peak. These figures known as λ^{Ex} and λ^{Em} are used throughout the experimental phases to measure the fluorescence of OTA in solution.

2.3.6.3 *Measurement of OTA in solution for SPE experimental phase*

The standard protocol for measuring OTA in solution for SPE experiments was as follows.

All positive, negative standards and experimental samples were collected for each measured probe. The positive standard is spiked to the required concentration of OTA. Each sample is placed into a 4 ml uPVC cuvette and measured at $\lambda^{\text{Ex}} = 379$ nm and $\lambda^{\text{Em}} = 434$ nm. The fluorescent signals are

tabulated and the percentage of extracted OTA is calculated by equation 2.1. This is repeated in triplicate to obtain standard error.

$$\frac{(\text{Experimental OTA signal} - \text{Negative standard})}{(\text{Positive standard} - \text{Negative standard})} \times 100$$

Equation 2.1: Method for obtaining percentage of OTA present in experimental solution by comparative signal measurement.

2.3.6.4 Fluorescent analysis of OTA in membrane

A fibre optic cable with lens designed to emit and collect light, was attached to the Fluoromax-2 fluorimeter (Figure 2.4). All measurements were made in dark box. The cut membranes, produced as described in section 2.3.4 are then placed on a support under the lens, so the light focused onto the membrane (This was performed under a visible wavelength ~550 nm).

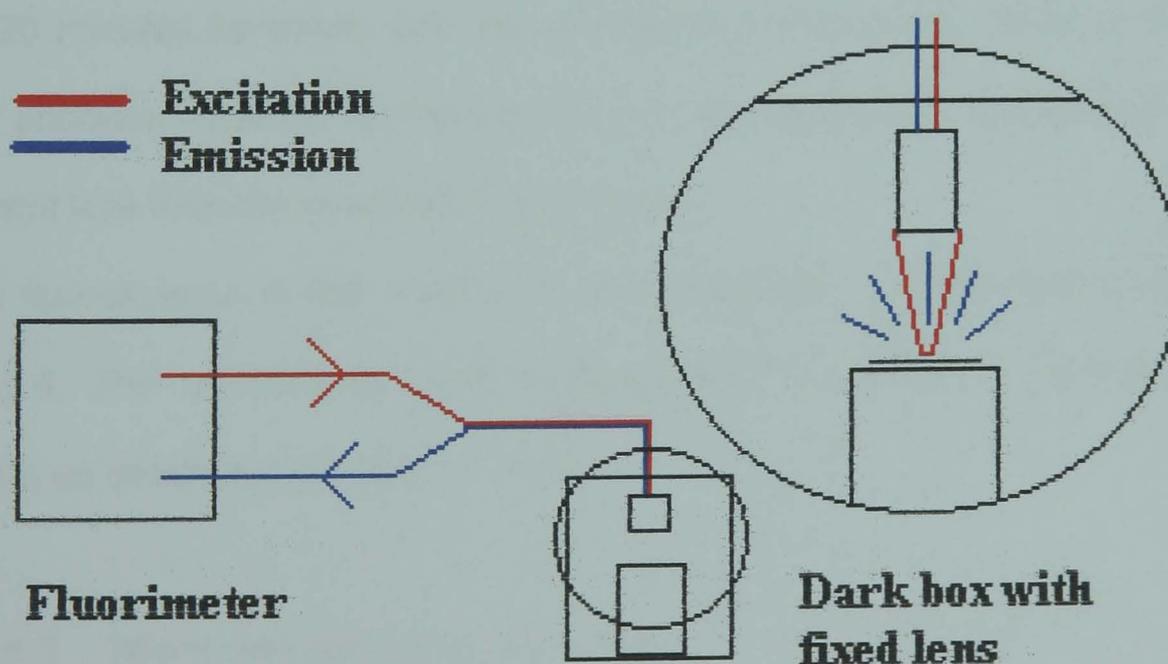


Figure 2.4: Instrumentation set-up for membrane polymer study.

The spectra of the membranes were measured using an emission scan at $\lambda^{\text{Ex}} = 379 \text{ nm}$ and $\lambda^{\text{Em}} = 434 \text{ nm}$. This was repeated several times with the position of the membrane been changed under the light, on both planes, and on both sides. This allows for the homogeneity of the polymer to be considered in the results.

The general protocol for membrane measurement is as follows.

Prior to measuring the prepared membranes were allowed to soak in solvent used in the experiment which does not contain OTA for 20 minutes and then excess of the solvent removed using tissue paper.

The polymer was measured as described above to obtain background fluorescence.

Each polymer was then allowed to soak in 1 ml of the experimental solution for 20 minutes as before and dipped quickly in the same solvent to remove any unbound material from the surface of the membrane. The excess of the solvent was then removed with tissue paper.

The fluorescence of the membrane was measured as described in section 2.3.6.4. The membranes were regenerated by washing in methanol and water, as described in section 2.3.4.2

2.3.6.5 Membrane specificity and affinity

The specificity of the membranes was demonstrated using the protocol in section 2.3.6.4.

The fluorescent backgrounds of both imprinted and non-imprinted polymer A were recorded and these membranes were exposed to a series of different concentrations of OTA (between 10-500 ppb) in 100 mM sodium phosphate buffer pH 7.5. The signals were recorded and the results plotted (Figure 3.15).

2.3.7 Nuclear Magnetic Resonance (NMR)

All measurements were made in deuterated acetonitrile using a Jeol JNM-EX270 FT-NMR (Japan) fitted with a Jeol NM-EVTS3 liquid nitrogen-based variable temperature system.

1 mg of OTA was dissolved into 600 μ l of deuterated acetonitrile, to which 1 μ l of TMS (tetramethylsilane) was added to act as a reference standard.

3.4 mg of acrylamide was dissolved into 1 ml of deuterated acetonitrile. 4.3 mg of methacrylic acid was dissolved into 1 ml of deuterated acetonitrile. 9.2 mg of DEAEM was dissolved into 1 ml of deuterated acetonitrile.

Using these solutions a series of titrations against the template OTA were performed using dilutions in molar ratios for monomer-template running from 1:1 to 5:1.

The shifts seen in the NMR spectrum of OTA are presented in Section 3.2.7.

2.3.8 Particle Size Analysis

The polymer particles were dried, sieved between 25-38 micron and collected. These particles were then placed in the solution of interest for 5 minutes and then dispersed onto a glass microscope slide. A "Labram 1B" Raman Microprobe with integrated microscope and a high stability BX40 frame with objectives up to 100x was used to analyse size of the particles. The image

was taken with a linked CCD-camera, permitting the positioning of the sample and the visualisation of attenuated laser spot as a focus aid. Images of the particles were taken with 10x objectives and 1.4x optic magnification. These images were then analysed using LABVIEW Imaq Builder (National Instruments, USA) to obtain size distribution. On average 7 images were studied with ~ 50 particles in each, giving an average size distribution of ~350 particles. The results are presented in Section 3.2.8.

2.3.9 Solid Phase Extraction (SPE) Analysis

The basic protocol used in SPE analysis is described in Figure 2.5. The cartridge was conditioned with same solvent as used in loading (1). Then the sample is loaded in this solvent (2) and washed to remove the unwanted material bound (3). The required material is then eluted (4). For this project, several different protocols were used in order to explore the binding properties of the materials studied, all of them working around these general principles.

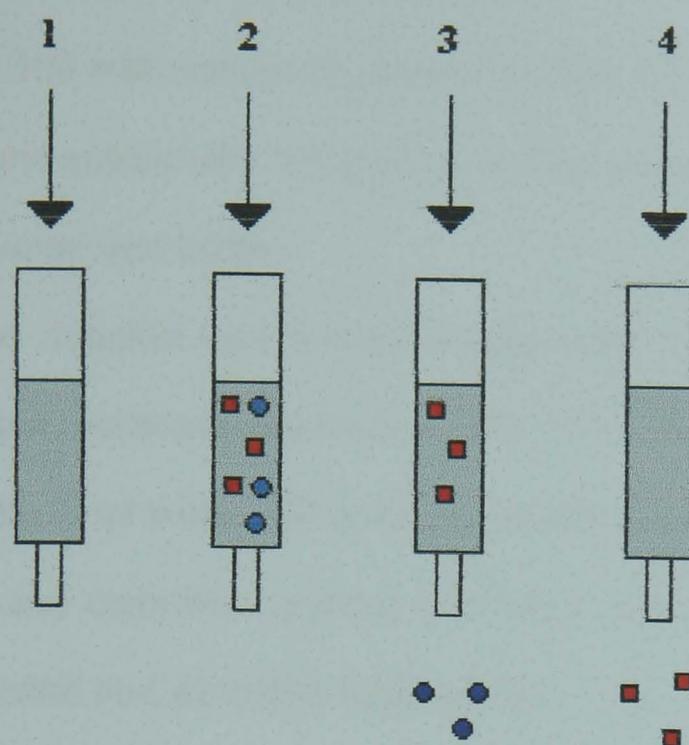


Figure 2.5: Schematic of the SPE process.

1) Cartridge preparation. 2) Loading. 3) Washing. 4) Elution.

2.3.9.1 MIP-based SPE

The cartridges with Polymer A were conditioned with 3 ml methanol followed by 3 ml water and 3 ml buffer. The solutions were filtered through the cartridges under reduced pressure using a Supelco Preppy vacuum manifold powered by a vacuum pump. The samples were collected using 4 ml Supelco vials.

Due to the number of different experiments utilising similar protocols I have described the major experiments and any alterations to these protocols will be listed in the results and discussion section.

2.3.9.2 *Binding of OTA in different pH*

A series of 100 mM sodium phosphate buffers with pH ranging from 4-9 were produced and spiked with 100 ppb OTA. The cartridges were conditioned with methanol, water and buffer.

3 ml of the solution was loaded slowly under reduced pressure onto the cartridge and collected afterwards. The OTA recovery was performed by washing with 3 ml water. All solutions were collected and their fluorescence measured and compared against a positive and negative standard. Results were calculated and plotted in Figure 3.18.

2.3.9.3 *Binding of OTA in different ionic concentrations*

A series of sodium phosphate buffers with concentration ranging from 0 to 200 mM pH 7.5 were produced and spiked with 100 ppb OTA. The cartridges were conditioned with methanol, water and buffer.

3 ml of the solution was loaded slowly under reduced pressure onto the cartridge and collected afterwards. The OTA recovery was performed by washing with 3 ml water. All solutions were collected and their fluorescence measured and compared against a positive and negative standard. Results were calculated and plotted in Figure 3.19.

The experiment was repeated in triplicate after careful washing of the polymer and then for each solution.

2.3.9.4 *Binding of different OTA concentrations*

A 100 mM sodium phosphate buffer, pH 7.5 was spiked with a range of OTA concentrations of 0-500 ppb. The cartridges were conditioned with methanol, water and buffer.

3 ml of the solution was loaded slowly under reduced pressure onto the cartridge and collected afterwards. The OTA recovery was performed by washing with 3 ml water. All solutions were collected and their fluorescence measured and compared against a positive and negative standard. Results were calculated and plotted in Figure 3.20.

The experiment was repeated in triplicate after careful washing of the polymer and then for each solution.

2.3.9.5 *Binding of OTA in varied concentration of acetonitrile (MeCN)*

A series of buffer solutions (100 mM sodium phosphate buffer, pH 7.5) with varying concentration of MeCN (0%-100%) were spiked with 100 ppb OTA. The cartridges were conditioned with acetonitrile, water and loading solution. 3 ml of the solution was loaded slowly under reduced pressure onto the cartridge and collected afterwards. The OTA recovery was performed by washing with 3 ml water. All solutions were collected and their fluorescence measured and compared against a positive and negative standard. Results were calculated and plotted in Figure 3.21.

The experiment was repeated in triplicate after careful washing of the polymer and then for each solution.

2.3.10 Cross reactivity studies.

The cross reactivity of MIPs was analysed for a series of compounds, structurally similar to OTA (Figure 2.6). Solutions of these compounds were produced at a concentration of 100 ppb in 100 mM sodium phosphate buffer, pH 7.5.

3 ml of the solution was loaded slowly under reduced pressure onto the cartridge and collected afterwards. The OTA recovery was performed by washing with 3 ml water.

For recovery experiments, the quantification of ochratoxin, its analogues and their positive and negative standards was performed by Waters HPLC in tandem with a bench-top triple quadrupole mass spectrometer model Micromass Quatro Micro (Waters, Milford, MA, USA) equipped with electrospray probe. The values of the voltages applied to the sampling cone (40 V), capillary (3.2 V), extractor (1 V) were optimised by continuous infusion in order to achieve the highest possible sensitivity for each analyte.

The HPLC conditions were: mobile phase- methanol, flow rate- 0.2 ml/min, column- Luna 3 μm (i.d.- 3 mm, length- 50 mm) (Phenomenex, UK). The quantification was performed using the MassLynx software.

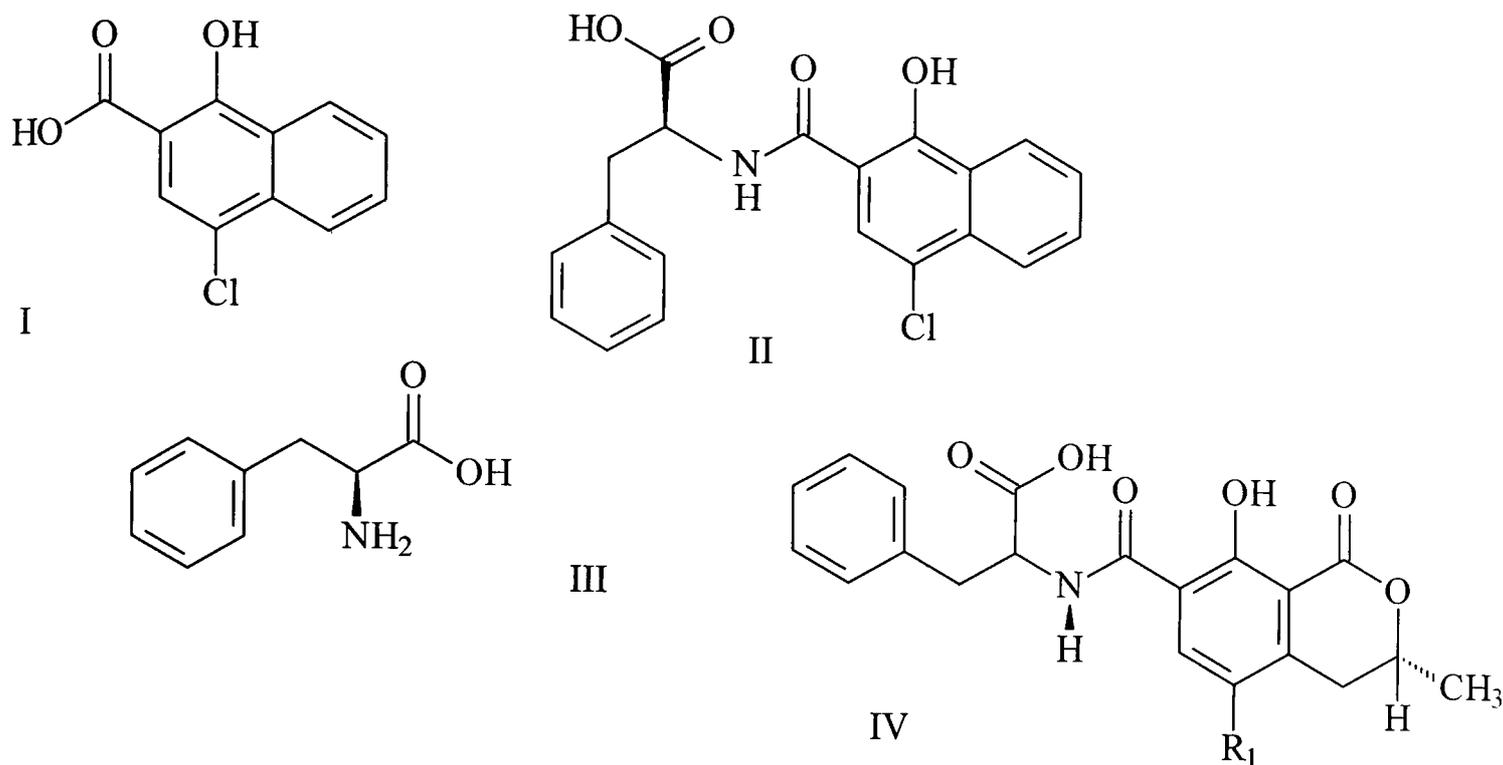


Figure 2.6: Analogues used for cross reactivity studies.

I: 4-chloro-1-hydroxy-2-naphthoic acid (CHNA). II: N-(4-chloro-1-hydroxy-naphthoylamido)-(L)-phenylalanine (L-Phe-CHNA). III: Phenylalanine (Phe). IV: Ochratoxin A (OTA).

2.3.11 Sensor design and analysis

This section is divided into two parts. The study of the polymer membranes with respect to grain, grape juice and wines samples is described, then the experimental attempts to isolate an extraction and identification protocol for SPE from grain is described.

2.3.11.1 Preparation of grain extract

The maize was milled and stored at -20 °C until needed. 1g of this was taken and placed within a test tube. 10 ml of MeCN was added to the ground maize and the mixture was shaken vigorously, and sonicated for 1 min and then

filtered through No.4 Whatman paper. The collected extract was then used for subsequent binding experiments. For a binding study extract was spiked with 100 ppb OTA.

2.3.11.2 Membrane study with model solutions

The MIP membrane (polymer A) fluorescence was measured before loading to obtain a background level as described in section 2.3.6.2. This membrane was allowed to soak in a fresh acetonitrile for 20 mins and then re-measured. This was then repeated with a fresh membrane. The whole experiment was repeated with a MIP of polymer B.

2.3.11.3 Membrane studies with wine and grape juice

Utilising the protocol as described in 2.3.6.2, the binding of OTA to the polymer A membrane in a white wine and in a white grape juice was performed. The membrane was measured before to obtain a background level and allowed to soak in fresh white wine for 20 mins and then re-measured. This was then repeated with a fresh membrane and wine spiked with 100 ppb OTA. The whole experiment was repeated with a blank membrane of Polymer A. This whole experiment was then repeated with white grape juice.

A further experiment was performed to isolate the lower detection limit. Using the same method as described in section 2.3.6.2 the experiment was repeated with a MIP membrane of Polymer A using a range of OTA concentrations.

2.3.11.4 Membrane study with grain extract

The MIP membrane fluorescence was measured before loading to obtain a background level as described in section 2.3.6.2. The membrane of polymer B was allowed to soak in a fresh extract as prepared in section 2.3.11.1 for 20 mins and then re-measured. This was then repeated with an extract spiked with 100 ppb OTA.

This was then repeated with a blank of polymer B and a MIP, which had not undergone the UV burning, to act as a control.

2.3.12 SPE experiments in grain samples

2.3.12.1 Identification of suitable materials for extraction of OTA from grain

Several different materials were tested for extraction suitability of OTA from maize extract:

Commercial SPE ion exchange cartridges (IEX) (50 mg) (4 types)

50 mg of kieselguhr silica (Celite 545)

50 mg of activated carbon /activated aluminium oxide (1:1)

30 mg cartridge of the polymer B blank

Each cartridge was conditioned with 1 ml methanol, and then 1 ml acetonitrile.

A series of experiments were performed on each material to prove its suitability for OTA extraction.

3 ml of 100 ppb OTA solution in acetonitrile was loaded onto the cartridge, the eluate was collected and its fluorescence measured. A 1 ml acetonitrile wash was performed and then the cartridge was washed with 3 ml of 100 mmol pH

7.5 phosphate buffer. This was collected and measured for fluorescence using the method described in. This analysis was repeated in triplicate.

The same protocol was repeated using an extract of maize, and an extract spiked with 100 ppb OTA. An experiment was repeated with 1% Sodium dodecyl sulfate (SDS) added to the elution step was also performed.

2.3.12.2 IEX extraction combined with MIP extraction

An NH₂ cartridge was conditioned with 1 ml methanol, and then 1 ml acetonitrile. 3 ml of acetonitrile was loaded onto the cartridge and then the cartridge was washed with 3 ml of 200 mM sodium phosphate buffer, pH 7.5. This sample was then spiked with 100 ppb OTA.

This solution was measured for fluorescence and then loaded onto a prepared cartridge of polymer A. The eluate was collected, and its fluorescence measured. A 1 ml 200 mM sodium phosphate buffer, pH 7.5 was used to wash cartridge and then the cartridge was washed with 3 ml of water, which was collected and measured for fluorescence.

This whole procedure was repeated with an extract of maize.

2.3.12.3 Study on the effect of the extract on binding of OTA to polymer A

Using the standard SPE protocol as described in Section 2.3.9 the following samples were tested to provide insight into the effect of the extract on polymer A. 3 ml of 100 mmol pH 7.5 phosphate buffer, spiked with lecithin (1%) (a phospholipid) and 100 ppb OTA was prepared and loaded on the polymer A MIP cartridge. The binding and release were calculated.

Attempts with other surfactants (Tween 20 and SDS) were made but were not suitable due to interference with the fluorescent signal.

Chapter 3

Experimental Results

3.1 Introduction

This chapter is split into two sections. The first describes the design, synthesis and characterisation of polymeric materials, specific for OTA. The second describes the use of these materials in development of protocols for use as sensors and in solid phase extraction. The results are presented in order following the experiments laid out in the materials and methods section.

3.2 Polymer Characterisation

3.2.1 Molecular Modelling

The choice of monomers and determining their optimal ratio in monomer mixture is one the most important aspects of the imprinting process. Traditionally, scientists selected monomers by analysing the chemical features of template and trying to match them with complementary functionalities of corresponding monomers.

As described in Section 2.3.1 a protocol utilising molecular modelling software to predict the interactions between template (OTA) and monomers was used to identify and optimise the composition of two polymers for high affinity and specific recognition of OTA.

3.2.1.1 *Design of molecular model of OTA*

A 2D structure of the template was drawn using the sketch molecule command in the Sybyl software and was translated into a 3D representation of the molecule (Figure 3.1). The chemistry of the template was checked to

ensure that the program had correctly identified the type of chemical groups and chemical bonds present in the molecule and that the chirality is correct. It was found that the automatic charge assignment for the -COOH group was incorrect, with the charge unevenly spread between the two oxygen atoms. Also the same problem was found in the cyclic structures in the dihydroisocoumarin portion of the molecule. Using the atom assignment function with the SYBYL 6.9 software the correct charge assignments were given.

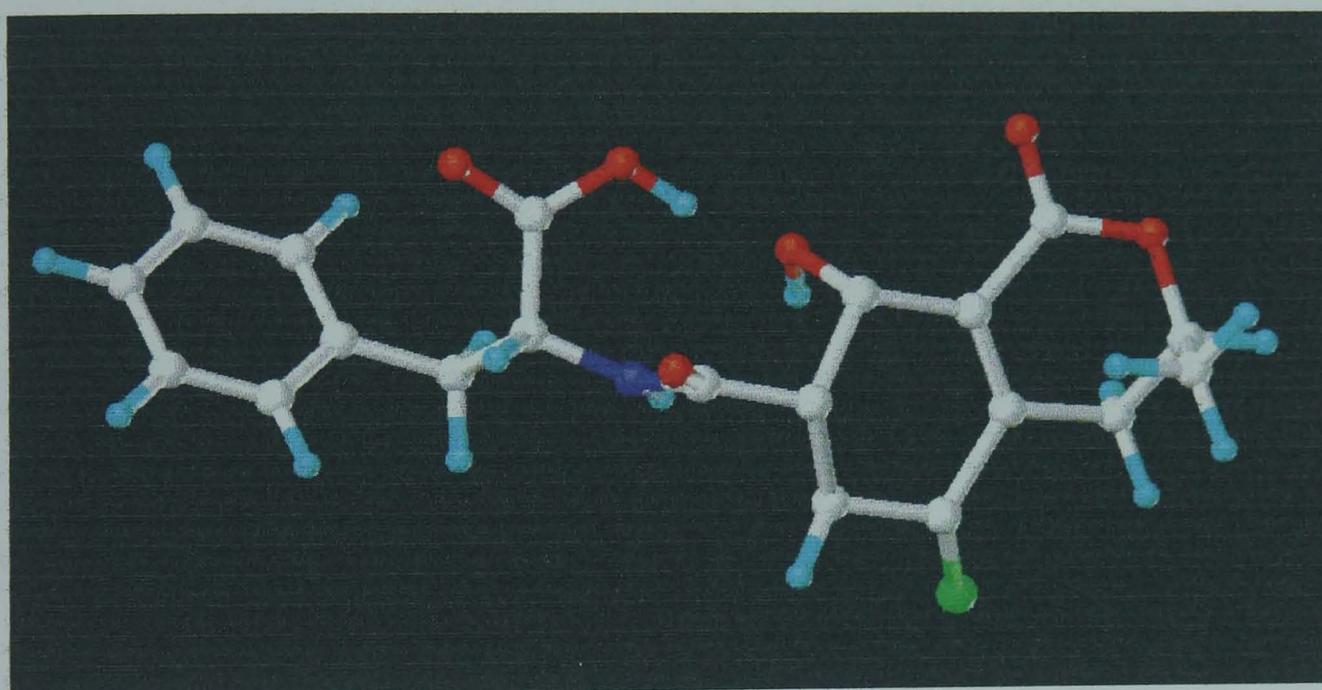


Figure 3.1: A minimised 3D representation of ochratoxin A, as simulated by the SYBYL 6.9 molecular modelling software.

The molecule was then refined by energy minimisation, as described in Section 2.3.1. The resulted conformation of OTA, presented was then used for all further modelling experiments.

3.2.1.2 *Rational design of polymer using molecular modelling*

A computer-aided rational design was used to optimise the composition of a molecular imprinting polymer. After the creation of a virtual library containing both charged and uncharged functional monomers, Leapfrog was applied to screen the library: each of the monomers was probed for the possible interaction with OTA. The results of Leapfrog screening in a vacuum (dielectric constant =1) are reported in Table 3.1. The positioning of the monomers interactions with respect to OTA are portrayed in Figure 3.2a and 3.2b. From these results two non-charged monomers were selected (methacrylic acid and acrylamide) which were shown to have reasonable binding scores to the template. Having charged monomers such as vinylimidazole in intended experimental conditions would be highly unlikely. The methacrylic acid was seen twice in the chart however the uncharged form is more likely to be seen in DMF, The polymerisation solvent, in comparison to the negatively charged form.

The process was repeated using a higher dielectric constant (37.5) in the calculations to mimic the effects of the presence of acetonitrile. The results are shown in Table 3.2. Two monomers – allylamine and DEAEM gave the highest binding score. Unfortunately it was found that allylamine has poor inclusion into a growing polymer (<30%). From this and the initial Leapfrog experiment DEAEM was selected as a monomer for practical work. The positioning of the DEAEM monomer interaction with respect to OTA is portrayed in Figure 3.3.

Table 3.1: The binding scores between OTA and monomers in a vacuum (dielectric constant = 1).

Abbreviations: DEAEM (diethylamino) ethylmethacrylate; EGDMA (ethylene glycol dimethacrylate); 1-VI (1-Vinylimidazole).

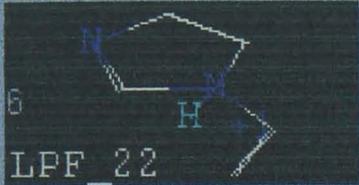
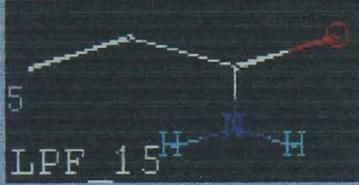
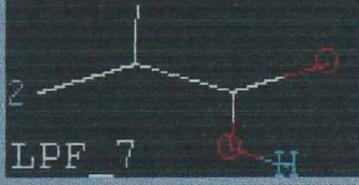
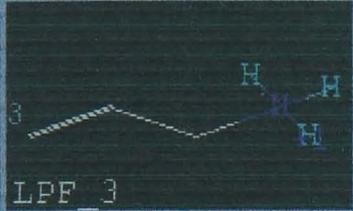
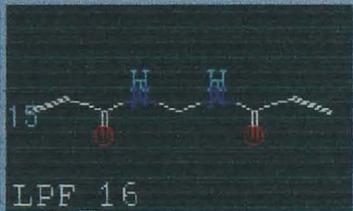
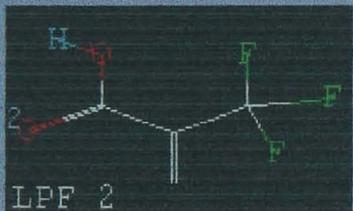
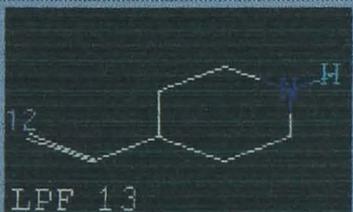
	1: SLN	2: BINDING
 <p>6 LPF 22</p>	+1-VI	-59.23
 <p>5 LPF 15</p>	Acrylamide	-35.32
 <p>4 LPF 14</p>	+DEAEM	-30.59
 <p>2 LPF 7</p>	Methacrylic	-20.99
 <p>8 LPF 26</p>	DEAEM	-10.96
 <p>1 LPF 5</p>	1-VI	-9.41
 <p>3 LPF 9</p>	EGDMA	-7.31
 <p>7 LPF 24</p>	-Methacrylic	-6.48

Table 3.2: The binding scores between OTA and monomers in acetonitrile (dielectric constant = 37.5).

1: (+) Allylamine. 2: (+) DEAEM. 3: N,N-methyl bisacrylamide (Bis). 4: Tri-fluoroacetic acid (TFA). 5: 4-vinylpyridine (4-VP). 6: 2-vinylpyridine (2-VP).

	1: SLN	2: BINDING
 <p>LPE_3</p>	$N[+1]H_3CH_2CH=CH_2$	-174.90
 <p>LPE_20</p>	$N[+1]H(CH_2CH_2OC(=O)C$	-81.58
 <p>LPE_16</p>	$NH(CH_2NHC(=O)CH=CH_2$	-48.81
 <p>LPE_2</p>	$FC(F)(F)C(=CH_2)C(=O)O$	-39.63
 <p>LPE_13</p>	$N[1]HCH:CH:C(CH:CH@1$	-35.21
 <p>LPE_9</p>	$N[1]HC(CH:CH:CH:CH@1$	-33.06

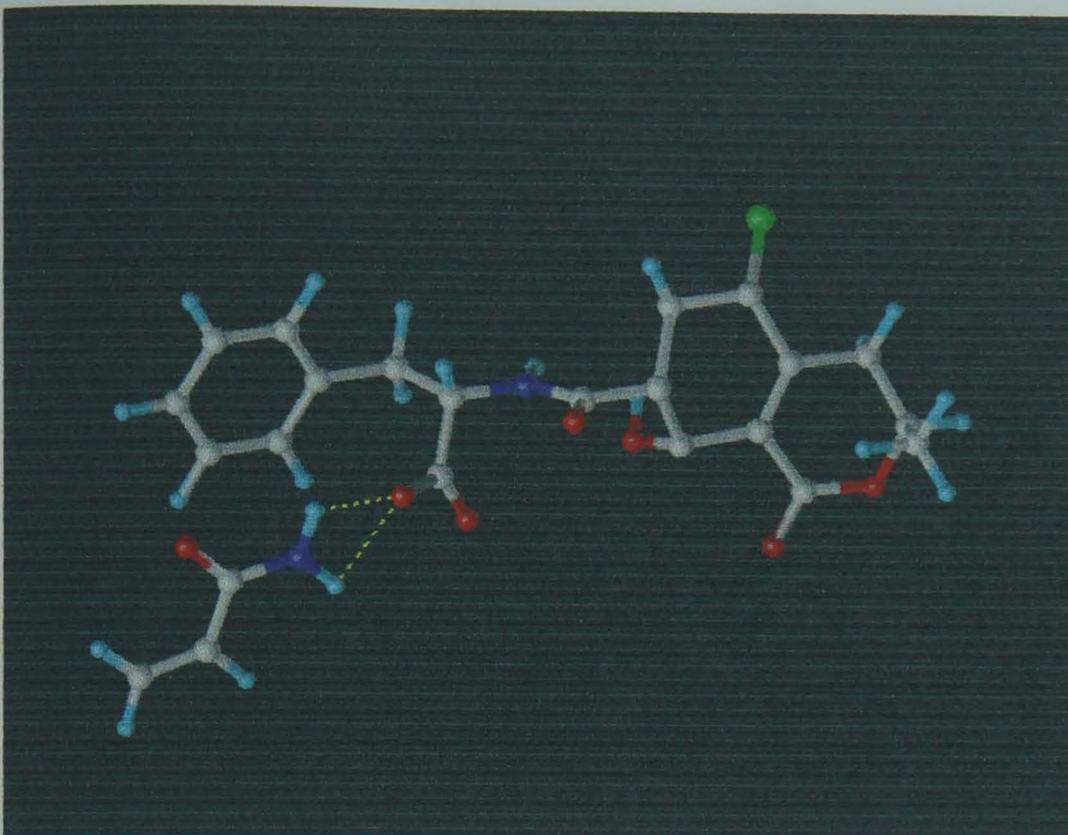


Figure 3.2a: Predicted interaction between OTA and acrylamide from Leapfrog screening performed in vacuum. The yellow dotted lines signify hydrogen bonding between templates.

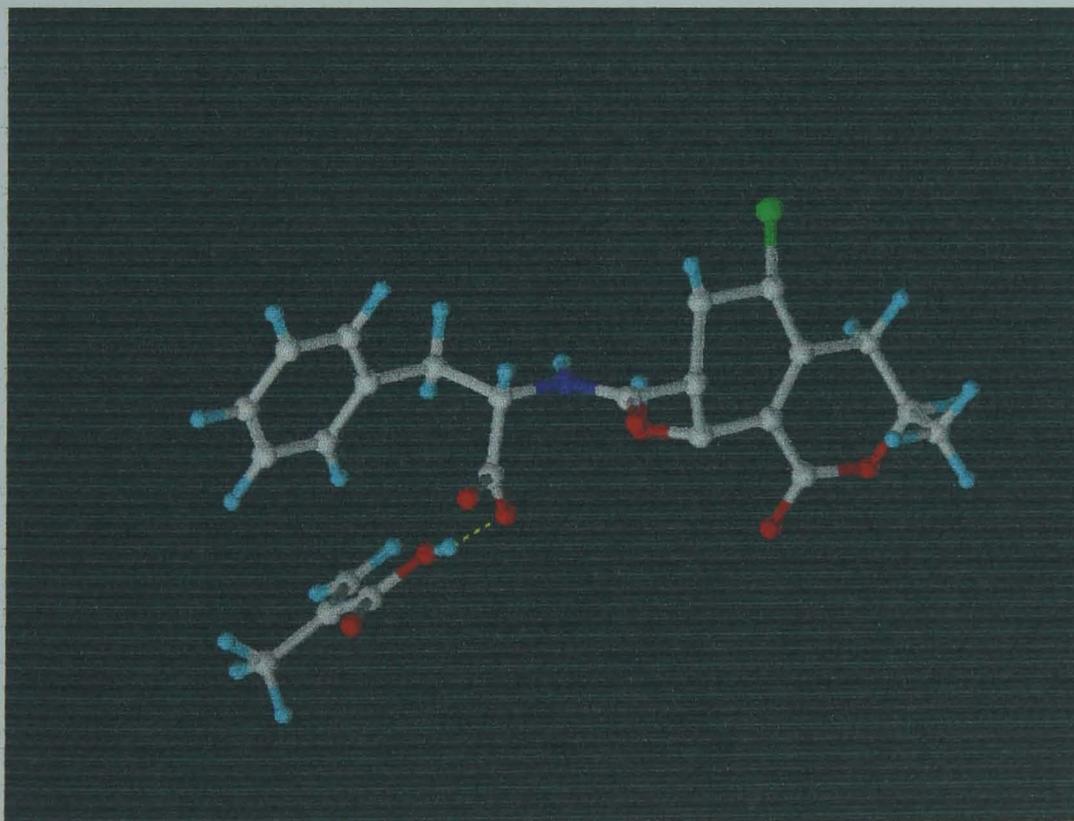


Figure 3.2b: Predicted interaction between OTA and methacrylic acid from Leapfrog in vacuum. The yellow dotted lines signify hydrogen bonding between templates.

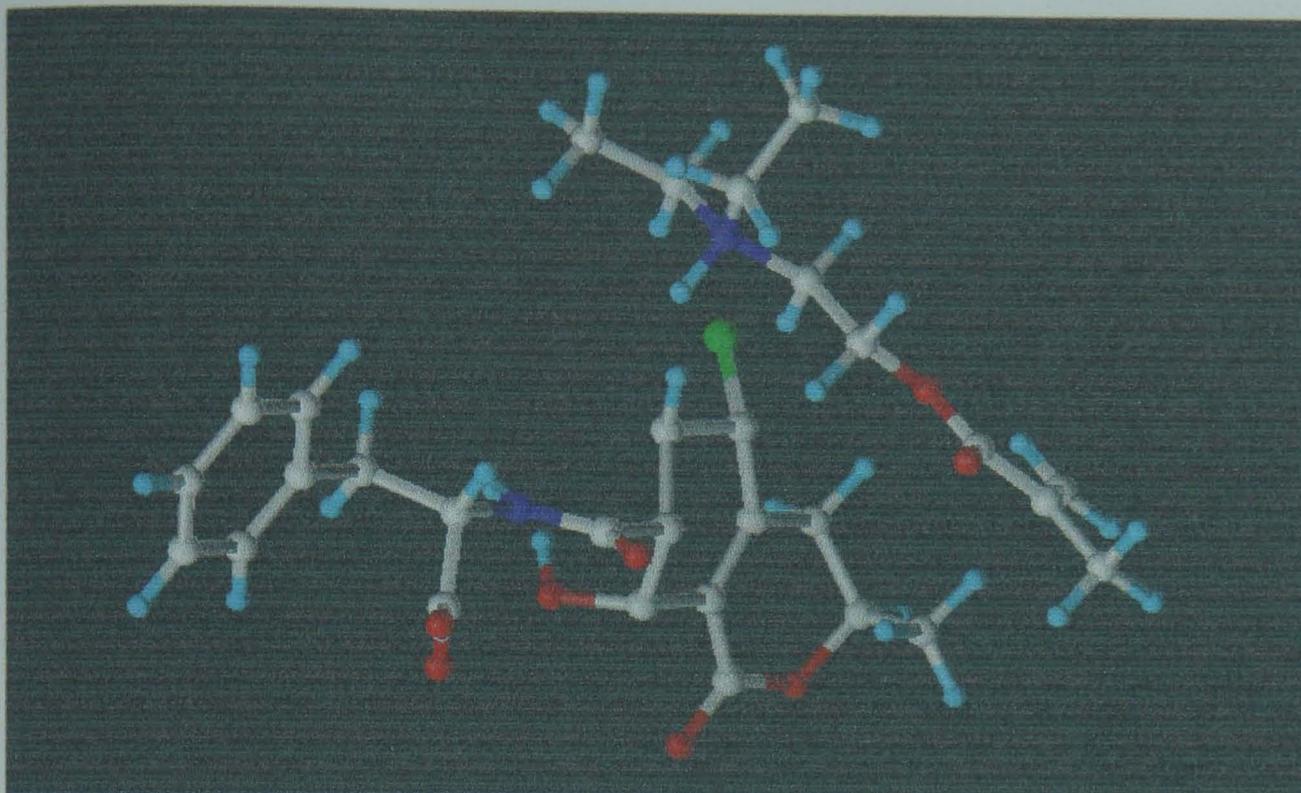


Figure 3.3: Predicted interaction between OTA and DEAEM from Leapfrog in acetonitrile.

The Leapfrog results are then used to simulate the pre-arrangement of functional monomers and template in the mixture prior to polymerisation, multiple copies of the chosen monomers were placed around OTA using solvation experiments. Then, an energy minimisation of the obtained box was applied (Figure 3.4).

The simulated annealing process as described in section 2.3.1.4 was applied to the minimised box as shown in Figure 3.4, to obtain the predicted monomer/template ratio. The results of this are described in Figures 3.5.

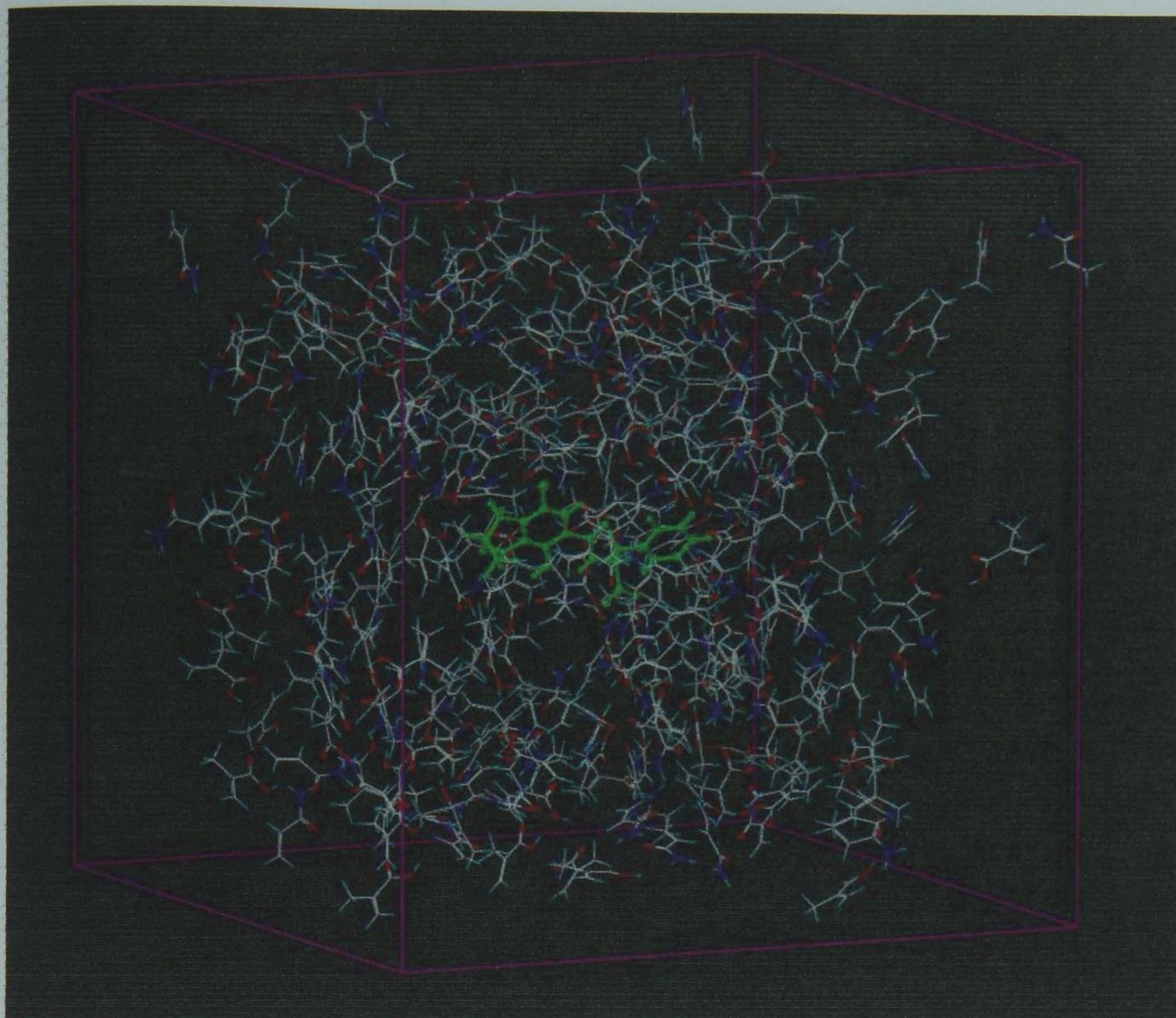


Figure 3.4: Example of the refining step solvation box. OTA (green) surrounded by methacrylic acid and acrylamide molecules in solvated box. This system has been minimised.

The complex between OTA and the first shell layer of monomers consists of one methacrylic acid molecule and one acrylamide molecule (Figure 3.5). Thus the predicted ratio of monomers is (1:1:1 OTA: Acrylamide: Methacrylic acid). This result was then used for polymer synthesis as described in section 3.2.4.

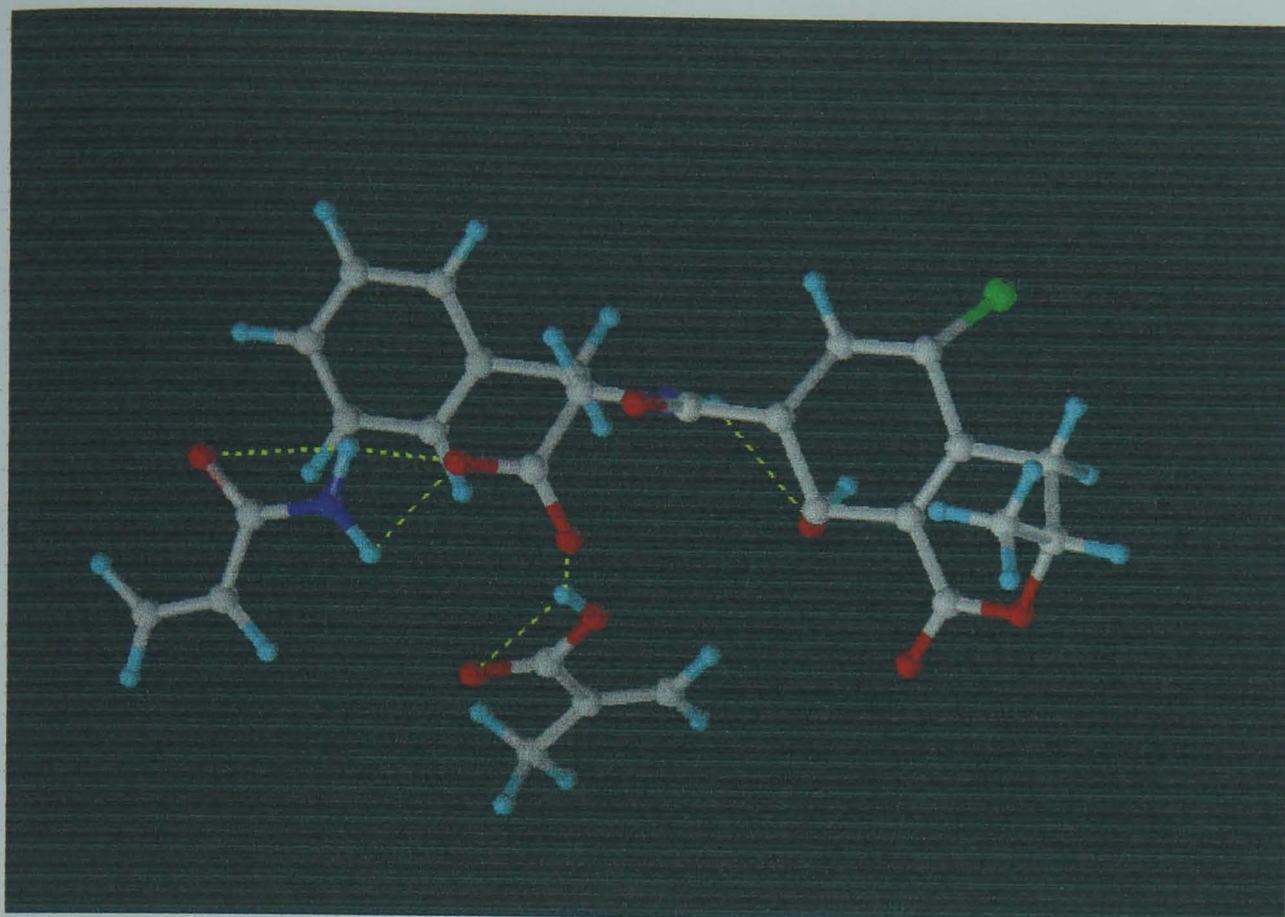


Figure 3.5: Final result of refining step. This figure shows the predicted interactions between OTA, acrylamide and methacrylic acid.

The refining step for DEAEM was performed exactly as the previous experiment. The final result of this experiment is shown in Figure 3.6.

The complex between OTA and the first shell layer of monomers consists of four DEAEM molecules (Figure 3.6). However, previous work based with other polymers (data not shown) had demonstrated that an excess of strong anion-exchange monomers in the polymer led to high non-specific binding, which complicated the elution step in SPE. This possibility was supported also with the NMR data (shown in section 3.2.2). Thus the decision was made to use a ratio of monomers of (1:1 OTA: DEAEM), initially to test this theory and if required to increase the ratio if the polymer proved successful.

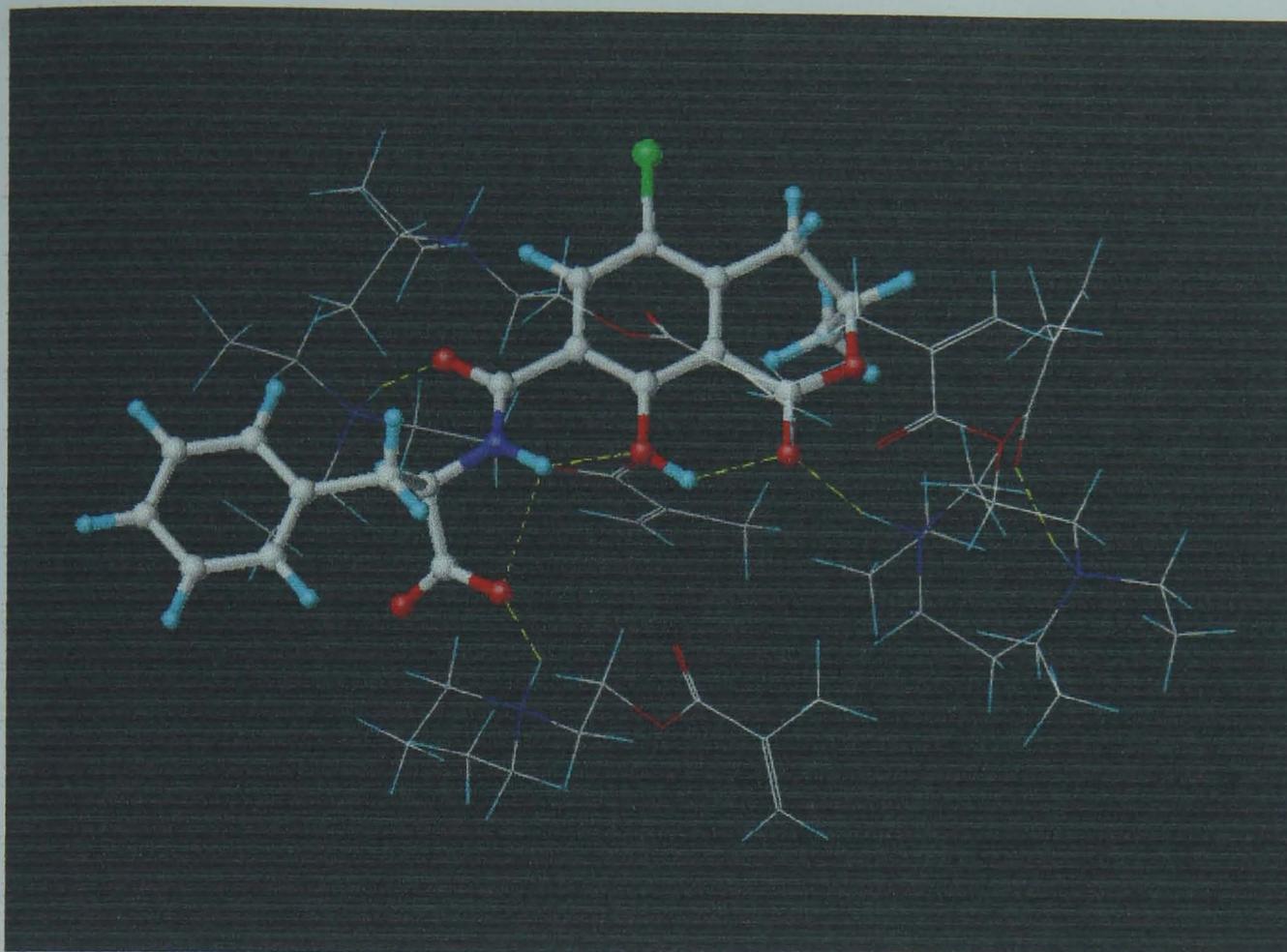


Figure 3.6: Final result of refining step. This figure shows the predicted interactions between OTA and DEAEM.

3.2.2 Nuclear magnetic resonance

The nuclear magnetic resonance (NMR) experiments were used to verify the interactions of the chosen monomers and OTA in acetonitrile (the solvent used for extraction and potentially in sensing). Results from binding experiments of both polymers shown throughout this chapter, demonstrated that in this solvent the polymer B (based on DEAEM) bound OTA, but polymer A (methacrylic acid and acrylamide) did not. Therefore the aim was to analyse the nature of interactions for future optimisation of binding conditions.

The peak designation of OTA is described in Table 3.3, using the notation in Figure 3.7. Figures 3.8 and 3.9 show the interactions between OTA and

acrylamide and OTA and methacrylic acid respectively. From the comparison with standard OTA spectra above (Figure 3.8a and 3.9a) it is seen that there is no strong interaction taking place between the monomers and template in MeCN.

Figures 3.10a and 3.10b portray the interactions between DEAEM and OTA. The close up section of the NMR spectra shows the shift of a peak (designated peak 2). Clearly a strong interaction is shown to exist between these two molecules. This suggests that the DEAEM interacts with the COOH, causing a shift in the peak at position 2.

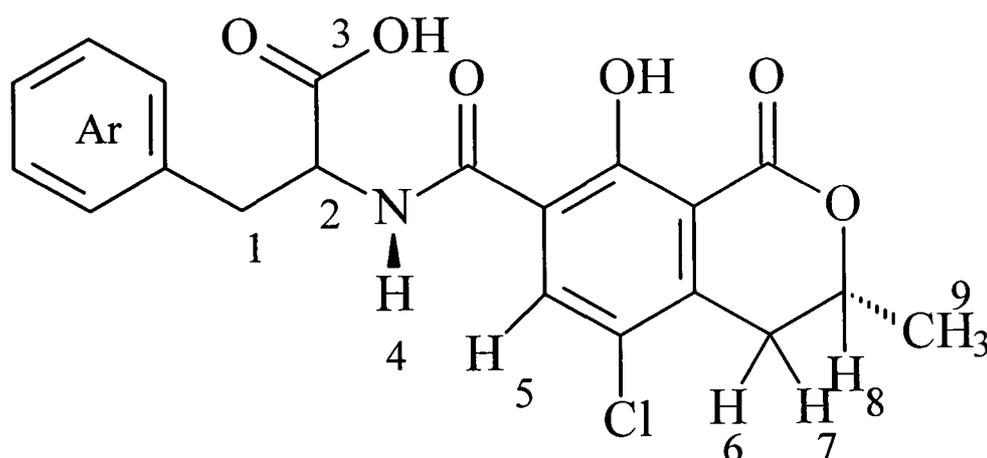


Figure 3.7: OTA, numbered for NMR identification.

Table 3.3: Peak designation of OTA (270 MHz). Based on the numeration in Figure 3.7.

ppm	Notation	Number
1.49	3H, d, CH ₃	9
2.87	1H, dd, CH	6 or 7
3.14	1H, dd, CH	6 or 7
3.26	2H, dd, CH ₂	1
4.7 – 4.9	2H, m, CH + CH	2/8
7.3 – 7.4	%H, m, CH-Ar	Ar
8.32	1H, br d, NH	4
13.5	1H, s, COOH	3

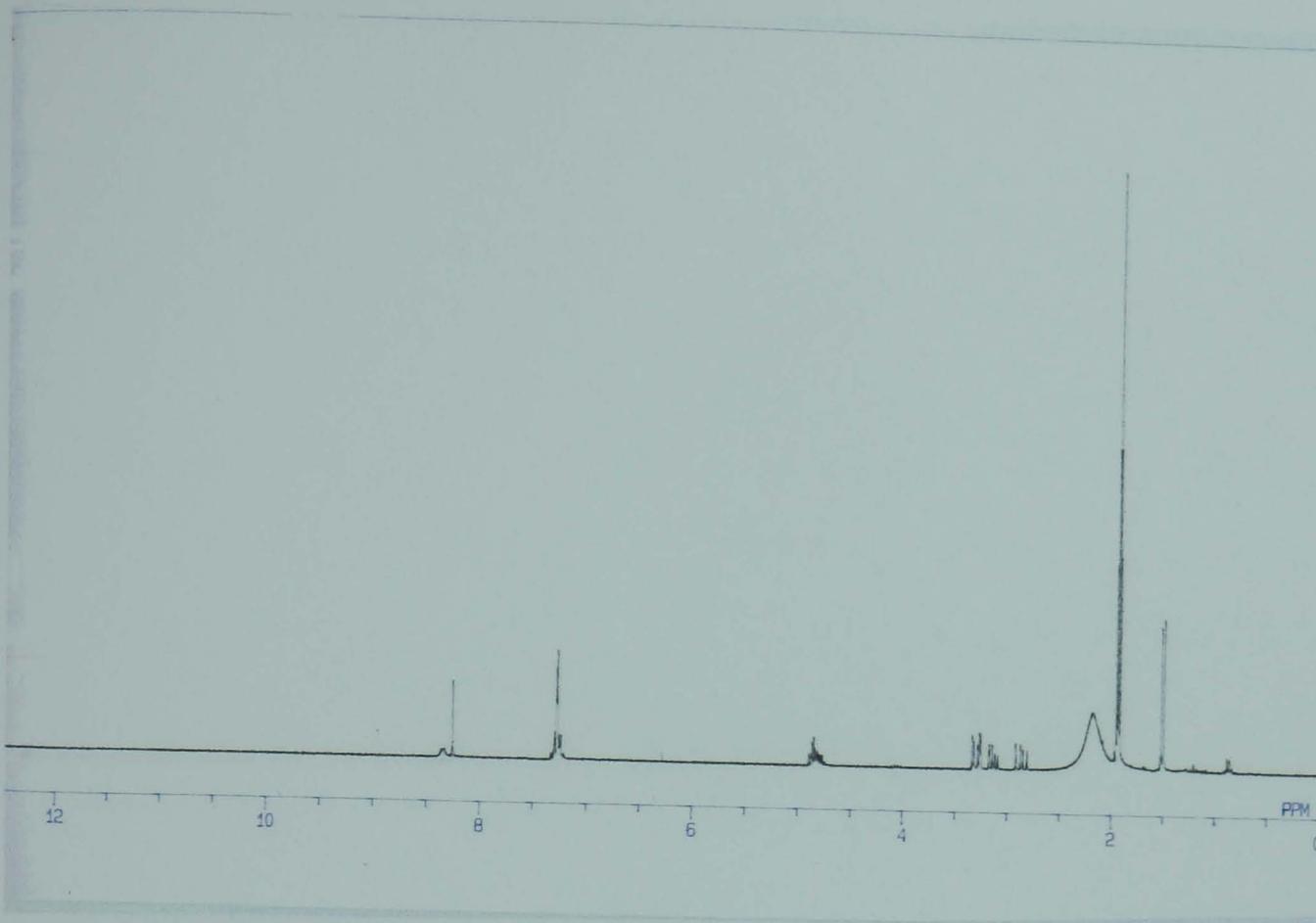


Figure 3.8a: NMR spectra at 270MHz of OTA in acetonitrile (MeCN).

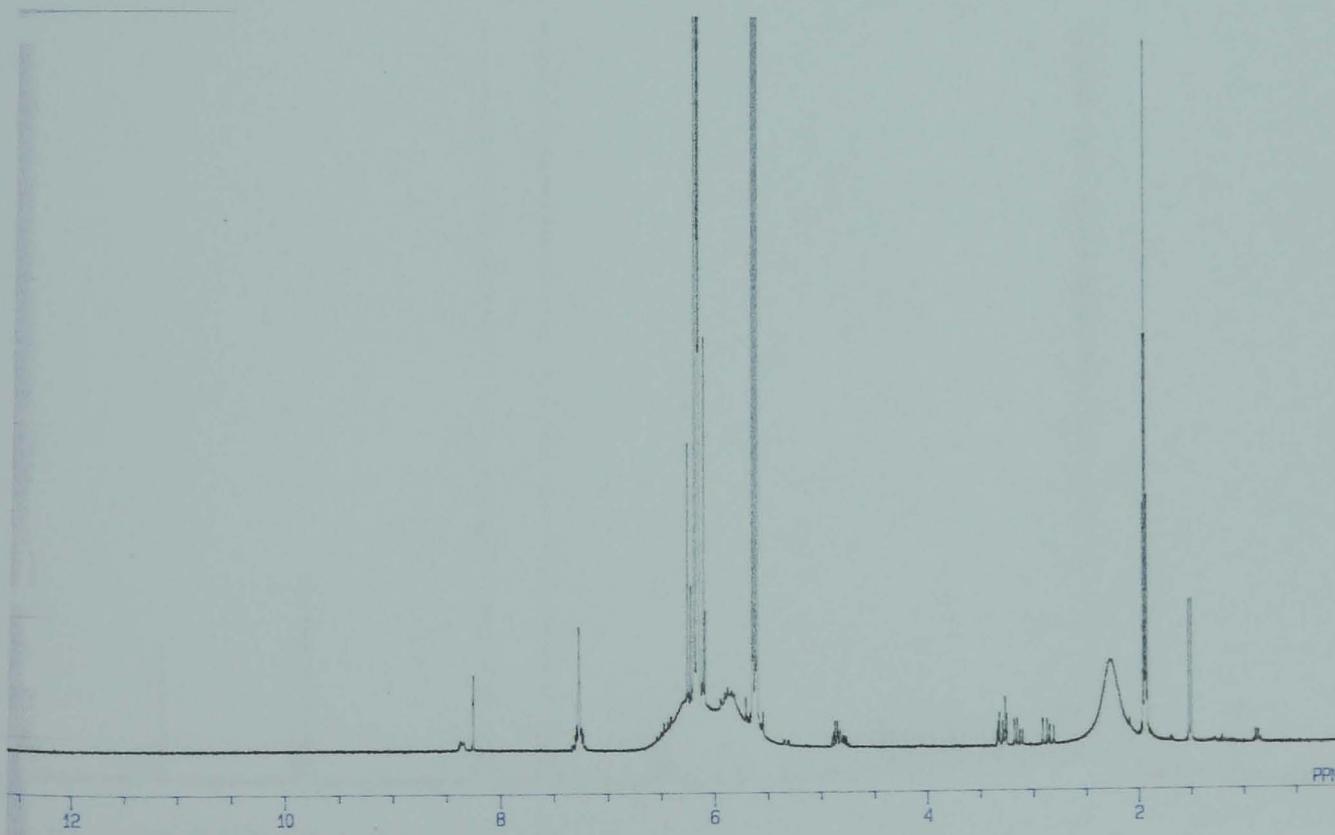


Figure 3.8b: NMR spectra of OTA and acrylamide in MeCN (1:5).

The twin large peaks in the centre of the figure (~6 ppm) represent acrylamide. The other peaks correspond as in Figure 3.8a for OTA.

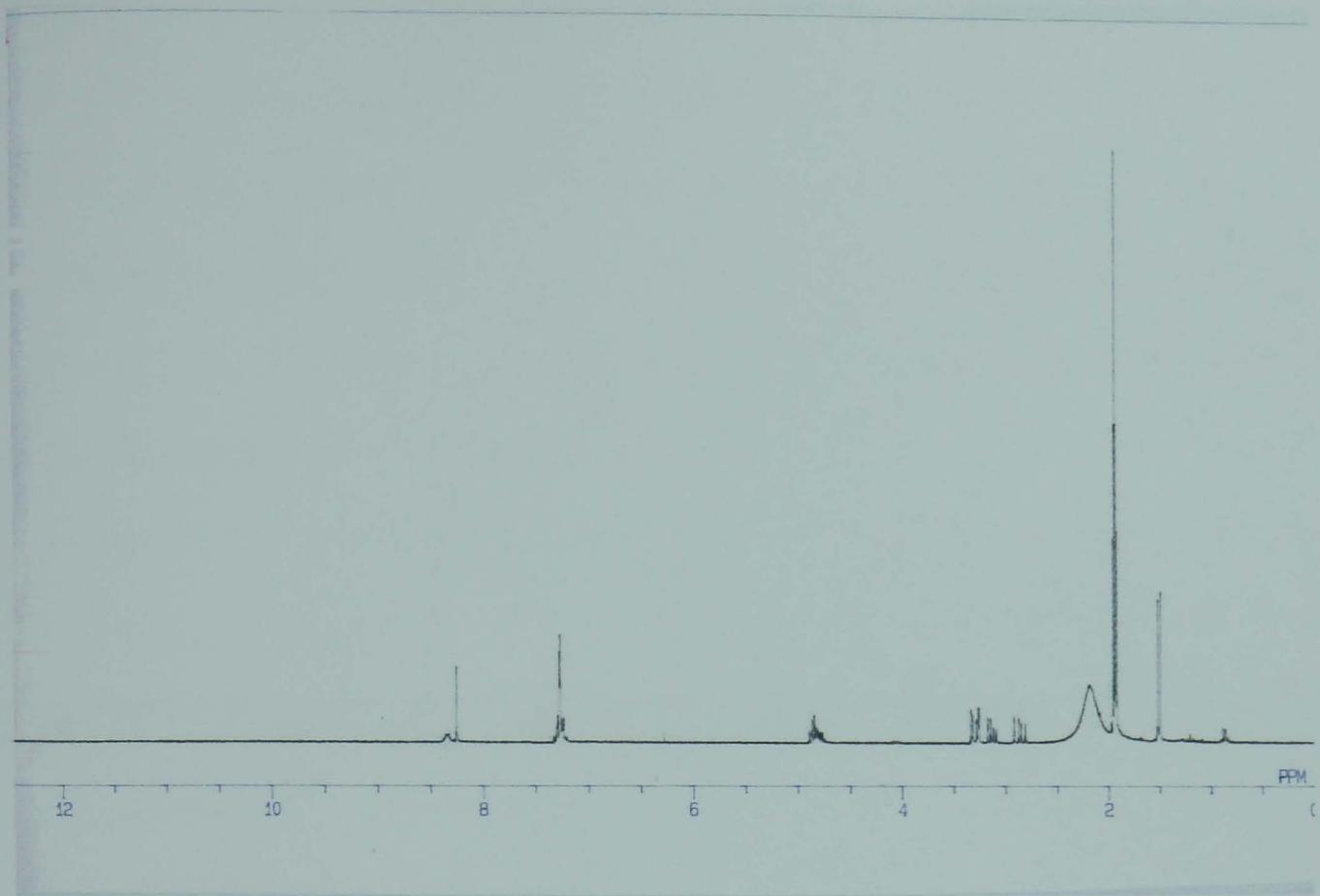


Figure 3.9a: NMR spectra at 270MHz of OTA in acetonitrile (MeCN).

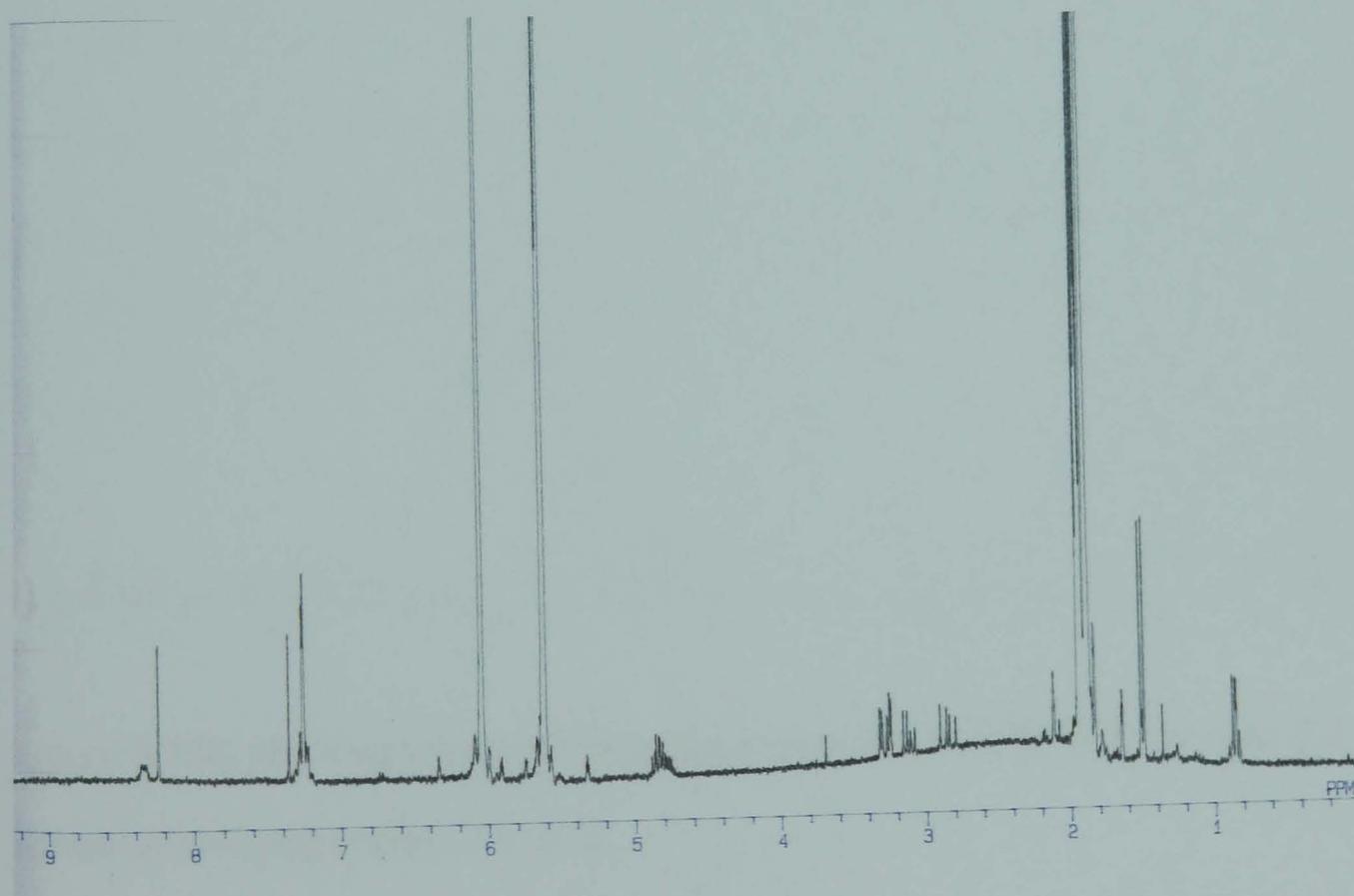


Figure 3.9b: NMR spectra of OTA and methacrylic acid in MeCN (1.5).

The twin large peaks in the centre of the figure (~ 6 ppm) represent methacrylic acid. The other peaks correspond as in Figure 3.9a for OTA.

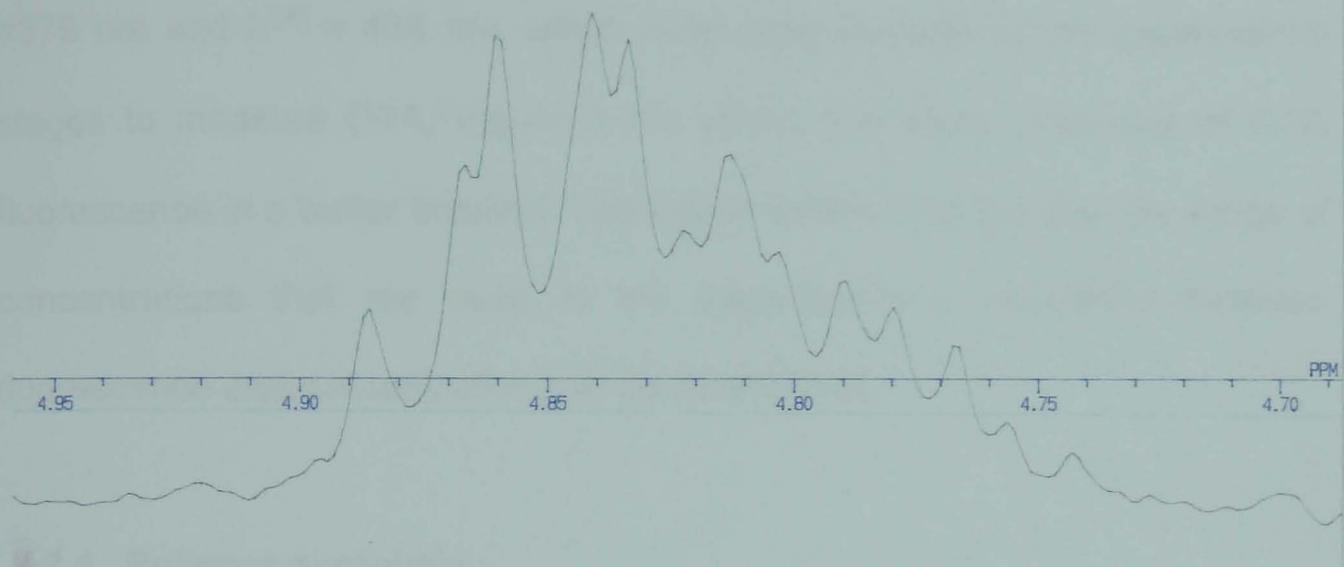


Figure 3.10a: NMR spectra of OTA (close up showing groups 2 and 8).

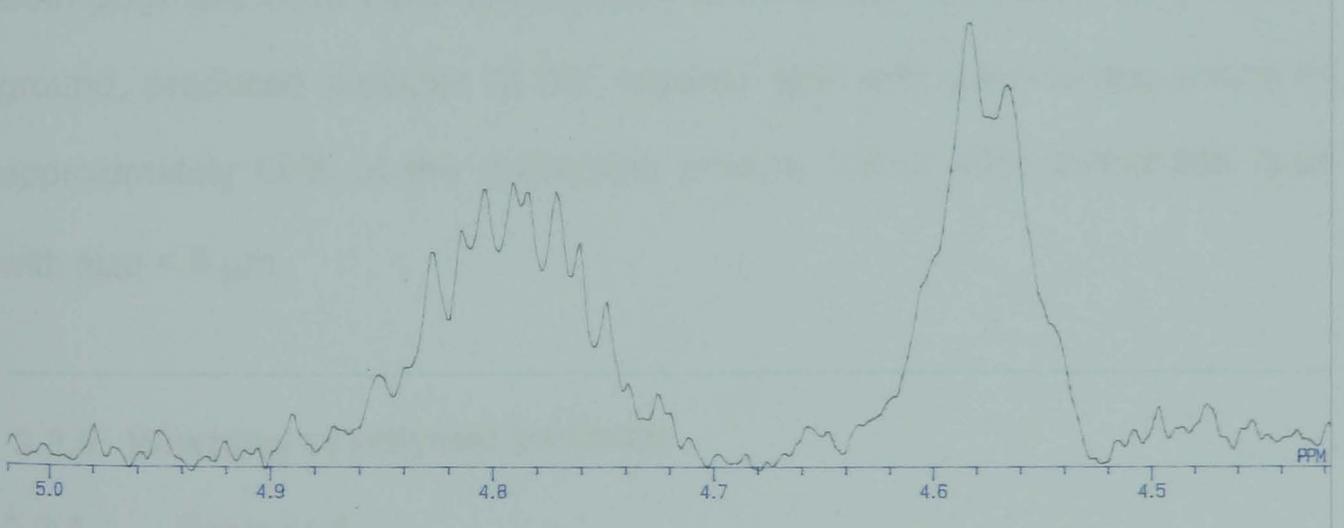


Figure 3.10b: NMR spectra of OTA in the presence of DEAEM in the ratio 1:4 (close up showing groups 2 and 8).

3.2.3 Measurement of OTA by fluorescence

Initially it was required to identify the emission and excitation wavelength of OTA in solution. Using the protocol as described in 2.3.6.2 we identified the maximums for both (Figures 3.11a and 3.11b). These were identified as $\lambda^{\text{Ex}} = 379 \text{ nm}$ and $\lambda^{\text{Em}} = 434 \text{ nm}$, which were used throughout the experimental stages to measure OTA. Figure 3.11c shows the linear properties of OTA fluorescence in a buffer solution. This demonstrates that over the range of concentrations that are used in the experiments a correlation between fluorescence and concentration can be determined.

3.2.4 Polymer synthesis

Two polymers were synthesised according to the modelling results by using protocols described in section 2.3.2.

Both polymers were hard semi-transparent monolith structures, which, when ground, produced particles of the required size with yield corresponding to approximately 60% of the theoretical amount. Other 40% constituted fines with size $< 5 \mu\text{m}$.

3.2.5 Washing of polymer particles

3.2.5.1 Polymer A

During the experimental testing it was found that methanol and water elute loaded OTA efficiently. Some amount of OTA was eluted from the polymer during grinding and sieving, which complicated measurement of the precise quantity of eluted template. The packed cartridges were shown to have slight fluorescence under UV light, suggesting that some OTA remained trapped in

the polymer matrix. Comparable experiments described in the literature suggest >90% of the template is removed (Mena *et al*, 2002).

Very little amount of OTA were still detected in elution buffer after intensive washing, which corresponded to <1 ppb. This relatively low leaching was acceptable for practical goals for both sensor and SPE work.

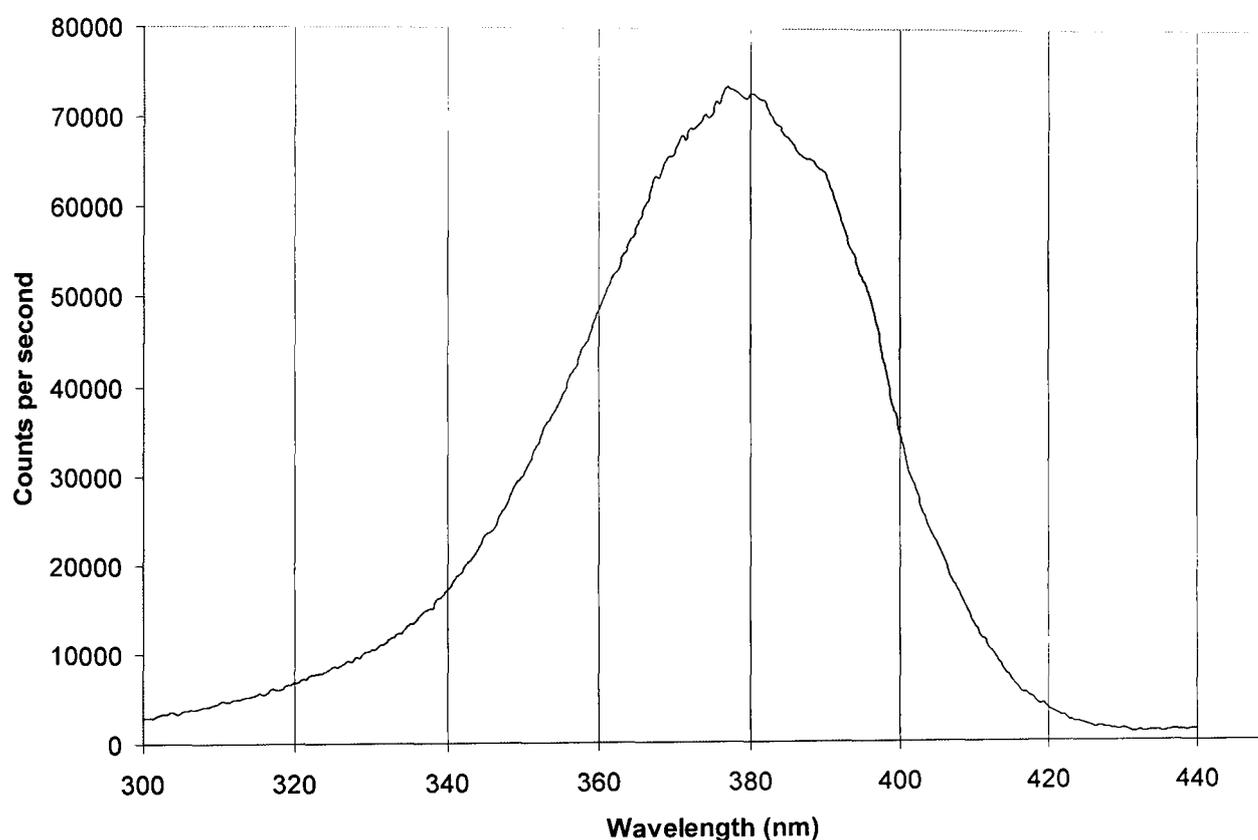


Figure 3.11a: Fluorescent emission spectra for OTA in 50 mM sodium phosphate buffer (pH 7.5).

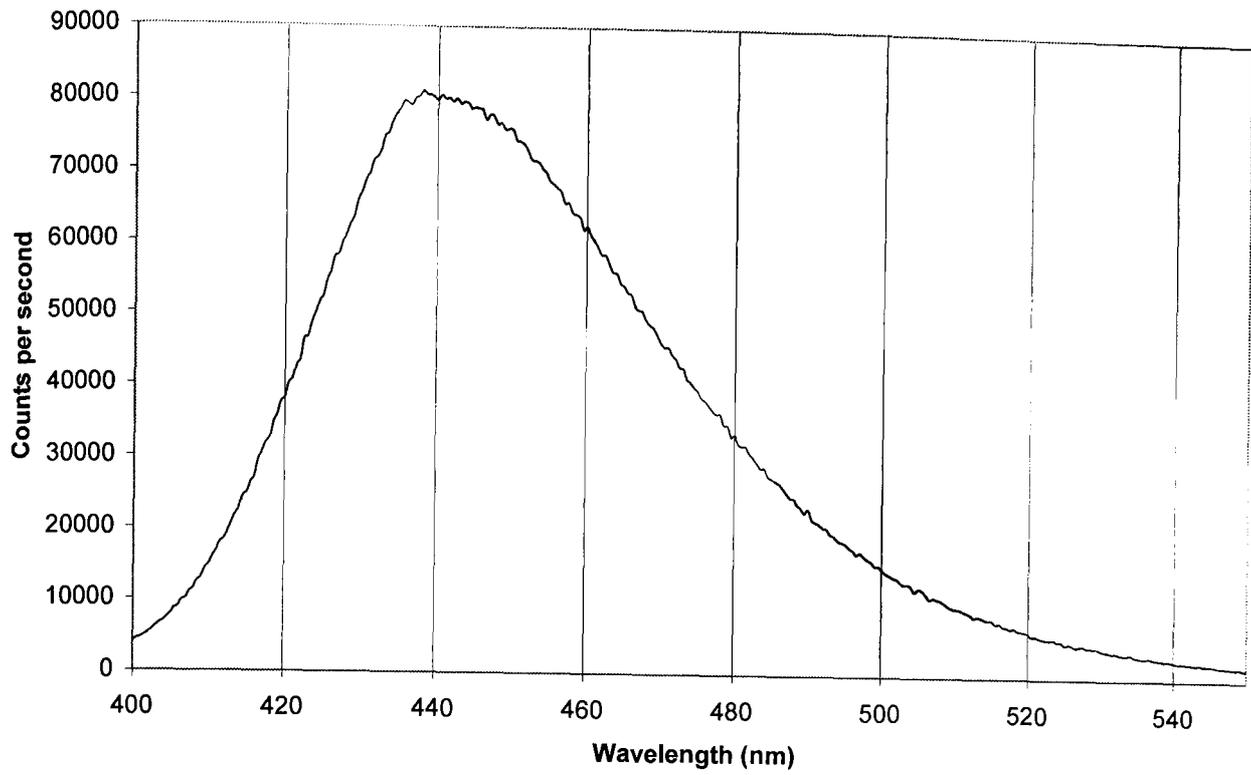


Figure 3.11b: Fluorescent excitation spectra for OTA in 50 mM sodium phosphate buffer (pH 7.5).

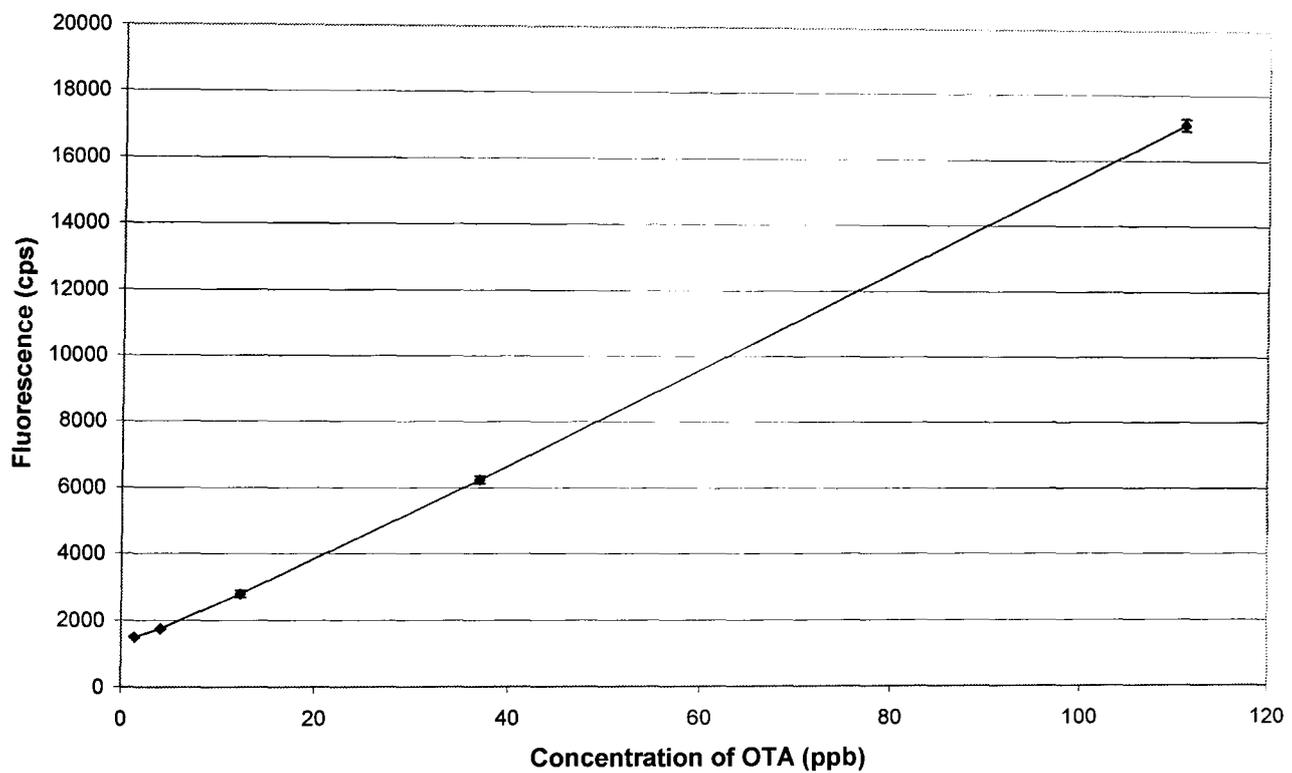


Figure 3.11c: Linear properties of OTA fluorescence in sodium phosphate buffer solution (100 mM, pH 7).

3.2.5.2 Polymer B

The same washing procedure as described in section 2.3.2 was performed to clean the polymer B. However unlike polymer A, a steady leaching of template equating to approximately 10 ppb OTA was continuously seen in all washes. This did not drop with an increased number of washes, suggesting that a large amount of template was still trapped in the material. Washes with acidified and basic solutions failed to improve the leaching problem, therefore an electrophoretic technique was employed (section 2.3.3.1).

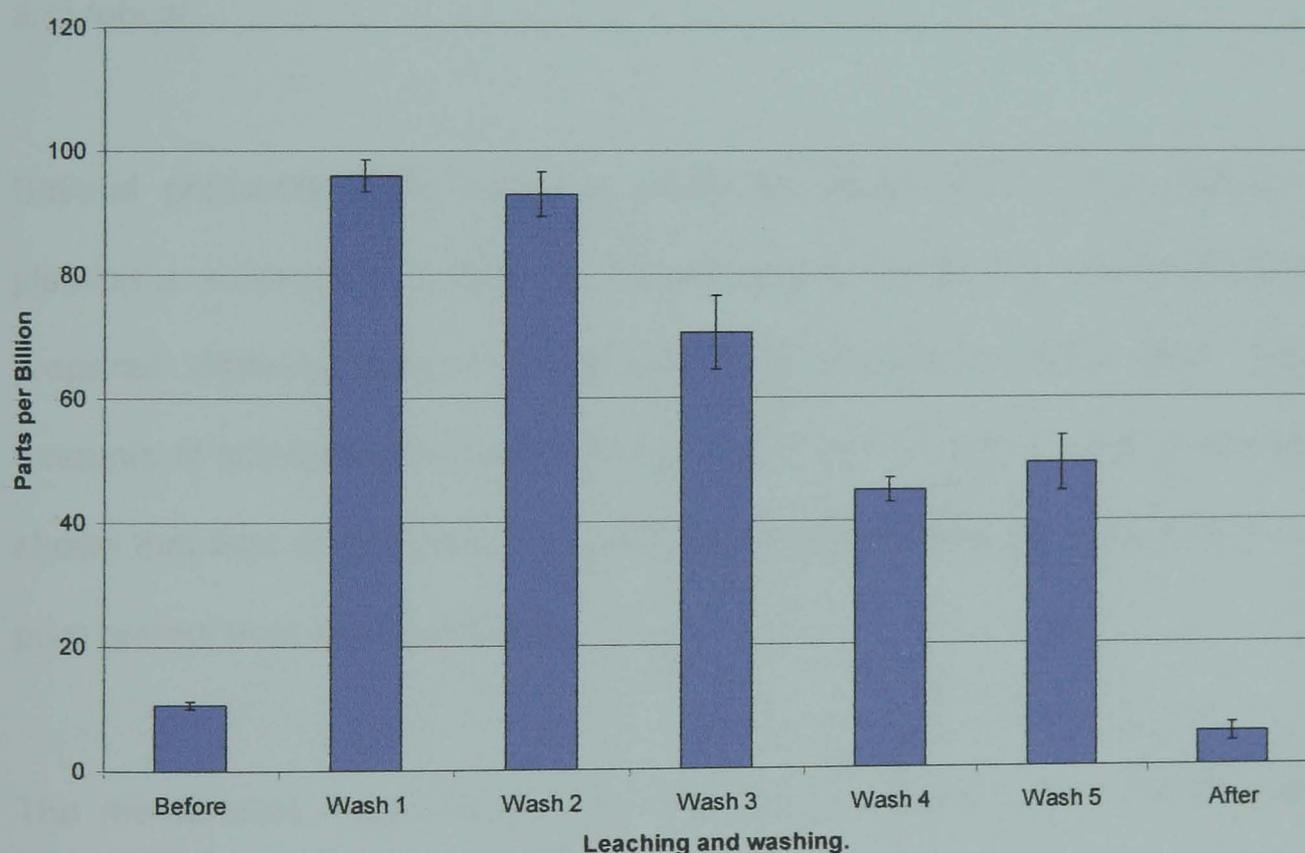


Figure 3.12: The presence of OTA in successive eluted solutions during electrophoresis, measured by fluorescence. Bars indicate standard error.

The washing solutions were collected in 3 ml aliquots and compared against a standard as described in section 2.3.6.3 (Figure 3.12).

This technique was successful in removing larger amounts of OTA from the MIP (Figure 3.12), however despite repeated washing the leaching of OTA continued on relatively large scale (>5 ppb). This leaching precluded the use of the polymer B MIP in the SPE applications; however the composition was still used in sensing.

3.2.6 Imprinted membrane production

Using the protocol described in section 2.3.4 several membranes were produced. The resultant materials were homogenous and optically transparent (possessing an even thickness across the material) as well as been flexible and robust.

Several protocols were tested in order to obtain the correct mixture of plasticiser, solvent and to find the correct polymerisation time. Some polymers prepared demonstrated a brittle nature if contained <15% OUA, lower amounts of solvents and if polymerised for longer periods of time. It was also shown that less solvent led to non-homogeneous membranes, possibly due to poor control over heat exchange.

The membranes were washed as described in section 2.3.5. Synthesised membranes still demonstrated high fluorescence due to trapped OTA within the membranes. Therefore each membrane was irradiated with UV to “burn” residual OTA and decrease the fluorescent signal.

Each membrane was exposed to the UV source and the kinetics of OTA decomposition was monitored fluorimetrically every minute for 10 minutes as described in Section 2.3.6.4. The results are shown in Figure 3.13.

After UV treatment each membrane was washed again in methanol and water, to remove any decomposed material from the polymer.

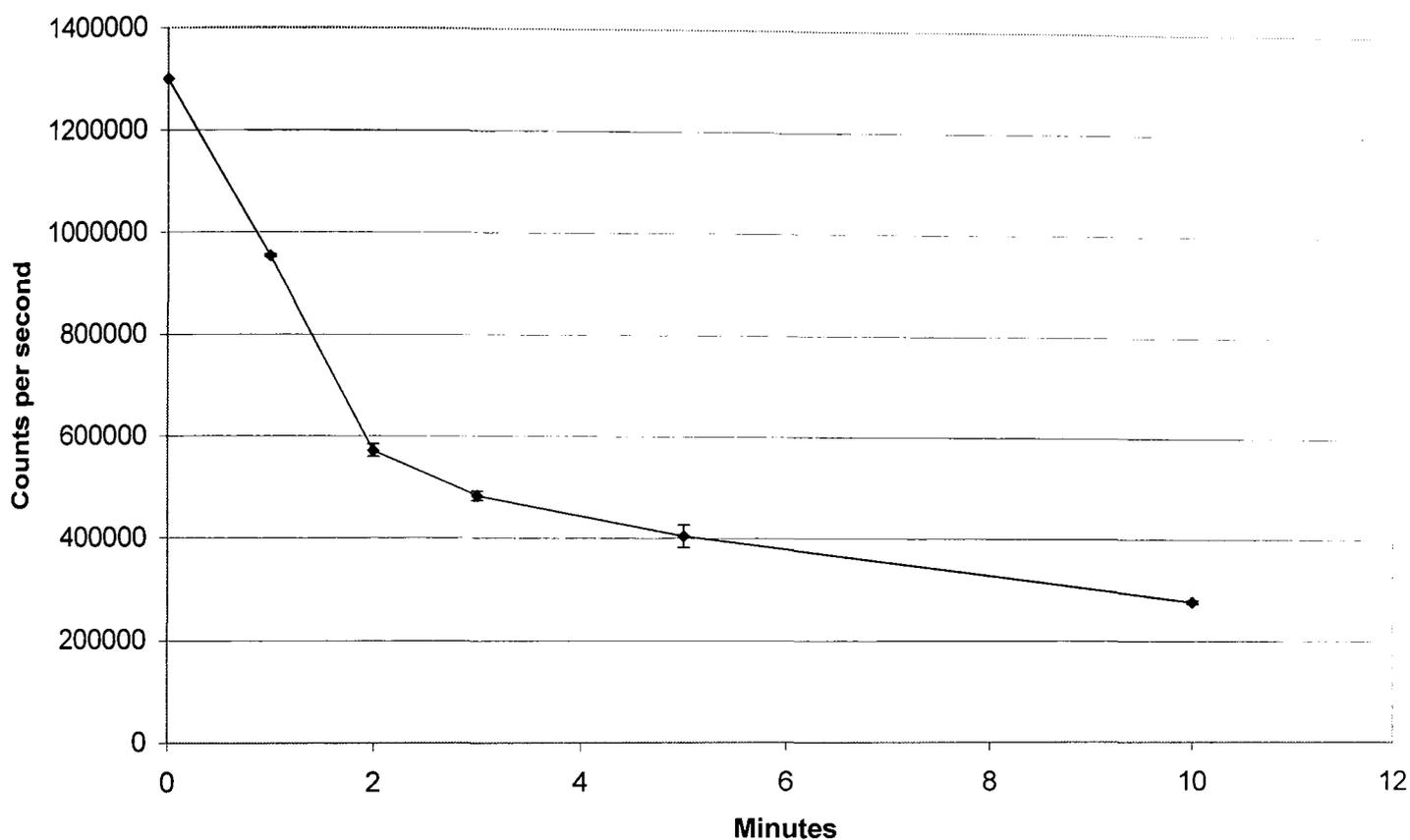


Figure 3.13: Degradation of OTA fluorescent signal with UV exposure, performed on polymer B membrane. Bars indicate standard error.

By “burning” membrane for 15 minutes we achieved a substantial reduction in membrane fluorescence, which allowed us to continue with sensor work.

3.2.7 Fluorescent analysis of OTA in membrane

Using the protocol described in section 2.3.6.4 the presence of OTA was detected in the free-standing membranes. Examples of the background fluorescence for both MIP and NIP of polymer A are shown in Figure 3.14. Clearly the residual OTA trapped in the polymer matrix affects the background signal however any OTA bound to the polymer can be measured by subtracting the baseline.

The baseline on the fluorescence of NIP membrane was not flat which can be attributed to reflectance of the excitation signal by the membrane. For comparative readings all following measurements were made at $\lambda^{\text{Em}} = 424 \text{ nm}$ for both MIP and NIP.

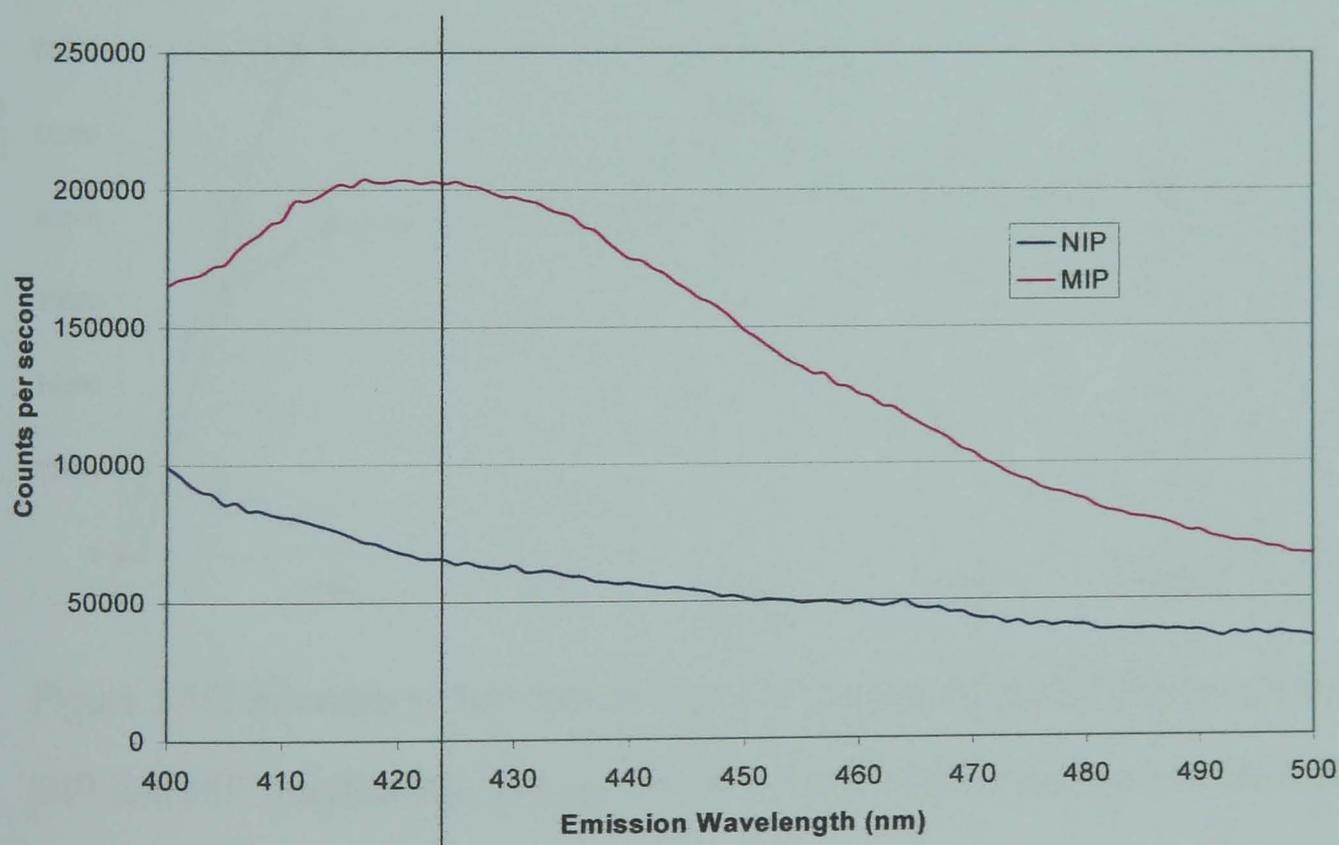


Figure 3.14: Examples of the background fluorescence for both MIP and NIP of polymer A. $\lambda^{\text{Ex}} = 379 \text{ nm}$. The line at 424 nm is used for comparative measurements.

The affinity of the same membranes was studied as described in section 2.3.6.5. The signal corresponding to at each concentration was measured at 424 nm and from this the value of the background signal for each membrane was subtracted to leave the signal caused by the bound OTA. These were then plotted to demonstrate the difference in binding affinity for MIP and NIP (Figure 3.15).

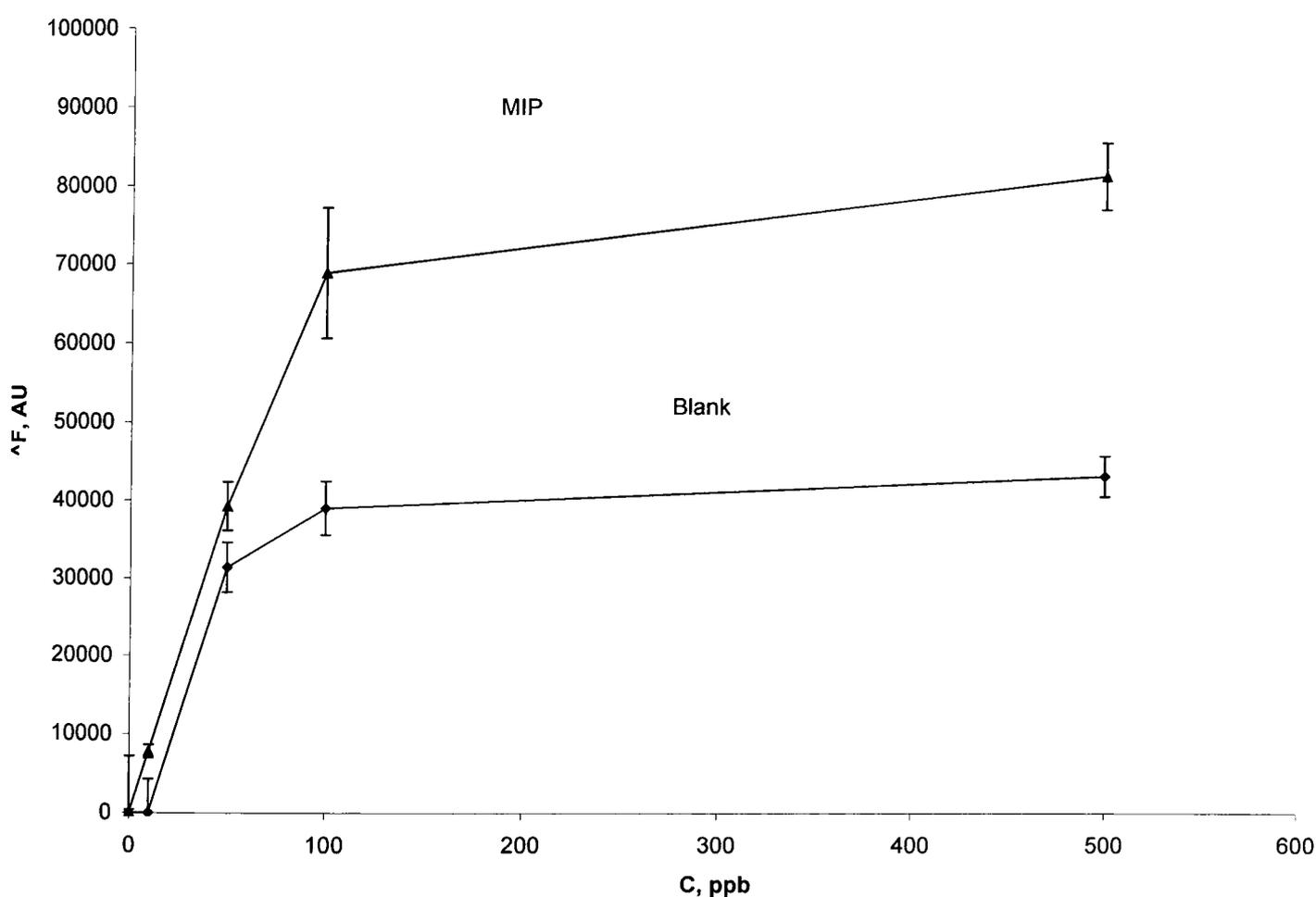


Figure 3.15: Increase in fluorescent signal relating to bound OTA to polymer A MIP and NIP respectively, dependent of concentration. Bars indicate standard error.

This clearly demonstrates the higher affinity of the MIP over the NIP and also demonstrates the possibility of using the polymer for detection of OTA in solution. This is further described in section 3.3.1.

3.2.8 Particle size analysis

It was noted during several of the SPE experiments that the particles swelled and shrunk, depending on the solvent. In order to study polymer swelling we attempted the use of a laser scatter method. The glassy nature of the polymer however caused intensive reflection, which vastly affected the results, therefore an image analysis method had to be used for characterisation of size of particles (section 2.3.8).

Figure 3.16 is an example of the photographs taken by the CCD system using the microscope. Each particle is clearly defined and the software allows for the area to be calculated against the known area of the photograph. By this method the particle size can be ascertained.

Samples of polymer A (both MIP and NIP) were ground, sieved and the fraction between 25- 38 micron size was collected and placed in solutions of water, methanol and 100 mM sodium phosphate buffer (pH 7.5), and described in the protocol in section 2.3.8. The changes in average particle size were calculated and presented in Figure 3.17.

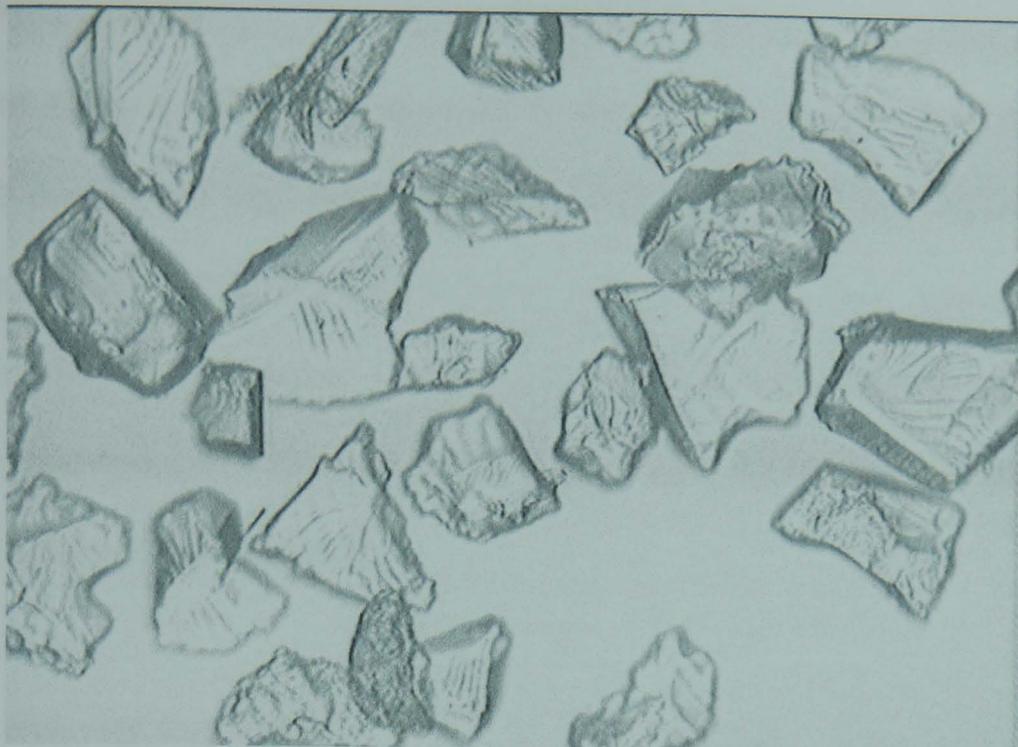


Figure 3.16: Example of image taken during particle size analysis. This is a picture of polymer A in the presence of water at 10x magnification.

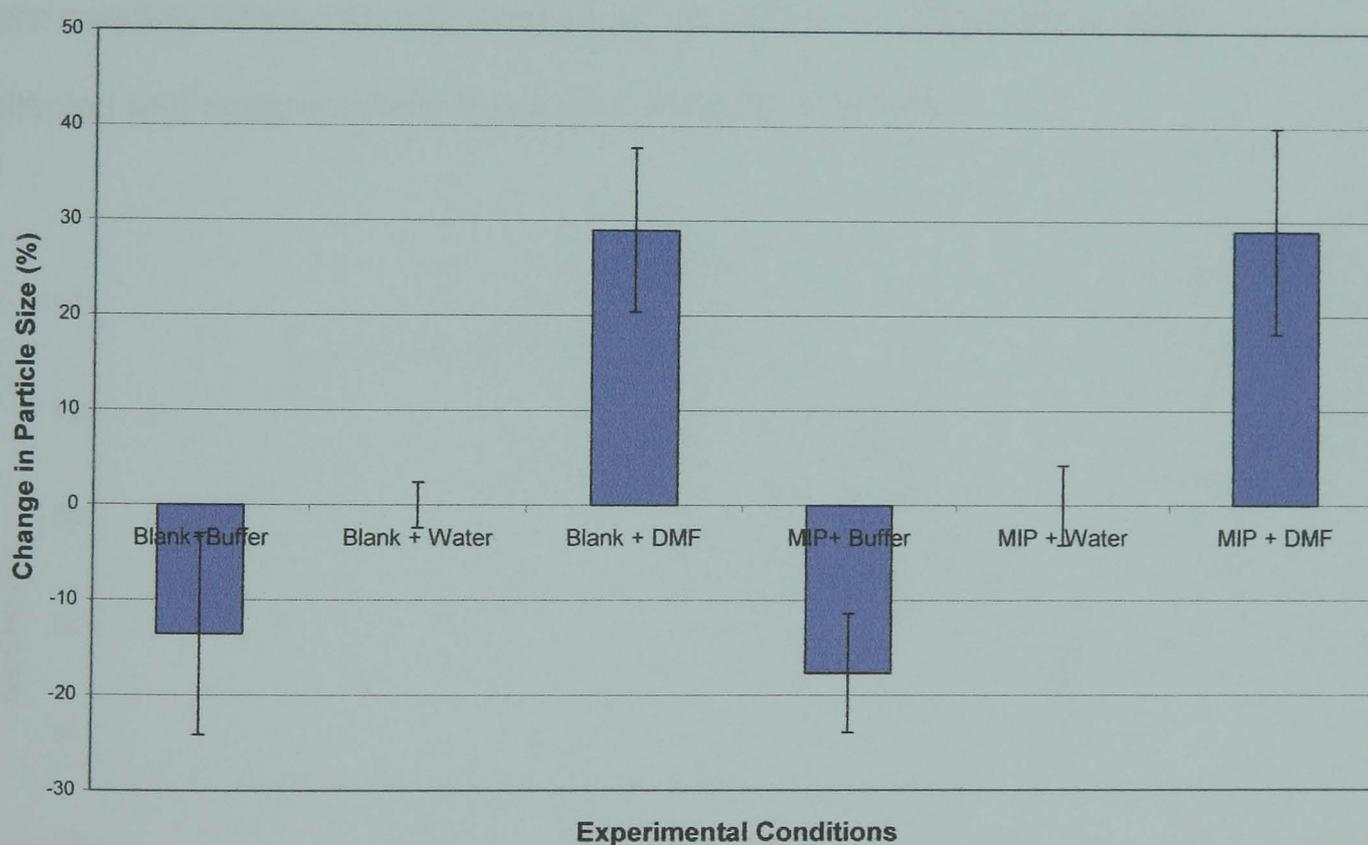


Figure 3.17: Relative changes (%) in average size of polymer particles (polymer A) in different solvents as compared with the size of the particles in water. The bars indicate standard error of particle size distribution.

3.2.9 SPE analysis

The use of fines in SPE was precluded as filtration under vacuum proved impossible due to too high back pressure. Therefore the larger particles had to be used. Due to the small amount of MIP prepared the cartridges were packed with 30 mg of material, which meant that each monolith preparation (5 mg OTA) gave two cartridges for study. These cartridges were conditioned as described above (section 3.2.3).

3.2.9.1 Polymer behaviour

Figures 3.18 – 3.21 show the binding characteristics of OTA to polymer A. Following the protocols described in sections 2.3.9.2 - 2.3.9.5, the solutions were carefully filtered through cartridges of polymer A. All solutions were collected and comparatively measured using fluorescence.

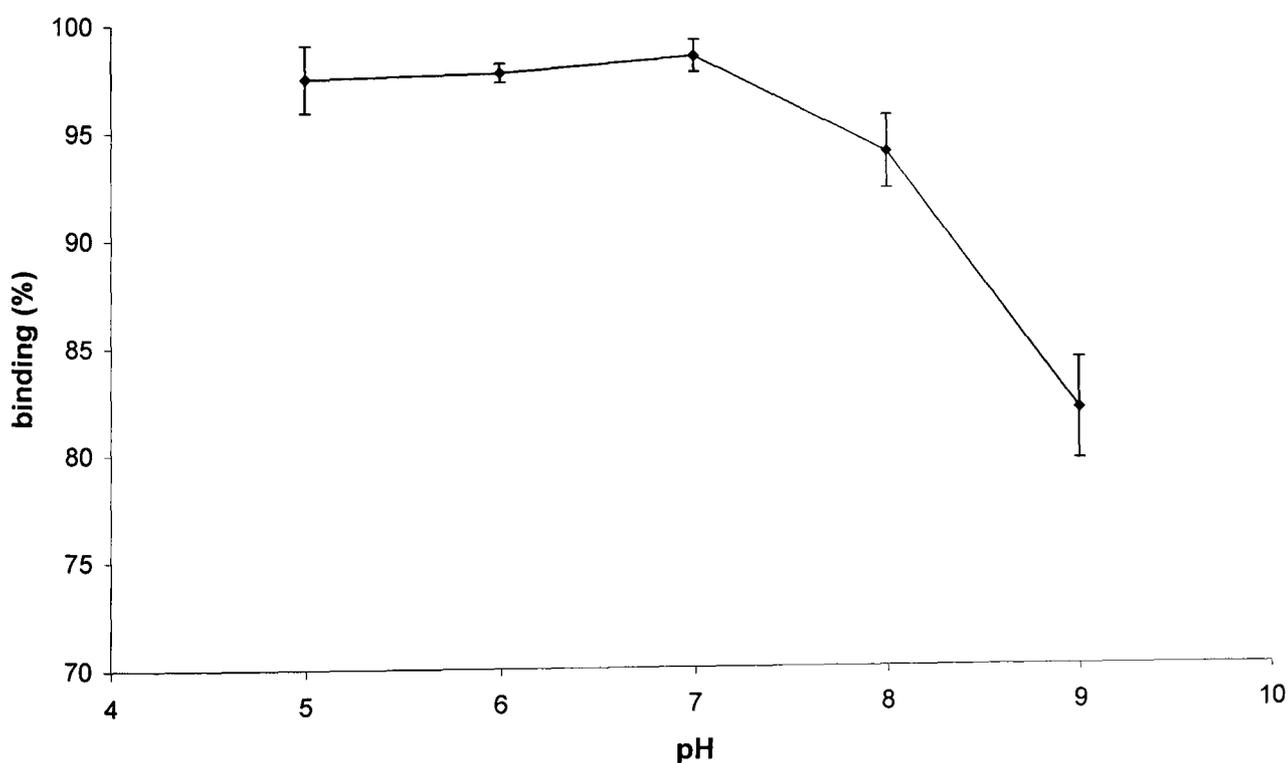


Figure 3.18: Effect of pH on binding of OTA to MIP of polymer A. Bars indicate standard error of measurement.

Figure 3.18 shows that binding is affected by changes in pH. As fluorescence of OTA is low in acidic solutions, a compromise of pH 7.5 was selected for all future experiments. The buffer with this pH provides good binding conditions (~95% binding) whilst guaranteeing a high fluorescent signal. This is also similar to pH of many biological solutions, which might prove useful in further application of this material.

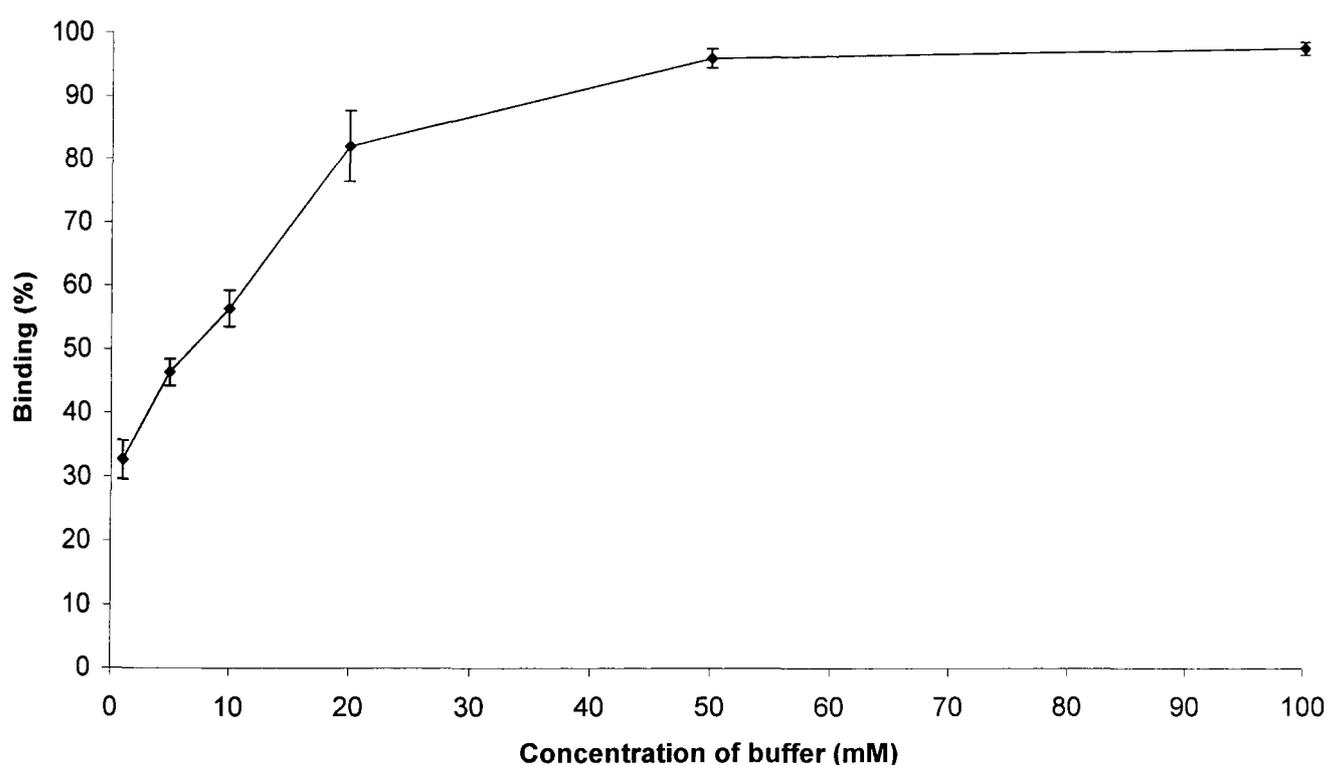


Figure 3.19: Effect of concentration of sodium phosphate buffer on binding of Ochratoxin A to the MIP of polymer A at pH 7.5. Bars indicate standard error of measurement.

Figure 3.19 demonstrates the importance of buffer concentration on the polymer binding properties. Clearly the presence of ions affects the system and aids binding of OTA to the polymer.

Figure 3.20 describes the affinity of the polymer for different concentrations of OTA. With the binding exceeding 95% for all studied concentrations this shows that the polymer works well within the range required in the project (2-100 ppb).

Figure 3.21 shows the negative impact on OTA binding to polymer A caused by the presence of organic solvents.

Figure 3.22 demonstrates the release of OTA from the polymer in water.

It is shown that OTA is released in organic solvent and in de-ionised water (~70% can be recovered in the first 3 ml wash and ~85% in 6 ml).

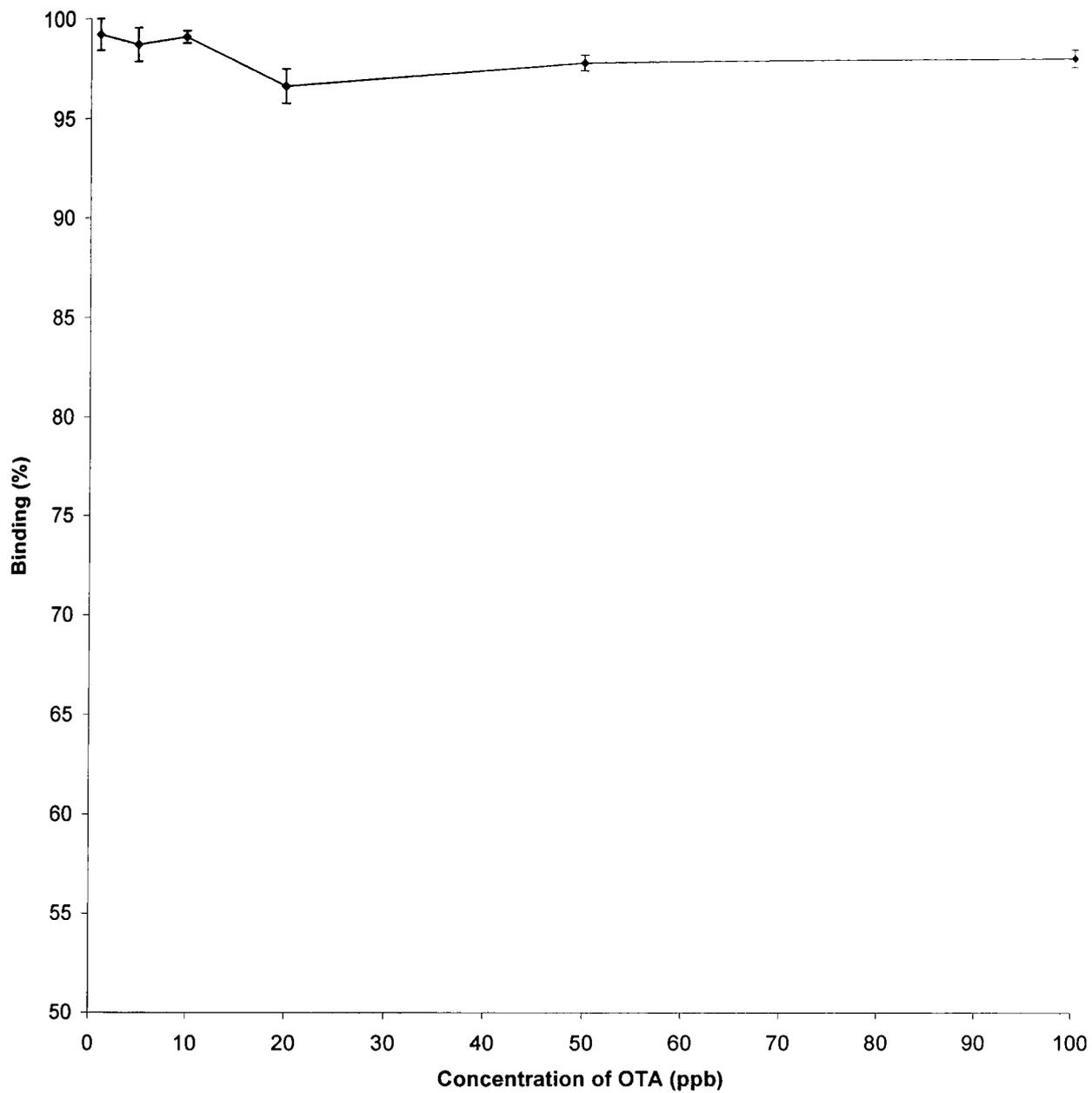


Figure 3.20: Binding of different concentrations of OTA to polymer A MIP. Solutions prepared with 100 mM sodium phosphate buffer pH 7.5. Bars indicate standard error of measurement.

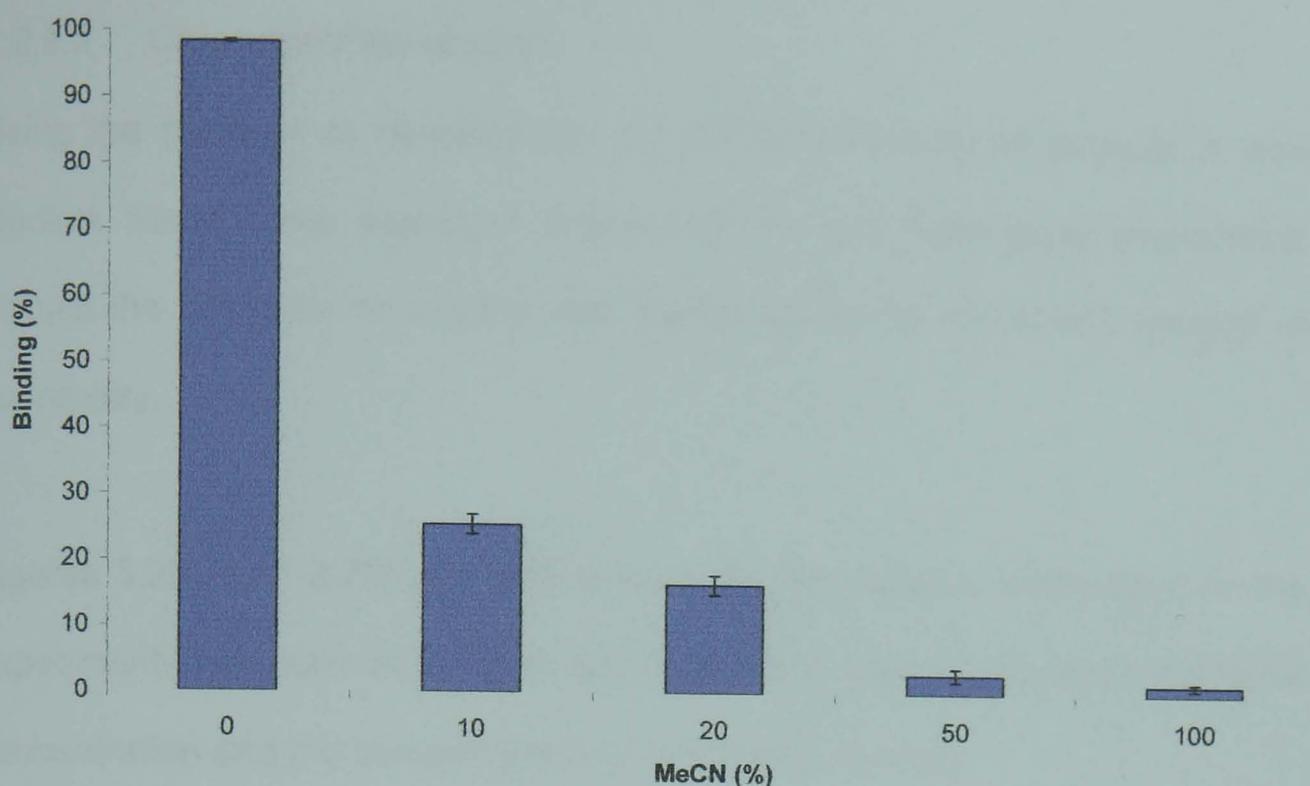


Figure 3.21: Effect on binding of OTA to MIP of polymer A in the presence of acetonitrile (MeCN). Solutions prepared with 100 mM sodium phosphate buffer pH 7.5. Bars indicate standard error of measurement.

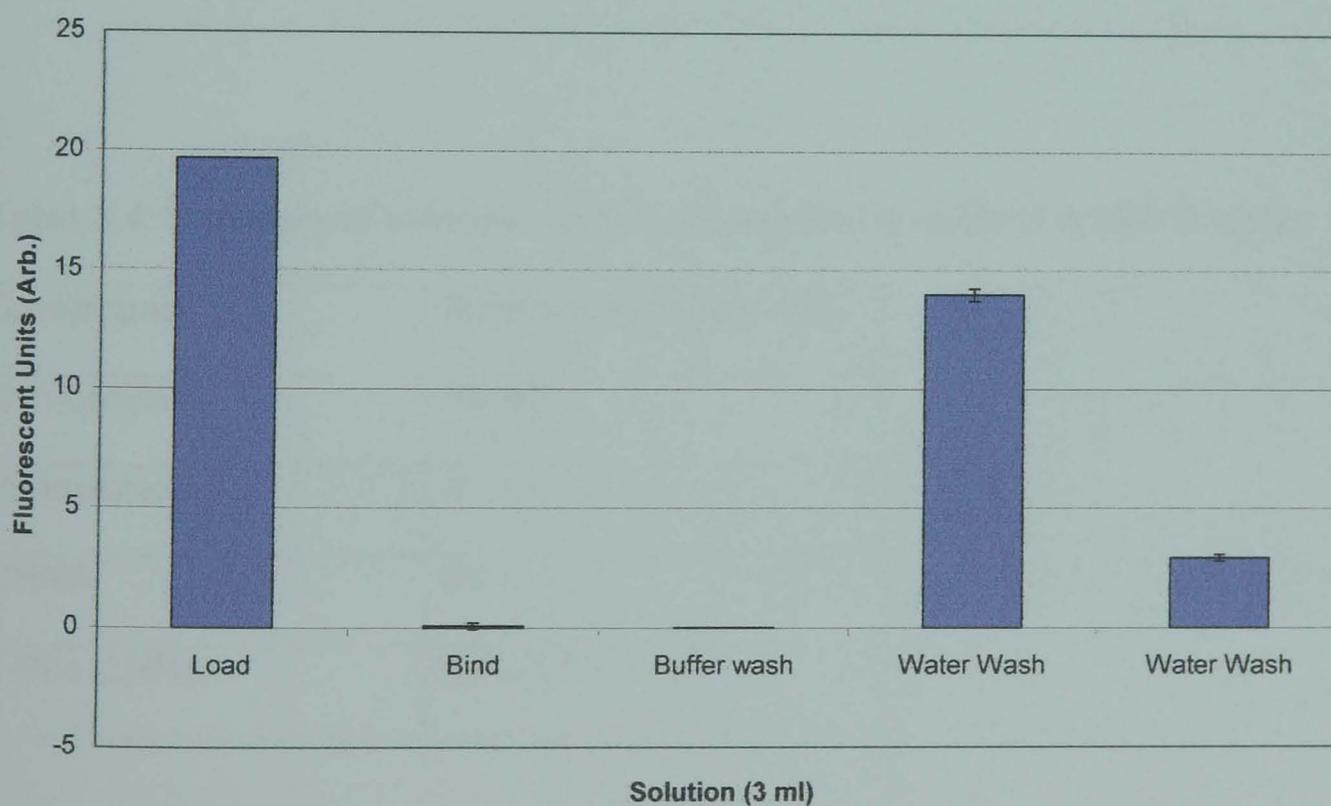


Figure 3.22: Release of OTA from polymer A MIP in successive washes. 100 mM sodium phosphate buffer pH 7.5 and de-ionised water were used as eluents. Bars indicate standard error of measurement.

3.2.9.2 Cross reactivity studies

Using the protocol as described in 2.3.10 the selectivity of polymer A was studied. Since some analogue compounds did not have good fluorometric signals the detection of binding was performed using HPLC-MS, instead of fluorimetry.

Figures 3.23a and 3.23b are examples of the MS spectra obtained from the experiment. The peak at 3.79 (a) and 3.65 (b) is used to compare the initial concentration and the concentration of recovered material.

Table 3.4 shows the recovery results of the cross reactivity studies. It shows that the concentrations of recovered analogues are less than that of OTA, which demonstrates the specificity of the polymer.

Table 3.4: Recovery of template and its analogues by polymer A MIP in water.

Compound	Relative recovery (%)
Ochratoxin A	75 ± 2
Phenylalanine	37 ± 2
CHNA	48 ± 2
L-Phe-CHNA	54 ± 1.5

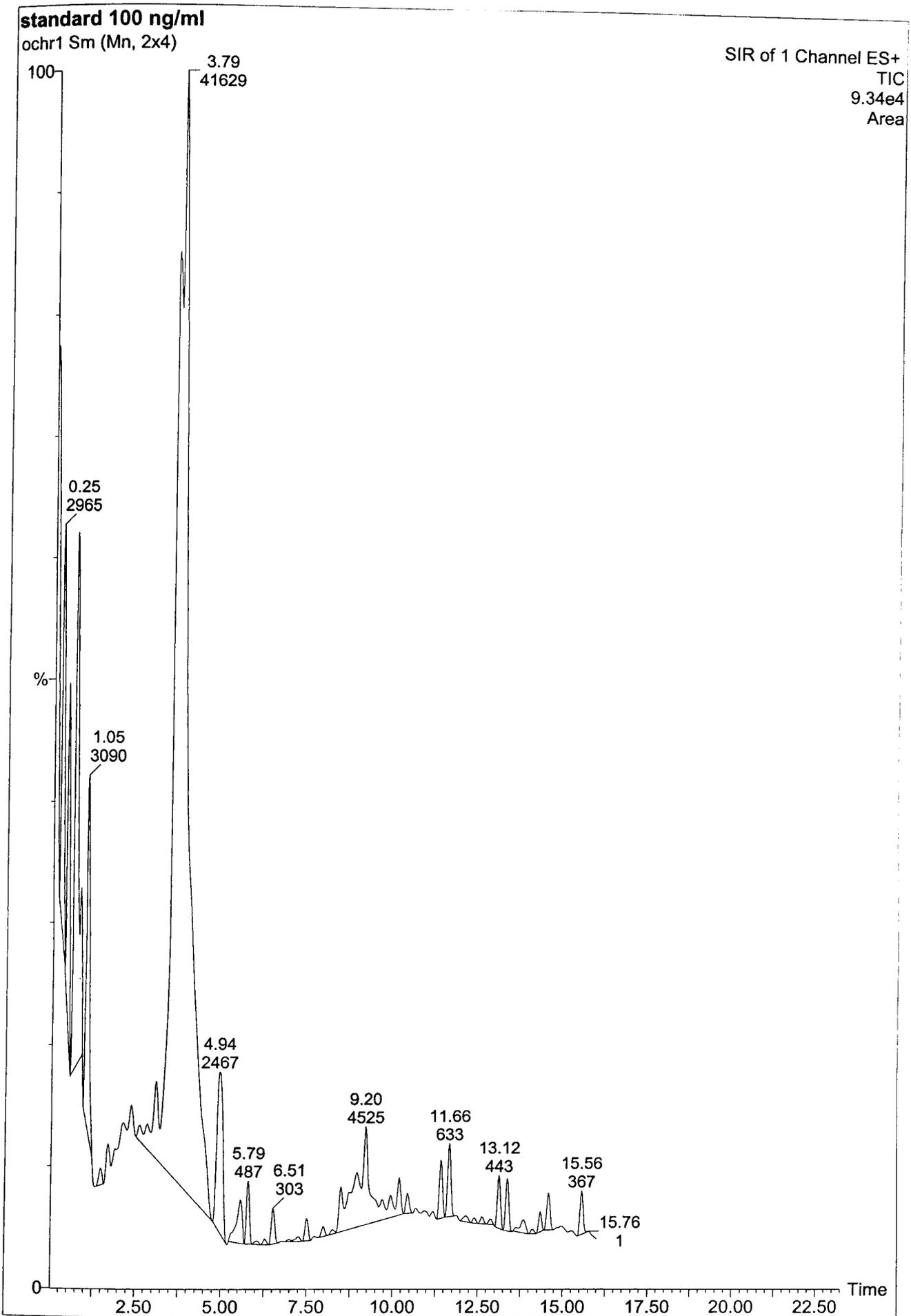


Figure 3.23a: Chromatogram of L-Phe-CHNA standard 100 ng/ml in 100 mM sodium phosphate buffer. The peak at 3.79 indicates the fragment used for concentration comparison.

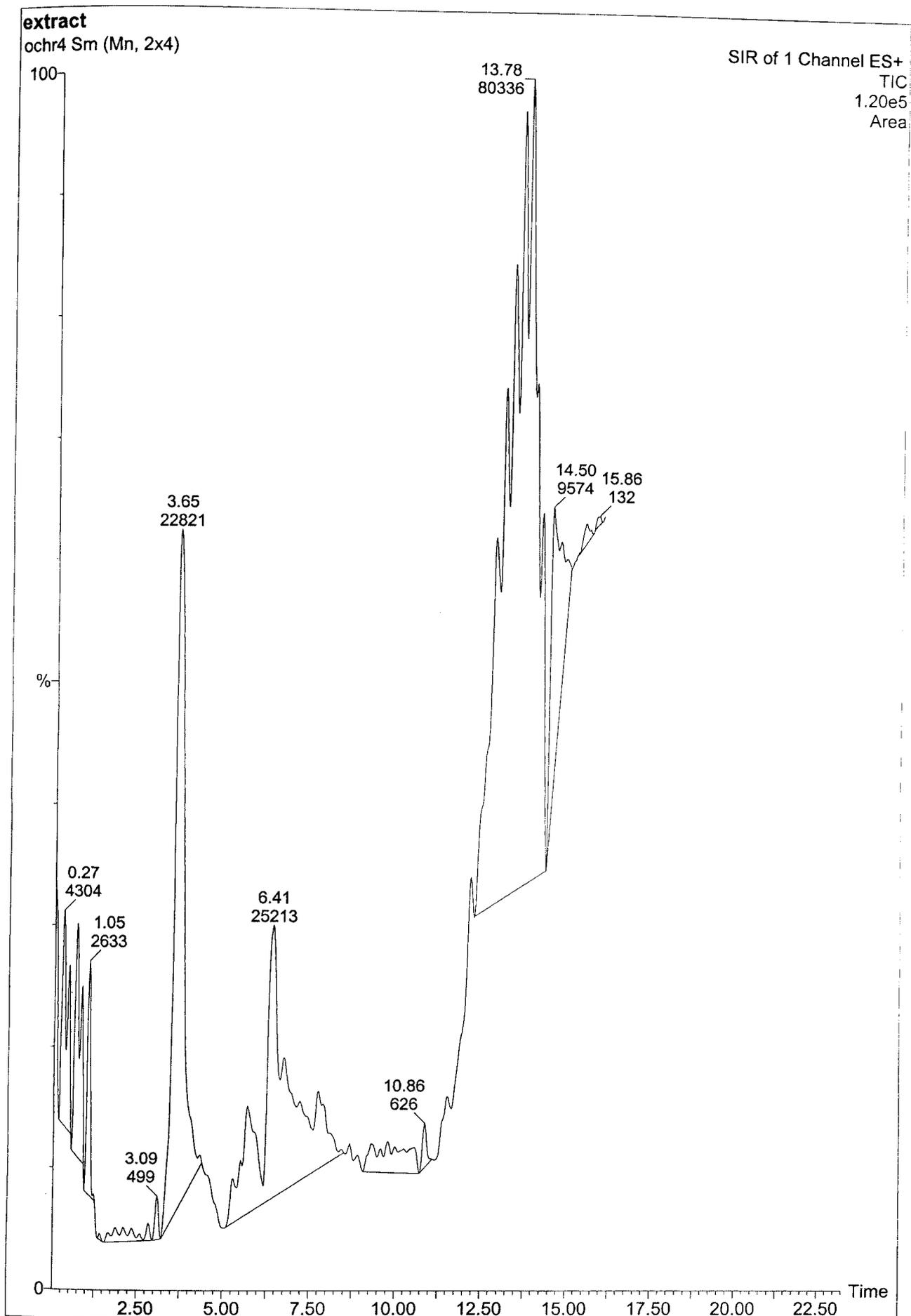


Figure 3.23b: Chromatogram of L-Phe-CHNA after elution in water from MIP polymer A. The peak at 3.65 indicates the fragment used for concentration comparison.

3.3 Sensor design and analysis

3.3.1 MIP membranes in optical sensing

3.3.1.1 Membrane study in model solutions

We have already shown that the membrane made of polymer A shows affinity towards OTA in aqueous buffers as shown in Figure 3.15. However extraction of OTA from grain should be performed in acetonitrile and it would be preferable for any sensor material to work in this solvent to make an analysis faster. To test the polymers suitability for this we performed the experiment as described in section 2.3.11.2. The results are shown in figures 3.24 and 3.25.

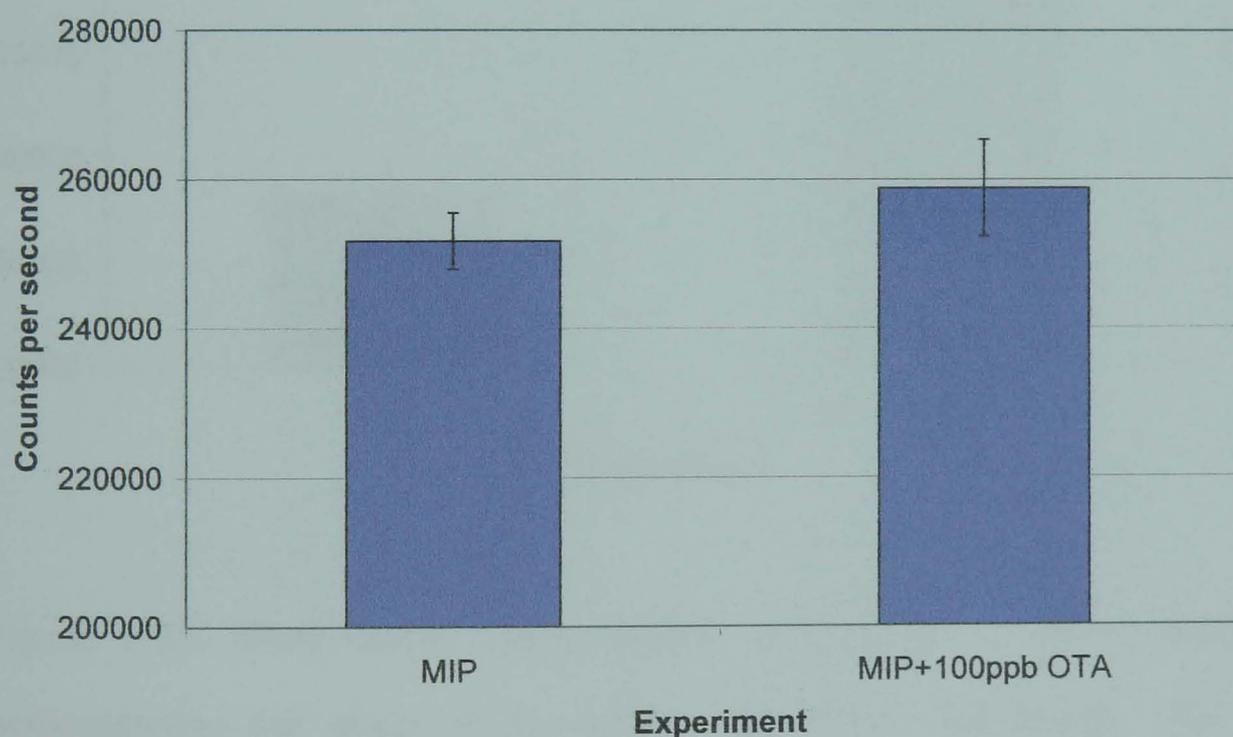


Figure 3.24: Fluorescent measurement of polymer A MIP membrane demonstrating the effect of the binding of OTA in acetonitrile. The bars indicate standard error of measurement.

Figure 3.24 demonstrates that in acetonitrile, membranes made of polymer A do not exhibit any enhanced binding to OTA (results which are in agreement

with SPE and NMR data as described in sections 3.2.9 and 3.2.2 respectively).

Figure 3.25 shows the results of the similar experiment, using the membrane made of polymer B. It clearly proves that the polymer is able to bind OTA in organic solvent as was expected from NMR data.

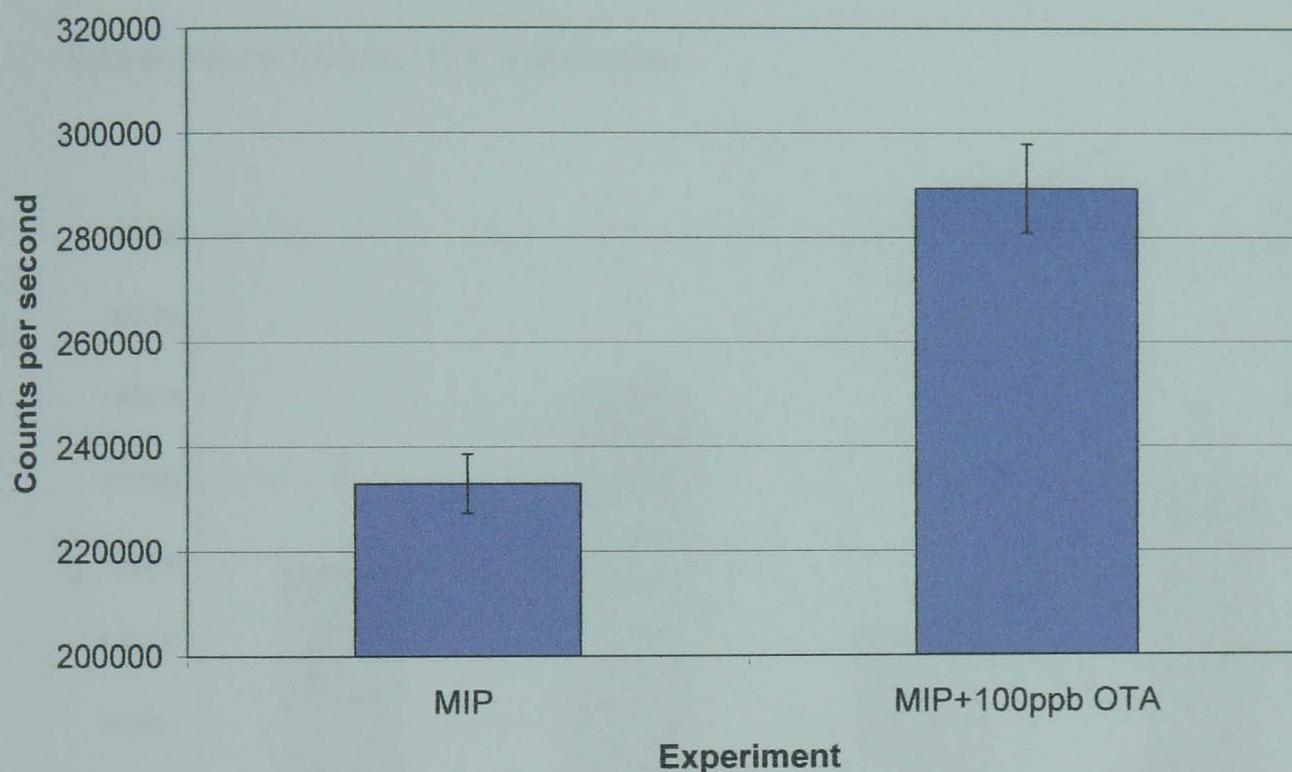


Figure 3.25: Fluorescent measurement of polymer B MIP membrane demonstrating the effect of the binding of OTA in acetonitrile. The bars indicate standard error of measurement.

On the basis of these results and also the results shown in section 3.2.6, the membrane made of polymer A was selected used to develop a possible sensor in aqueous media, namely grape and wine juice, both possible carriers of OTA, and the polymer B system was used in an organic system, namely an acetonitrile extraction from maize.

3.3.1.2 Membrane studies with grape juice and wine

As described in section 2.3.11.3 membranes made of polymer A were used to study the uptake of OTA from white wine and grape juice. Both blank and MIP membranes were studied for comparison. The membrane was measured in solution and then in a spiked solution in an attempt to view any changes in fluorescence due to OTA adsorption.

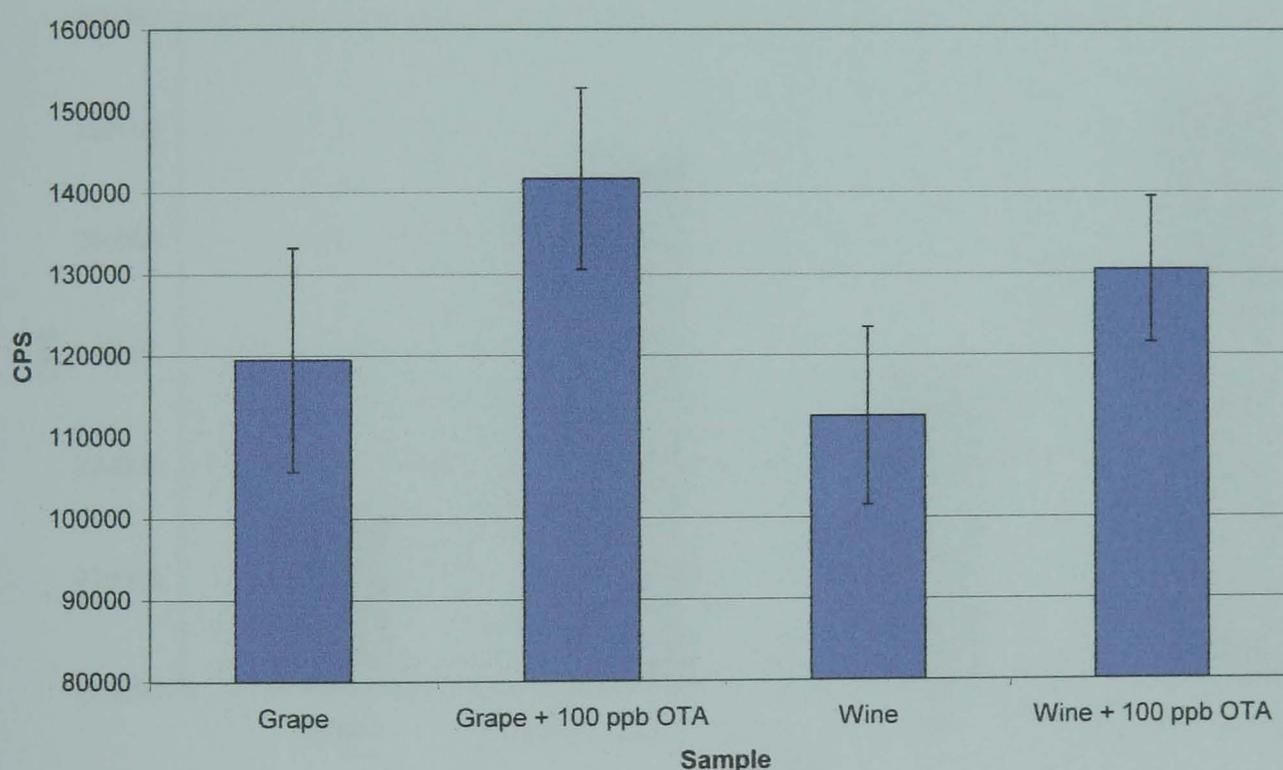


Figure 3.26: Fluorescent measurement of polymer A blank membrane demonstrating the effect of the binding of OTA from grape juice and white wine. The bars indicate standard error of measurement.

The blank membrane registers an increase in signal between the standard and the spiked membrane, indicating binding of OTA to the membrane in both wine and grape juice (Figure 3.26). This supports the results shown in Figure

3.15, however the signal was too low to justify use of the blank membrane in sensors.

Figure 3.27 shows the binding on OTA in grape juice and wine to the MIP of polymer A. The difference in signal due to the increased affinity for the template is clearly seen. This, combined with the reasonably low standard deviation (which can be further decreased by standardising membrane fabrication procedure), allows the possibility to use this material in possible sensor use.

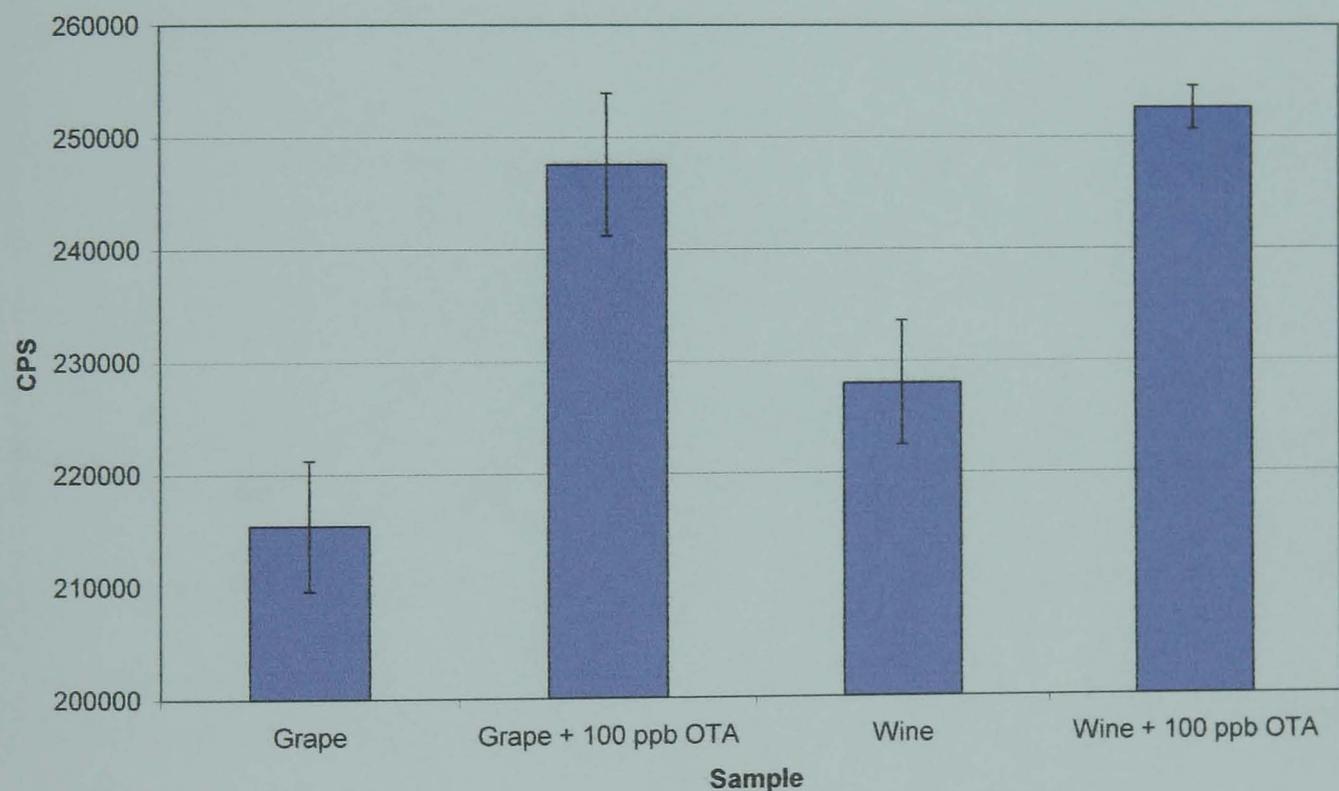


Figure 3.27: Fluorescent measurement of polymer A MIP membrane demonstrating the effect of the binding of OTA in grape juice and white wine. The bars indicate standard error of measurement.

Therefore, experiments were performed in order to determine a detection limit. Figure 3.28 shows the results of this experiment made with grape juice.

The calibration curve shown here proves that it would be impossible to detect OTA in the concentrations below 100 ppb without further improvement in homogeneity of formed MIP membranes and optimisation of fibre optic interface.

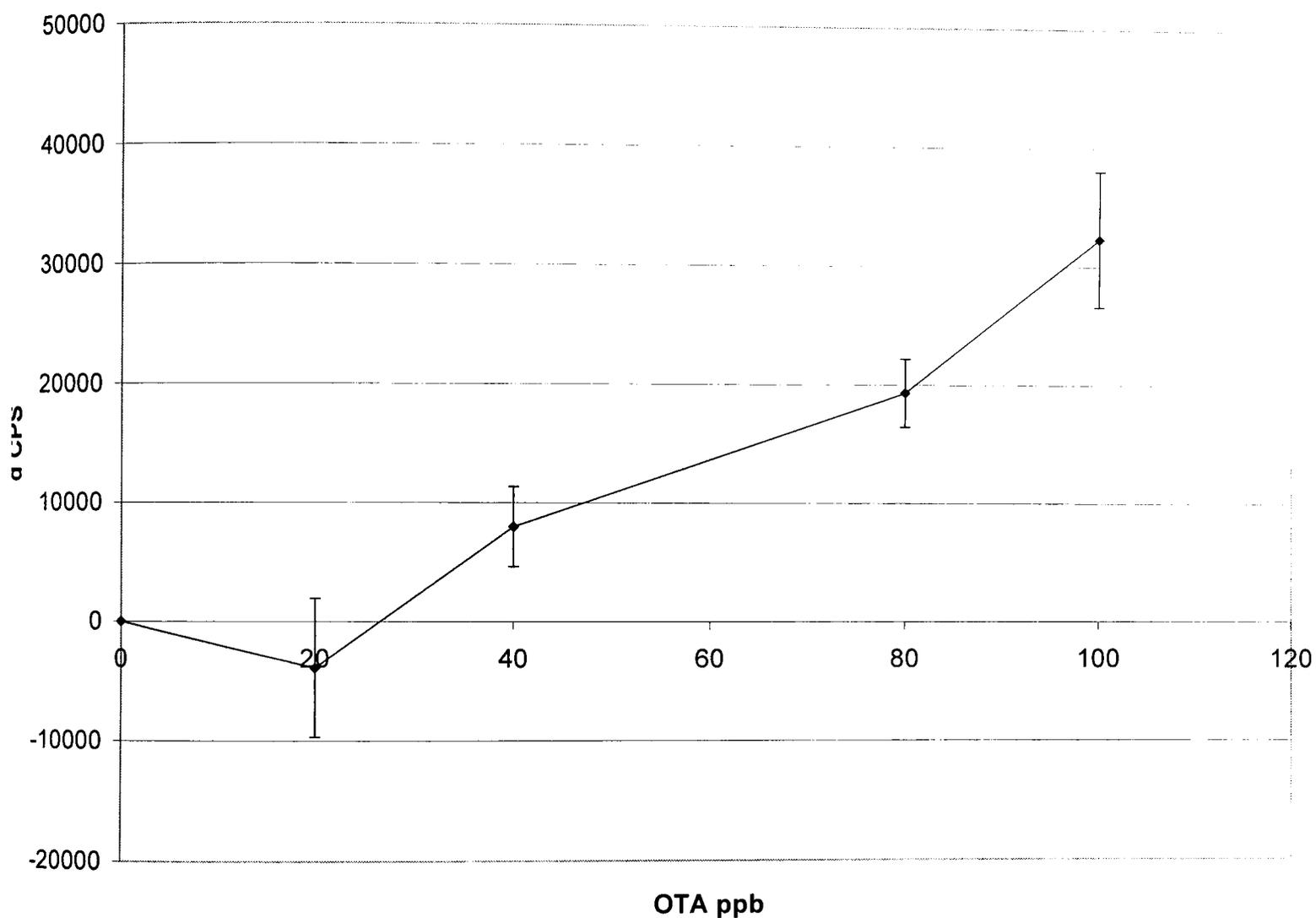


Figure 3.28: Calibration curve of detection of OTA in grape juice using a fibre-optic set-up integrated with MIP membrane. Bars indicate standard error in measurement.

3.3.1.3 Membrane studies with grain extract

Using the protocol as described in section 2.3.11.1 an extract of maize was prepared and tested as described as in section 2.3.11.4.

The results of the experiments are presented in Table 3.5.

Table 3.5: Results of spiked extract binding to MIP and blank membrane.

Membrane	Blank extract	Spiked extract
Blank	105581	109585
Std. Dev	+/- 10169	+/- 12098
MIP	305247	325815
Std. Dev.	+/- 16099	+/-17054
MIP (burn)	222731	226882
Std. Dev.	+/- 12293	+/- 24814

The results show no appreciable change in membrane fluorescence in the presence of spiked sample.

3.3.2 SPE experiments in grain samples

3.3.2.1 Identification of suitable materials for extraction of OTA from grain

Several SPE materials were studied for their ability to recover OTA from an acetonitrile solution in model and real samples using the protocols described in section 2.3.12.1.

The chosen materials were as follows. Celite 545, a kieselguhr silica, and activated carbon/activated aluminium oxide, which had shown good results with cleaning grain samples for deoxynivalenol (DON) analysis. Four commercially available ion exchange cartridges (strong and weak anionic and cationic) and blank polymer B were also used in SPE experiments.

In this section the results of these experiments for each material are present, in tabular form and an explanation of the results accompanies them.

3.3.2.2 *Celite 545*

Table 3.6: Fluorescence of maize extract components (measured at 434 nm) filtered through Celite 545 column.

Celite 545	Cartridge 1	Cartridge 2
Fluorescence of sample	2.62	2.62
Sample after filtration	2.37	2.59
<i>Bound Material (%)</i>	9.5	1.5

Table 3.6 shows that the silica does not bind much of the extract at all, therefore if the material binds high quantities of OTA then it could be useful. However, as shown in Table 3.7, the silica does not have the affinity to bind OTA in sufficient quantities suitable for practical quantitative analysis.

Table 3.7: Fluorescence of 100 ppb OTA solution (measured at 434 nm) filtered through Celite 545

Celite 545	Cartridge 1	Cartridge 2
Fluorescence of 100 ppb OTA	3.36	3.36
Fluorescence after filtration	1.47	1.36
<i>Bound OTA (%)</i>	<i>56.55</i>	<i>59.4</i>

3.3.2.3 Activated Carbon/ AlO_3

A method used by our laboratory, for removal of other fungal toxins from biological samples, namely DON, involves the use of an activated charcoal/aluminium oxide in a 1/20 ratio. This method has been tested for the recovery of OTA from maize extract.

Table 3.8: Fluorescence of maize extract components (measured at 434 nm) filtered through C/ AlO_3 column

C/AlO_3	Cartridge 1	Cartridge 2	Cartridge 3
Fluorescence of sample	2.62	2.62	2.62
Sample after filtration	0	0	0
<i>Bound Material (%)</i>	<i>100</i>	<i>100</i>	<i>100</i>

Table 3.9: Fluorescence of 100 ppb OTA solution (measured at 434 nm) filtered through C/AlO₃ column.

C/AlO ₃	Cartridge 1	Cartridge 2	Cartridge 3
Fluorescence of 100ppb OTA	2.96	2.96	2.96
Fluorescence after filtration	0	0	0
<i>Bound OTA (%)</i>	<i>100</i>	<i>100</i>	<i>100</i>

The results presented in Tables 3.8 and 3.9 show that this mixture bound both extract and OTA completely. A wash with water and several buffers, were employed in an attempt to remove the OTA provided no success with both acidic and basic solutions. This discounted the use of C/AlO₃ as an extraction agent.

3.3.2.4 Polymer B

Table 3.10 shows that the blank made of polymer B binds OTA, however it does not provide enough affinity for a practical, quantitative analysis.

Table 3.10: Fluorescence of 100 ppb OTA solution filtered through blank polymer B.

DEAEM blank	Cartridge 1	Cartridge 2	Cartridge 3
Fluorescence of 100 ppb OTA	17.04	17.04	17.04
Fluorescence after filtration	8.75	10.51	9.45
<i>Bound OTA (%)</i>	<i>51</i>	<i>61</i>	<i>55</i>

3.3.2.5 IEX cartridges

To test the commercially available materials the four major types (strong (SAX) and weak anionic (NH₂), and strong (SCX) and weak (CBA) cationic) cartridges were studied using the methodology as described previously.

100 ppb OTA in 3 ml acetonitrile was passed through the cartridges and fluorescence was measured.

Table 3.11: Fluorescence of 100 ppb OTA solution filtered through IEX cartridges.

IEX	SAX		NH ₂		SCX		CBA	
Fluorescence of 100 ppb OTA	10.38	10.38	10.38	10.38	10.38	10.38	10.38	10.38
Fluorescence after filtration	-0.26	-0.24	-0.26	-0.27	1.04	0.88	0.36	0.75
<i>Bound OTA (%)</i>	<i>100</i>	<i>100</i>	<i>100</i>	<i>100</i>	<i>90</i>	<i>92</i>	<i>96</i>	<i>93</i>

The OTA elution was performed using 50 mM sodium phosphate buffer, pH 7.5.

Table 3.12: Release of model OTA sample from IEX cartridges.

	SAX		NH ₂		SCX		CBA	
Positive sample*	22.79	22.79	42.16	42.16	4.04	4.04	18.94	18.94
Fluorescence with 3 ml buffer	1.55	2.85	32.46	32.15	0.38	0.28	1.87	4.15
<i>Released OTA (%)</i>	<i>7</i>	<i>12.5</i>	<i>73</i>	<i>73</i>	<i>9</i>	<i>7</i>	<i>10</i>	<i>22</i>

*The fluorescence of solution spiked after the filtration through the corresponding cartridge.

Table 3.11 shows that all four cartridges bind OTA in high quantities. The best results with OTA recovery were achieved with "NH₂" SPE material as shown in Table 3.12.

Therefore the experiment was repeated with this material as shown in Table 3.13. The results clearly show that this material is suitable for extraction of OTA in model solutions.

Table 3.14 shows the release data of OTA in buffer solution. The phosphate buffer concentration used for release was increased to 0.2 M at pH 7. This shows the release of ~70% of the bound OTA in 3 ml buffer, and ~85% in 6 ml, which is acceptable for both qualitative and quantitative sensor systems.

Table 3.13: Fluorescence of 100 ppb OTA solution filtered through NH₂ cartridge.

NH ₂	Cartridge 1	Cartridge 2	Cartridge 3
Fluorescence of 100ppb OTA	10.58	10.58	10.58
Fluorescence after filtration	0.15	-0.26	-0.21
<i>Bound OTA (%)</i>	<i>98.5</i>	<i>100</i>	<i>100</i>

Table 3.14: Release of OTA in 0.2 M sodium phosphate buffer (pH 7).

NH ₂	Cartridge 1		Cartridge 2		Cartridge 3	
	3ml	2 nd 3ml	3ml	2 nd 3ml	3ml	2 nd 3ml
Fluorescence of 100 ppb OTA*	31.64		31.64		31.64	
Fluorescence after elution	22.02	4.60	22.02	4.40	24.34	3.15
<i>Released OTA (%)</i>	69	14	69	14	76	10

* mean result taken from triplicate measurements.

In order to complete the study of the “NH₂” material an extract of maize was loaded onto the cartridge. The results presented in Table 3.15 show very little interference (< 9%).

In order to complete this series of experiments, a sample of extract spiked with 100 ppb OTA was prepared and filtered through the cartridges. The results are described in Table 3.16.

These results (Table 3.16) indicate that fluorescence of recovered OTA sample is very low, therefore the acetonitrile washing stage was removed (Table 3.17) to enhance the level of OTA recovery.

Table 3.15: Extract cleaning using NH₂ IEX cartridge.

NH ₂	Cartridge 1	Cartridge 2	Cartridge 3
Fluorescence of extract	3.02	3.02	3.02
Fluorescence after filtration	1.67	1.67	1.95
<i>Bound extract (%)</i>	45	45	35
Fluorescence after filtration	0.28	0.07	0.28
<i>Released interfering material (%)</i>	<9%	<2%	<9%

By excluding the acetonitrile washing step the recovery of OTA was enhanced. Table 3.18 shows an attempt to further enhance the elution stage by adding SDS to elution solution. This appears to work well, however it prevented binding of OTA eluted from IEX to the MIP. Therefore this step was removed from future protocols.

Table 3.16: Binding and recovery of OTA from real samples using NH₂ IEX cartridge.

NH ₂	Cartridge 1		Cartridge 2		Cartridge 3	
	Fluorescence of extract + OTA	2.51		2.51		2.51
Fluorescence after filtration	1.65		1.36		1.61	
Elution volume	3ml	2 nd 3ml	3ml	2 nd 3ml	3ml	2 nd 3ml
Fluorescence in buffer after recovery	4.81	4.53	5.79	5.26	6.67	4.62

Table 3.17: Recover of OTA using NH₂ IEX cartridge and modified protocol (MeCN washing stage is excluded).

NH ₂	Cartridge 1		Cartridge 2	
	Elution volume	3ml	2 nd 3ml	3ml
Fluorescence after filtration	13.55	3.12	11.49	5.12

Table 3.18: Binding and release of OTA and extract in the presence of 1% SDS

NH ₂	Cartridge 1	Cartridge 2	Cartridge 3
Fluorescence of extract + OTA	4.06	4.06	4.06
Fluorescence after filtration	1.62	1.35	1.19
Buffer spiked with 100 ppb OTA	33.02	33.02	33.02
Fluorescence in buffer.	34.10	28.70	28.05
<i>Recovery (%)</i>	<i>100</i>	<i>86</i>	<i>84</i>

This series of experiments obtained a practical working extraction protocol for OTA, from maize extract. Recoveries of ~70%, which is suitable for quantitative data regarding a sensor were obtained leading to the next set of experiments in which this protocol was combined with the SPE using polymer A MIP (section 3.2.9). This will attempt to produce a working qualitative sensor system.

3.3.2.6 *Combination of IEX and Polymer A MIP extraction*

As described in section 2.3.12.2 a buffer solution which had undergone IEX treatment was produced and loaded onto the cartridge of polymer A MIP.

Table 3.19 shows the binding and recovery of OTA from this model solution. The OTA is all bound, as expected, and the initial recovery in water is ~63%, which is suitable for a qualitative sensor system.

Table 3.19: Binding of OTA from IEX adjusted buffer solution to MIP polymer A cartridge.

	Cartridge 1	Cartridge 2	Cartridge 3
Initial Solution	24.15	24.53	23.96
After Filtration	0.17	0.00	0.45
<i>Bound Material (%)</i>	<i>99.9</i>	<i>100</i>	<i>99.9</i>
Buffer Wash	0.01	0.00	0.00
Water Elution	14.93	15.62	15.78
<i>Recovered OTA (%)</i>	<i>61.8</i>	<i>63.6</i>	<i>65.8</i>

This was repeated with an extract of maize, which had passed through the IEX cartridge. This sample was spiked with 100 ppb OTA and loaded onto the MIP of polymer A. Table 3.20 shows the results of this experiment. As shown nearly all the OTA passed through the cartridge, with only a small proportion binding to MIP. This shows strong interference of the components from maize extracts (possibly lipids) with MIP binding. To study this a buffer solution was taken (100 mM sodium phosphate pH 7.5) and spiked it with 100 ppb OTA. To this solution 1% lecithin was added. The addition of this phospholipid is to determine the effect of lipids on the binding ability of the polymer. Table 3.21 demonstrates the effect of this compound on the binding. An effect similar to that of the extract is seen.

These results confirm the inability of developed MIPs, even combined with IEX SPE material to be used for the detection of OTA in maize. Several

experiments, including extraction and re-suspension and addition of surfactants to the buffer solutions were attempted to remove the lipid from the extract, to allow the OTA to bind but these were unsuccessful (data not shown).

Overall these results explain the development of two polymers, their characterisation in both SPE and membrane format, and possible integration into usable sensor platforms for detection of OTA in solution. These results are discussed at length in the next chapter.

Table 3.20: Binding of OTA from IEX adjusted buffer solution containing maize extract to MIP polymer A cartridge.

	Cartridge 1	Cartridge 2	Cartridge 3
Initial Solution	27.62	28.34	28.96
After Filtration	24.36	26.16	25.17
<i>Bound Material (%)</i>	<i>11.9</i>	<i>7.7</i>	<i>13.1</i>
Buffer Wash	1.36	0.75	1.34

Table 3.21: Binding of OTA from buffer solution containing 1% lecithin to MIP polymer A cartridge.

	Cartridge 1	Cartridge 2
Initial Solution	29.63	27.67
After Filtration	25.16	26.13
<i>Bound Material (%)</i>	<i>15.1</i>	<i>5.6</i>

Chapter 4

Discussion

4.1 Introduction

In this chapter the methods presented in Chapter 2 and the results presented in Chapter 3 are discussed. Attention to the development of the two polymers from design and synthesis; to characterisation and use in sensor applications is covered, along with general comments, comparisons to other work in the field and notes about the work presented in this thesis.

4.2 Polymer design

4.2.1 Computer modelling

As it was mentioned in the literature review, OTA has been imprinted previously. The group of Lindner (Jodlbauer, Maier & Lindner, 2002) initially used well-established monomers such as methacrylic acid, HEMA and 4-vinylpyridine (4-VP), to produce a HPLC based protocol for detection of OTA, but without success. Therefore the authors used a series of tailored quinuclidine based monomers to enhance ion-pairing interactions between the template and polymer. The results showed only a moderate improvement. In another paper Baggiani (Baggiani, Giraudi & Vanni, 2002) used methacrylic acid to produce a series of polymers which demonstrated modest imprinting effects. Unlike the work presented in this thesis both groups used a mimic of OTA as the initial template. This method, known as “dummying” lowers the specificity of the resultant system, however removes the problems of template leaching (Matsui *et al.*, 2000a; Matsui *et al.*, 2000b).

The failure with these previous attempts could be related to two factors: (i) poor selection of the functional monomers unable to form high-affinity

interactions with the template and (ii) use of template analogues instead of OTA which decreases MIP affinity and specificity, therefore to improve the chances to develop a high performance MIP I decided to use OTA as template.

An encouraging new development by our group was the application of molecular modelling and computer simulation in design of imprinted polymers (Karim *et al.*, 2000; Piletsky *et al.*, 2002, Chianella *et al.*, 2002). This technology is proven to be much more effective than the subjective selection of monomers based on previously published experimental results, analysis of monomer-template complementarity, behaviour of similar systems and the individual experience of the chemist. In an example of this work related to mycotoxins Subrahmanyam (Subrahmanyam, 2002) imprinted aflatoxin-B1 using HEMA and allylamine as monomers, chosen by computational design. He was able to demonstrate enhanced affinity for this template as compared with traditionally used methacrylic acid. In other work he used the computational approach to develop MIP capable of differentiating between creatine and creatinine – the task which traditional MIP was unable to perform (Subrahmanyam *et al.*, 2001). As this method has been proved successful on several occasions and with several different templates, I decided to use it to select the monomers for OTA polymer synthesis.

The monomer choices predicted by the computer modeling for optimum interactions with OTA were split into two experiments, in order to compare the

theoretical data in vacuum with these in MeCN where binding was expected to take place.

4.2.2 Design of the molecular model

The template was drawn using a sketch command within the Sybyl software. At this stage several changes to the chemical notation were performed to ensure that the program had correctly identified the type of chemical groups and chemical bonds present in the molecule and that the chirality is correct. If the charges assigned to the structure were incorrect then any screening based on electrostatic forces (Van der Waals, hydrogen bonding etc.) will be inaccurate, leading to reduced ability within the final polymer. After this stage the template was minimised as described in the protocol developed in our laboratory (Chianella 2003). The monomer library was produced in similar fashion. The monomer library contained different types of compounds capable to form electrostatic, dipole-dipole and Van der Waals interactions with OTA molecule. I deliberately avoided using expensive commercially unavailable monomers, which could further increase the cost of polymer manufacture.

In this work the energy minimisation of the monomers and template was performed in two environments. The first experiment was performed by using default parameters for Dielectric constant corresponding to vacuum conditions as described in earlier work performed by our group. The second experiment was performed in the environment expected if acetonitrile is used as the solvent. This allowed the prediction of the influence of solvent on monomer-template interactions.

The DREAM mode, allowed the program to identify which monomers purport to have a good binding with the receptor site (in this case the template). As described in the protocol most of the parameters were left as default, with exception of the inclusion of the H-bonding. The selections of the Move Frequencies (as described in Table 2.2) stopped the program from the formation of covalent monomer-monomer complexes. All the possible toxin sitepoints were considered for the interactions with the monomers.

The results of the first experiment (dielectric constant = 1, vacuum) showed that the monomers acrylamide ($-35.32 \text{ kcal mol}^{-1}$) and methacrylic acid ($-20.99 \text{ kcal mol}^{-1}$) in theory represent good candidates for polymer preparation. The positioning of the monomers interactions with respect to OTA are portrayed in Figure 3.2a and 3.2b. Having charged monomers such as vinylimidazole in intended experimental conditions, would be highly unlikely, therefore they were discounted.

The process was repeated using a higher dielectric constant (37.5) in the calculations to mimic the effects of the presence of acetonitrile. Two monomers – allylamine and DEAEM gave the highest binding score. At first glance the allylamine looks exceptionally promising ($-174.90 \text{ kcal mol}^{-1}$), unfortunately, work performed previously in our laboratory found that allylamine has poor inclusion into a growing polymer (<30%), therefore it was discounted.

From this and the initial Leapfrog experiment in vacuum, DEAEM was selected as a monomer for practical work. The positioning of the DEAEM monomer interaction with respect to OTA is portrayed in Figure 3.3. In this environment (acetonitrile) the possibility to have ionised DEAEM molecules, as seen in the Leapfrog results, is high, therefore it warranted its selection as a monomer. The positioning of the TFA, in the predicted interactions, was the same as that of DEAEM; therefore I did not choose to select two monomers as in the experiment in vacuum.

However the results from the Leapfrog algorithm only show the highest binding energies and the rest are discarded. It is entirely possible that the chosen monomers can interact with other part of the molecule, and the effect may be more complex due to the presence of an excess of monomers to template, which in synthesis can interact between themselves. Therefore, a further refining step (simulated annealing) was performed. The simulated annealing process allowed the molecular dynamics of the system (in this case the template surrounded by the chosen monomers within a solvated “virtual” box) to be subjected to an energy minimisation. This box (used as periodic boundary conditions to keep the monomers from moving away from the template) is charged under the same conditions (Gasteiger-Hückel) and the energy minimisation was performed as before (Powell) (Chianella, 2003). Due to the complexity of the system the minimal energy was set higher ($0.05 \text{ kcal mol}^{-1}$ instead of $0.001 \text{ kcal mol}^{-1}$) in order to ensure that the calculations and program run time were suitable. The whole dynamic process was performed

at the range of temperatures from high (1000K) to low (300K) to obtain the lowest energy conformation of a template/monomer complex.

The predicted choices in vacuum were one acrylamide and one methacrylic acid to each template. The second experiment, based in modeled conditions representing the presence of acetonitrile gave the results of four DEAEM molecules to each template molecule.

It is not always that molecular modeling identifies two different monomers as candidates for polymer preparation. For example, Piletsky and co-authors have shown that one monomer could be sufficient for chiral separation of ephedrine enantiomers (Piletsky *et al.*, 2001). For large template molecules, which have a high number of functionalities, the modelling often predicts a more complex composition. Thus Chianella and co-authors developed an optimised composition for microcystin L-R which contained six monomers per one template (Chianella *et al.*, 2002, Chianella, 2003). Since OTA is a moderately large molecule (403 Da) with several functionalities I expected to have more than one monomer interacting with corresponding functional groups. Thus having 2-4 monomers was considered as an acceptable result.

Previous work made in our laboratory (data not shown) shows that an excess of strong anion-exchange monomers in the polymer led to high non-specific binding, which complicated the elution step in SPE. This was due to the strength of the electrostatic interactions formed between charged monomers and template (as seen in the Leapfrog table from the corresponding binding

scores which are more than double for charged molecules). Due to this I chose to synthesise a polymer with a ratio of 1:1 instead of 4:1 (Polymer B). The aim was to test the theory and increase the ratio later if required.

This whole process enabled the selection of monomer/template ratios for two polymers. The first based on neutral acrylamide and acidic methacrylic acid. The second polymer offered a system tailored toward operation in organics where a basic monomer (DEAEM) was expected to provide high affinity.

These polymer compositions appeared to have a certain degree of similarity between polymers designed in other groups. Thus Baggiani (Baggiani, Giraudi & Vanni, 2002) employed methacrylic acid in his study of OTA specific polymers. Other mycotoxins (deoxynivalenol and zearalenone) have also been imprinted using acidic monomers such as methacrylic acid and TFA or weak basic such as 4-VP (Weiss *et al.*, 2003).

4.3 Nuclear magnetic resonance

Proton nuclear magnetic resonance ($^1\text{H-NMR}$) experiments have been used successfully by several groups to demonstrate the interactions of the chosen monomers with its corresponding template and to characterise the recognition properties of the resultant polymer. Thus Lu and co-workers (Lu *et al.* 2003) used a simple titration of the template (4-L-phenylalanyl-amino-pyridine) by methacrylic to prove their interaction in monomer mixture.

Following this example it was decided that a study of any self-assembly mechanism between OTA and the chosen monomers formed under the chosen experimental conditions would benefit the overall understanding of the binding mechanisms, relating to the synthesised polymers, as well as support the modelling predictions.

The practical interpretation of the spectra of interacting compounds did not appear to be straightforward. The signals of the groups at points 6 and 7 were hard to assign due to the inherent structural similarities of corresponding groups. The peaks corresponding to those at points 2 and 8 were viewed as a multiplet as the system (270 MHz) was unable to separate them clearly. As shown later, the separation of the signals defining these two groups can be assigned in the presence of DEAEM, where the peaks, assigned to group 2 moves upfield due to electrostatic interactions.

The results show that both acrylamide and methacrylic acid do not interact with OTA in MeCN. Despite the addition of excess monomers the peaks corresponding to the sites that would be possibly affected by binding (CH-2 COOH-3) do not move. The NH (at 4) moves slightly but this not necessary serves as indication of complexation and could be due to its labile properties in solution.

Idziak (Idziak, Benrebouh and Deschamps, 2001) have shown that a complex formation between methacrylic acid and template such as 17- α -ethynylestradiol could be followed by NMR titration in toluene. Similarly the

binding of 2-aminopyridine to a methacrylic acid was observed in chloroform in $^1\text{H-NMR}$ experiments (Jie and Xiwen, 1999). Thus it was possible to make a conclusion that in acetonitrile interactions between monomers and template were very weak. The situation might be however different in more hydrophobic solvents where hydrogen bonds are much stronger as suggested by molecular modelling data.

In the presence of DEAEM, the multiplet between 4.75 and 4.90 is seen to separate into two distinct peaks, marked as 2 and 8. Looking at the position of the two groups, the shape of the peaks and comparing the results to the interactions suggested by the modelling and by previous studies of the NMR spectra of OTA (Pohland *et al.*, 1982) I can ascertain that the peak moving is CH-2. This is due to the possible complexation of DEAEM with the NH group adjacent to the CH-2 resulting in an upfield shift of approximately 0.3 ppm. The NH group shifts as well (data not shown) which might or might not be related to binding since, as it was discussed before, this alone does not indicate interaction (movement might be linked to labile properties).

The Leapfrog results suggest that DEAEM forms stronger complex with the template compared to other monomers such as methacrylic acid and acrylamide. The NMR demonstrated this, showing a clear interaction between the template and monomer which to some degree correlates with modelling results. DEAEM, the monomer predicted in the computer modelling to interact with OTA in acetonitrile solvent, demonstrates a strong interaction with the template in this solvent.

4.4 Polymer synthesis

Two computational polymers were synthesised using the ratios taken from the modelling results. Polymer A was synthesised using the results of the modelling experiment in vacuum; one acrylamide, methacrylic acid to one template. Polymer B was synthesised with one DEAEM molecule to one template.

4.4.1 Synthesis

In these polymer syntheses the choice of DMF (dimethyl formamide) as a porogen for the polymerisation reaction was justified by its power to dissolve completely the monomers, the cross-linker and OTA. In principle toluene would have made a better choice, since it is a better environment for monomer-template interactions, however it would not dissolve acrylamide. The cross-linker (EGDMA), the initiator, 1,1'-azobis(cyclohexane-carbonitrile) (ACCN) and the reaction conditions (production of polymer by heating), were chosen in this work for the synthesis of the receptor, as they are the most commonly employed in molecular imprinting. This allows comparison with previous protocols and each other (Baggiani *et al.*, 2001; Jodlbauer, Maier & Lindner, 2002; Piletsky *et al.*, 2002).

Both polymers were hard semi-transparent monolith structures, which, when ground, produced particles of the required size with yield corresponding to approximately 60% of the theoretical amount. Other 40% constituted fines with size < 5 μm . As OTA is highly toxic, and relatively expensive small amounts were used (5 mg) to produce the monoliths. This led to the

requirement of careful wet grinding, and ensuring all materials can be used. The small amounts produced by the monoliths (total weight = 100 mg) precluded the possibility of packing HPLC columns or performing analysis which might lead to the loss of the material. Therefore it was decided to pack cartridges with 30 mg of each polymer, allowing each monolith to produce ~2 cartridges each.

4.4.2 Washing

The preparation of the cartridges of both polymers involved small amounts. This required the use of wet grinding, to ensure the particles were not lost as dust. This was performed in methanol as it made the polymers easy to dry.

4.4.2.1 Polymer A

During the experimental testing it was found that methanol and water elute loaded OTA efficiently from polymer A, therefore the assumption can be made that a fair amount of OTA was eluted from the polymer during grinding and sieving. This complicated the measurement of the precise quantity of eluted template. Initially I extensively washed the packed cartridges with a series of organic solvents and water, and measured each of the eluents until no fluorescence was seen. Later preparations involved washing the cartridges with methanol and water in excess, as it was shown that these elute OTA from the polymer successfully.

The packed cartridges were shown to have slight fluorescence under UV light, suggesting that some OTA remained trapped in the polymer matrix. This

template slowly leached out from the matrix, however it was at a rate which did not affect any measurements during rebinding experiments. Comparable experiments described in the literature suggest >90% of the template could be removed (Mena *et al*, 2002). It was impossible to determine excess of template elution in our experiments due to change in fluorescence of the template as result of its partial oxidation during polymerisation (as can be seen from darkening of the polymer and eluate solution) and large variations in OTA fluorescence in response to change in the environment.

4.4.2.2 *Polymer B*

A serious problem was encountered with the removal of template from Polymer B. Despite several solvents been tested for efficacy, not one solvent performed well enough to be used for extraction. A signal was still seen in all eluates at the scale of 10 ppb (enough to intervene with detection). Therefore an electrophoretic technique was employed to remove the template, using sodium perchlorate in acetonitrile as electrolyte (Piletsky, Butovich & Kukhar 1992). This salt dissolves in organic solutions (acetonitrile) allowing a charge to be passed through it. The aim was to drag the template (charged in the presence of the salt) out of the matrix. This method had a marked effect on the removal of template from the polymer, however despite numerous washes the leaching of template was still seen at the scale >5 ppb. This was enough to cause a problem for the detection as the required level of detection is between 2-5 ppb. Similar problems were encountered by other researchers who worked on the development of MIP based SPE materials (Andersson, 2000).

This result, although meaning that Polymer B could not be used for SPE analysis due to the leaching, offered support to the modelling and NMR results. The NMR results demonstrated that DEAEM interacts with OTA in acetonitrile and this led to the polymer's ability to retain the template, even under harsh washing conditions. As expected DEAEM interacted strongly with OTA and this mirrors other work, performed by our group, demonstrating that an excess of strong anion-exchange monomers in the polymer leads to high non-specific binding. The observed leaching would be even more pronounced upon increase in the concentration of monomer in the MIP. This prevented any continuation with further experiments in this direction.

4.4.2.3 *Membranes*

The membranes were formed between two glass sides, with spacers to ensure even thickness. The polymer underwent radical initiation by UV. Initial experiments with blank materials identified the length of UV exposure required, the amount of solvent and the need for a plasticiser. This plasticiser was added to cross linker to increase the flexibility of the final material (Sergeyeva *et al.*, 1999b). Once this had been completed imprinted and non-imprinted membranes were produced for both Polymer A and Polymer B. It was noted that the polymer membranes were stable, robust, and optically transparent and kept their flexible nature during the course of the experiments.

The removal of OTA from the membranes proved as difficult as from the polymer monolith. Therefore, despite extensive washing in a range of solvents, residual OTA remained in the membrane, giving a high fluorescent signal.

It was possible to decompose OTA by UV radiation into fragments that had significantly lower fluorescent signal. Therefore I decided to “burn” the membranes using the powerful UV source used for polymerisation. This was performed after a solvent wash, aimed to remove any initiator from the membrane, to stop any further polymerisation. After the “burning” the membrane was washed again to remove some traces of fragmented OTA.

4.5 Measurement of OTA by fluorescence

Fluorescence, be it natural as in the case of OTA, or with the addition of a marker (fluorophore), is one of the most common methods for the detection of compounds in chromatography. A fluorimeter can be incorporated into any simple HPLC protocol as the detector (Shephard *et al.*, 1990; Jimenez, Mateo and Mateo, 2000; Chiavaro *et al.*, 2001).

Fluorescent assays are some of the best adapted to use with MIP as they are highly sensitive and can be performed in practically all conditions, depending on the label and type of the polymer used (Haupt, 1998). For example MIP specific for template/tagging agent complex was used to detect homocysteine (Chow, Lam & Leung, 2002). Suarez-Rodriguez and Diaz-Garcia used a methacrylate MIP to develop a flow cell sensor system for chloramphenicol

with a detection limit of $8 \mu\text{g mol}^{-1}$. A similar technique was employed for flavonol (Suarez-Rodriguez & Diaz-Garcia, 2000; Suarez-Rodriguez & Diaz-Garcia, 2001). The group of Darling (Wandelt *et al.*, 2002) demonstrated the ability of using an in-built fluorescent marker (in this case a functional monomer, which emits a signal upon binding with the template) to measure the specificity and mechanisms of binding on a MIP specific for adenosine 3'5'-cyclic monophosphate (cAMP).

Since OTA has natural fluorescence its detection does not require use of any fluorescent tags or enhancers. The measurement of OTA in the eluted solution could be easily detected by fluorimetry.

It is known that the fluorescence of a compound in solutions is dependent on several factors. These include the structural properties of the compound, the solvent in which it is dissolved, the pH, the presence of hydrogen bonding, temperature and the presence of quenching materials, such as oxygen (Lakowicz, 1983). It has been shown that any changes in these will affect the signal of OTA, hence the measurement protocol was designed to counter these variations.

As the project required any solutions to be passed through the charged polymeric materials (for example MIP and IEX cartridges) and be exposed to natural materials which could quench (or enhance) signal, it was important that a method was used to ensure that all results could be comparative. To do this, I used control samples that were prepared by treating corresponding

solutions in exactly the same way (e.g. filtering through the polymer cartridge). These samples were spiked with the expected maximum concentration of OTA, defined as positive standards. Non-spiked samples were defined as negative standards. Therefore to analyse change in OTA concentration the fluorescence of each sample was compared to both, positive and negative standards.

This method relies on fact that the positive sample, treated under the same conditions as an experimental sample should have the same properties and therefore have the same effect on the fluorescence on any OTA present. The identification of OTA in solution was performed primarily in a sodium phosphate buffer. The excitation wavelength was 379 nm, as any changes, caused by alterations in solution in our experiments did not affect this value. The peak maximum in the positive sample was used as the reference point for the steady state fluorescent measurement.

4.6 Characterisation of polymer A

4.6.1 SPE analysis of polymer behaviour

The simple format of SPE is ideal to study the specific recognition, sample clean up and pre-concentration abilities of MIPs; therefore it is one of the most widely investigated. Numerous protocols in the literature demonstrated the benefits of replacing the traditional reverse phase material (C18), normal phase (silica) or specific anion or cation-exchanger material with a MIP adsorbents specific for the templates of interest (Andersson, 2000). These have been used mainly for bioanalysis and pharmaceutical applications, with

only a few examples of the application of MIP-SPE in environmental samples. (Masqué, Marcé & Borrull, 2001) For example, Bjarnason (Bjarnason, Chimula & Ramstrom, 1999) extracted, and concentrated triazines (herbicide) from urine and apple extract, in a SPE format with methacrylate MIP. The same family of molecules was shown to be able to be extracted from water samples (Matsui, 2000b) and this was furthered by the group of Ramstrom (Pap *et al.*, 2002) who demonstrated the extraction and clean up from river samples. The high affinity of these systems allowed detecting very low concentrations of samples. The past successes brought confidence in this approach for my work. This, combined with the cost prohibition of an OTA MIP for HPLC, meant that SPE was the ideal choice to analyse the properties of the material.

The choice of solvent is important for loading analyte and polymer washing. It was shown that this MIP did not bind OTA in organic solutions such as methanol, acetonitrile and chloroform. Even the addition of 10% of acetonitrile lowered the binding by ~70%. This is an uncommon phenomena as most polymers demonstrate binding in similar solvents to those used in polymerisation (Stevenson, 1999). Since all non-covalent forces are influenced by the properties of the solvent, non-polar solvents normally lead to the best recognition. The lack of binding could be explained however by the fact that due to solubility problem I was unable to make polymer in toluene which would be the ideal solvent for this polymer. Thus absence of binding in organic solvents was not totally surprising. The binding of OTA was found to be ~100% in a sodium phosphate buffer solution over the range of

concentrations required by the EU project. Excellent binding in aqueous environment is most likely resulting from the combination of two factors: (i) shape complementarity and (ii) enhanced Van der Waals and hydrophobic interactions. As OTA has to be extracted from maize in an organic solvent, any protocol had to incorporate the transfer of OTA from an organic medium to aqueous. However, it did not discount the use of the polymer in aqueous mediums such as grape juice and wine.

Further study of this system shown that the concentration and pH of the buffer solution affected the affinity of OTA to the polymer, with high pH (>8) and weaker buffer concentrations (<50 mM) causing a drop in polymer affinity.

As the fluorescence signature of OTA is low in acidic solutions, a compromise of pH 7.5 was selected for all future experiments. The buffer with this pH provides good binding conditions (~95% binding) whilst guaranteeing a high fluorescent signal. This is also similar to pH of many biological solutions, which might prove useful in further application of this material. From the washing steps I had seen that methanol worked well, however it was also noted that de-ionised water also removed OTA from the polymer. The buffer wash in between demonstrates that the material is not caught in the polymeric matrix by hydrophobic interactions. The effect of organic solvent elution was considered to be non-specific, similar for both MIP and blank polymers, unlike the elution of the template from the polymer in de-ionised water, which was significantly more pronounced for the MIP.

4.6.2 Cross reactivity

As described in the previous section, the selectivity of the elution step has been demonstrated, however the specificity of the system had yet to be proven. The cross reactivity studies were performed using HPLC-MS, as some of the analogue compounds did not have good fluorescence. The experiment was based on the recovery of the compounds in de-ionised water, as the mass spectrometry equipment is sensitive to salts.

Materials with sterical and chemical similarity to OTA were kindly donated by Professor Baggiani from the University of Turin. L-Phe-CHNA was the closest OTA analogue whereas CHNA and phenylalanine (Phe) resembled the different smaller fragments of the OTA structure.

As the results show the recovery of OTA, compared to the analogues is higher, suggesting that the polymer had specificity towards the template. Two other analogues had lower recovery suggesting that the recognition of the template by the polymer is not only sterical but also requires (or depends on) more than one point of interaction, leading to better interaction with the larger molecules.

It is not uncommon for the loading solvent to be an aqueous sample (Pap *et al.*, 2002) as the analyte would be bound by a combination of electrostatic forces and hydrophobic interactions. However, as shown previously, success in selective binding and recovery of the template relied on the presence of some concentration of organic solvent to obtain any specificity (Matsui *et*

al.,2000b; Bjarnason, Chimula & Ramstrom, 1999). The demonstration of selective binding and elution with water is novel.

4.6.3 Polymer conformation

The morphology of a MIP is affected by the surrounding medium. Thus, for the most of published MIP systems the swelling was most pronounced in chlorinated solvents, such as chloroform and dichloromethane, compared to more polar solvents such as acetonitrile. This swelling may lead to changes in the three-dimensional positioning (including spatial distance) of the functional groups taking part in the recognition in the sites resulting in poorer binding capability. The observations of the similar effects in MIP systems were made before (Wulff, Pol, & Minarik, 1986; Allender, Brain & Heard, 1997). The polymers studied here however were unique in the sense that change in swelling was used effectively to control binding/elution.

The effects of the solvents on the polymer conformation were studied using an image analysis method. This system was chosen, as the laser scatter method, normally used for particles analysis (Allen, 1990) had complications due to the glassy nature of the polymer, which caused intense reflection. Images of the polymer particles in solution were taken, and processed by software which calculated the surface area of the particles in the sample. Careful sieving allowed for a small fraction of particles to be isolated, which minimised the distribution in particle size.

The size of polymer particles in water was used as a standard. By measuring differences in size between solvents I was able to calculate swelling effect. A decrease in average particle size was seen in the presence of buffer. The results also show a greater inference of this trend in the MIP than in the blank, suggesting that the specific binding sites may be involved.

It was clear from the SPE experiments that the presence of ions in solution affected the affinity of the polymer. The binding was greater at low pH and weaker at high pH where carboxylic functional groups in the polymer were ionised. From the particle size analysis I can clearly see that the polymer shrinks in buffer where repulsive forces between charged polymeric groups are shielded. This could be explained by the Donnan effect (Alberty & Silbey 1997). This describes the effect of ionic strength and pH on the occurrence of localised charges within the structure (MIP). The Donnan effect explains satisfactorily the influence of pH and ionic strength on the degree of swelling and changes in binding.

From Figure 3.17 the conclusion can be drawn that the binding of template occurs in buffer where the polymer is shrunk. As a result the functional groups in buffer will be close enough to ensure multiple interactions between template and functional groups in the binding cavity. When exposed to water and DMF swelling is seen and some interactions will be disrupted due to increased distance between the functional groups on the polymer and the template. As a result the template is released.

A seeming contradiction appears between no binding in DMF and the polymerisation conditions, where DMF was used as solvent, where in theory the binding cavities are formed. An explanation for this would be in the

polymerisation mixture as well as DMF there is the presence of functional monomers, cross linker, initiator and template which obviously differs from pure DMF. It is also true that the polymer matrix is more compact straight after polymerisation and swells after removal of template (Wulff, 1995; Piletsky 1998). Therefore in DMF alone the distance between polymer functional groups will be larger than those that exist in newly formed polymer hence the swelling. It would be possible to speculate that the arrangement of polymer functional groups in the binding cavities in the presence of buffer are the same or similar to those in a newly formed polymer.

Therefore I can put forward the theory demonstrated in Figure 4.1.

1. The presence of the buffer ions affects the conformation of the polymer, by altering the net charge on the polymeric chains forming the matrix.
2. This conformational change directly affects the structure (size of cavities) of binding sites specific for OTA, making them favourable or unfavourable for binding of template (depending on environment).
3. In opposite to this the presence of de-ionised water or organic solvent reverses the effect that buffer has, altering the conformation to a state unfavourable for binding, hence elution is possible.

In addition, binding in water could be more complicated due to increase in repulsion forces between similarly charged template and polymer functional groups.

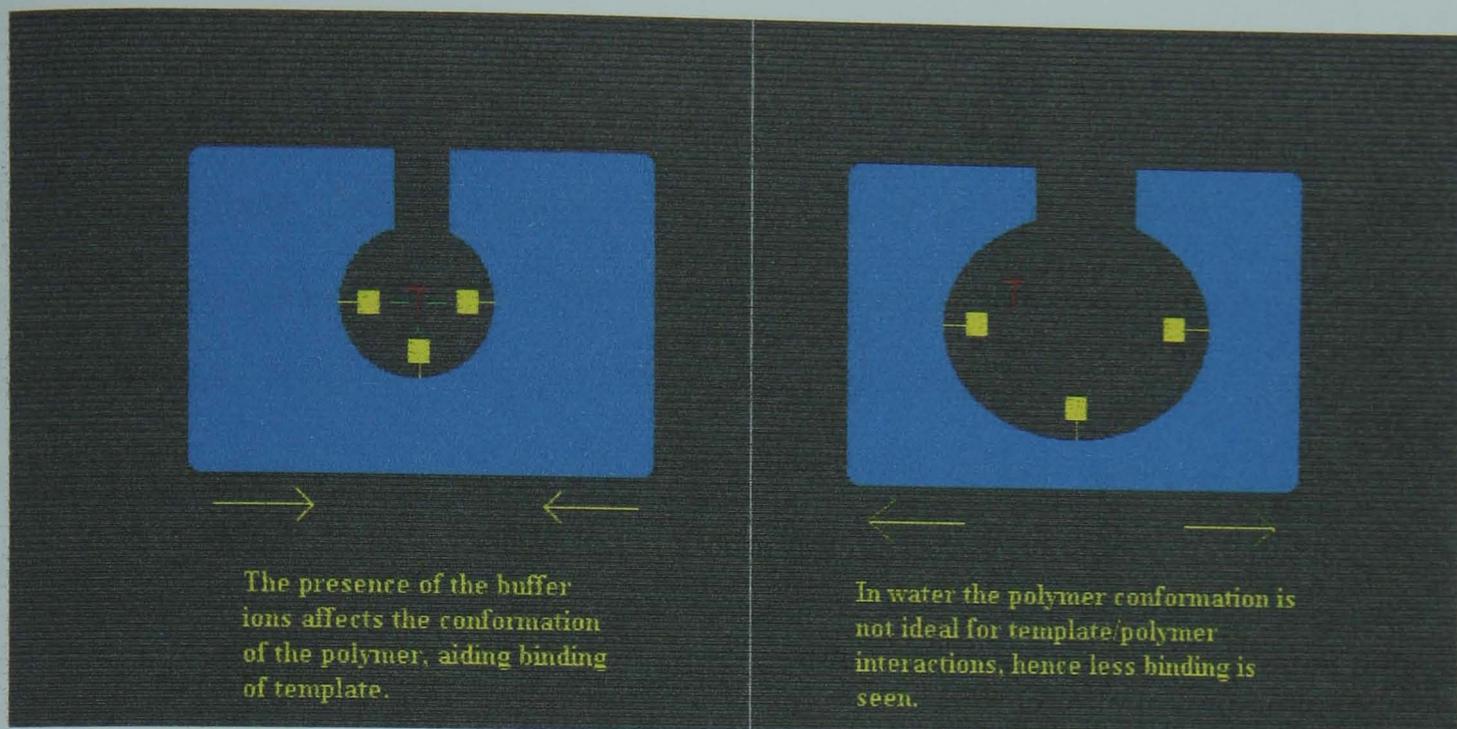


Figure 4.1: Representation of the switch between conformations caused by presence of buffer or water on the polymer.

MIP recognition properties in particular depend on shape, size and structure of binding sites or “pockets” formed during polymerisation. These pockets are subject to the flexible nature of the polymer, which can be affected by environmental changes (Sellegren & Shea, 1993). This is however, the first example of such nature where binding in MIP systems can be completely and reversibly “switched” by changing ionic properties of the solution.

In the future development the role of changes in pH and buffer concentration on particle size and on the binding of the template could be studied to find a way to maximise this effect. As shown in the binding studies, pH and ionic concentrations play an important role and we can expect that the morphology of the polymer will be affected by these. This is due to the pH and ionic strength of solution affecting the net charge on the polymer, which in turn will affect the conformation hence net size of polymer particle.

This method (image analysis) can only produce a qualitative result, demonstrating the change in polymer size. To attempt an analysis to produce a scale change against pH or ionic strength would prove difficult due to the standard errors seen between sieved particle sizes when compared with net change in polymer size. When sieving, a range of particle sizes can be obtained and this range will cancel out any small changes in size caused by different solutions. Only when demonstrated with great differences can the phenomena be seen effectively.

Other techniques such as surface potential measurement, measurements of electrokinetic effects, solid state NMR, and neutron diffraction could be used to understand the mechanisms of selective recognition and exploiting them for the development of new type of sensors, assays and SPE materials.

4.7 Membrane analysis

The selective properties of MIP membranes have been well documented and exploited (Piletsky et al., 1999). The permeability and transport properties of the membrane, linked with its unique selectivity has been used in a wide range of chemical separations, such as amino acids (Yoshikawa et al., 1995; Yoshikawa et al., 1998), herbicides (Kochkodan, Weigel & Ulbricht, 2002) and cAMP (Hilal et al., 2003). The selective permeability of the membrane, in the presence of its template (gate effect) was demonstrated by both Hattori and Yoshimi (Hattori, Yoshimi & Sakai, 2001; Yoshimi et al., 2001). Sergeyeva developed a herbicide sensor, based on the conductometric properties of a

membrane specific for atrazine (Sergeyeva et al., 1999a). However, as OTA is naturally fluorescent, the membrane's transport or permeability properties were not needed as the aim was to use the membrane as a freestanding material, robust enough to withstand the rigour of been used as a "dip" sensor. The bound material would be directly measured optically using a fibre optic probe, similar to the system employed by Yusof and Ahmad (Yusof & Ahmad, 2002) for the detection of lead.

To measure OTA adsorbed by membrane I had to find a protocol that was simple and offered minimal room for experimental error, such as the possibility of interference of reflected light by the membrane. The platform employed a simple support made from black plastic, which adsorbed light passing through the membrane. The fibre optic probe was placed within a dark box, which stopped the interference of external light. The distance between the lens of the probe and the support was kept constant to ensure that results were comparable. The fibre optic bundle was split into two types of fibres; half of which emit light and the other half collect and carry the light emitted from the polymer back to the fluorimeter.

The λ^{Ex} of 379 nm was used to excite the OTA and the emission peak was measured at the highest point of the curve (~424 nm). This decrease in emission wavelength, compared to fluorescence of OTA in solution was due to the influence of the solid phase (Lakowicz, 1983).

By using this technique I was able to measure OTA bound to the polymer, without the need for a comparative standard, as the amount bound to the polymer during an experiment can be derived from measurements of the membrane, before and after. In the future a differential signal can be obtained by measuring simultaneously MIP and blank membrane which can increase the selectivity of the measurements.

The work in the previous section shows the unique properties of polymer A, and the aim was to exploit them in membrane/sensor format in aqueous media. Likewise I intended to test Polymer B membranes (not applicable for SPE analysis due to leaching problems) in acetonitrile, the solvent used for OTA extraction from maize. I expected that the problem of leaching would be irrelevant, since only the binding would be measured (leaching occurs during elution step) and membranes will be made disposable. This makes this polymer recipe suitable for sensor use in organics.

4.7.1 Polymer A

Before any experiments with natural samples could be performed the polymer A membrane was used to demonstrate the difference in binding between the MIP and NIP. A series of solutions, containing increasing concentrations of OTA in sodium phosphate buffer were used to show that the MIP has superior affinity over its blank, as described in Figure 3.15. As well as performing this task experiment demonstrated the relative simplicity of the system and the possible applications MIP membranes in a “dipstick” sensor. Further analysis

proved that the polymer A membrane did not bind OTA from acetonitrile, supporting the data from the SPE analysis and NMR.

Having proven its affinity to OTA in buffer solutions, it was decided to test abilities of Polymer A in grape juice and white wine. These foodstuffs could be possibly contaminated with OTA (Visconti, Pascale & Centonze, 2000; Leitner *et al*, 2002) and a sensor system for them would be a useful tool.

Both blank and MIP were tested and proved to be successful in binding OTA from these solutions. The MIPs greater affinity was evident and this, combined with reasonably low standard deviation offered a level of detection ~100 ppb, which, for such a simple set-up was acceptable.

Although the results made with grape juice, chosen as it did not contain any ethanol (negative effects of organic solvent on binding has been shown in the SPE analysis) were better than the results with wine. The calibration curve shows that it still would be impossible to detect OTA in the concentrations below 100 ppb without further improvement in homogeneity of formed MIP membranes and optimisation of fibre optic interface.

4.7.2 Polymer B

The characterisation of polymer B by SPE was complicated by leaching of the template, however from the binding experiments performed with this material I have seen that it possesses strong affinity for the template allowing testing of the membrane in acetonitrile. The initial results demonstrate that the MIP was

successful in binding OTA from acetonitrile and a reasonable level of detection was attained (~100 ppb) in model solution.

The next experiment attempted to use corresponding MIP and NIP to bind OTA from a maize extract. This experiment gave negative results, with no increase in signal, as observed in experiments with the model sample. A further experiment with a membrane, which had not undergone OTA “burning”, was performed to see if this part of the protocol had affected the polymer affinity. Again this was unsuccessful.

The conclusion was drawn that components of the extract were interfering with the chemical interactions required for selective binding between OTA and polymer. This corresponds to results obtained with polymer A and maize extracts as described in section 4.8.

Overall the outcome of the membrane work was considered successful. I had demonstrated that the membrane has potential application in a “dipstick” sensor, and I have developed a protocol, which can detect OTA in grape juice and in wine, although the levels of detection were 50x greater than required. Further optimisation of the membrane manufacture could provide better homogeneity of formed MIP membranes and this would lower the standard error. Optimisation of fibre optic interface (fixed support for the membrane) will improve reading and increase accuracy of the measurements along with optimisation of the distance between lens and membrane will allow for maximum signal to be collected (Yusof & Ahmad, 2002).

It would also be possible to deposit the MIP onto the fibre optic sensor producing a specific probe, using UV light emitted from the fibre optic to “grow” the polymer on its tip. This guide would be capable of measuring analyte by dipping tip of the sensor into the test solution.

4.8 SPE experiments for maize analysis

The extraction from maize is an important step in OTA detection protocol. Hydrophobic toxins, such as OTA require the use of organic solvents (Holcomb, 1992; AOAC, 1997). The direct extraction with acetonitrile was chosen as the use of chlorinated solvents, such as chloroform, are gradually been phased out due to ecological pressure (Montreal Protocol, 2003).

A buffer solution for elution was selected as the extraction from maize by these materials was intended as the first step in a full extraction, clean-up and quantification protocol for OTA in maize. As the MIP had demonstrated specific binding in buffer, the elution solution had to be the same else complications and time issues would arise transferring the OTA between solvents.

Once this had been performed, several materials were tested to transfer the OTA from organic into aqueous solution compatible with polymer A. Four types of commercial SPE ion exchange cartridges (IEX) (50 mg) were chosen which were used before for sample clean up (Pelegri *et al*, 1997). In addition

50 mg of kieselguhr silica (Celite 545) and 50 mg of activated carbon/activated aluminium oxide, used previously in our laboratory for clean-up of mycotoxins such as Deoxynivalenol were tested on their ability to recover OTA. Lastly I tested 30 mg cartridge of the polymer B blank which had demonstrated strong affinity for OTA in organic solvents.

The silica (Celite 545) bound very little of the interfering fluorescent material from the extract, however when binding OTA from a model solution it performed poorly, with only ~60% of the OTA adsorbed by the material. Overall recovery level was very poor, below 30%. This was deemed not sufficient, as the limits for detection set by the EU are low, and as much material as possible needs to be recovered.

The activated carbon/aluminium oxide bound all of the interfering extract as well as all the OTA from the test solutions, however the required elution solutions with water or buffers failed to remove any of the material, therefore this was discounted for use as an extraction agent.

The blank (NIP) of polymer B was tested and found that it bound OTA from acetonitrile, however not in sufficient quantities to warrant its use in an extraction protocol. It was also noted that, in previous washing experiments the recovery of OTA from the polymer was difficult, which would have led to problems in later stages.

The IEX cartridges were tested in the same conditions as the other materials.

Firstly a model sample of OTA was loaded on to the cartridges, and it was found that all materials bound OTA over 90%. The elution step, performed with sodium phosphate buffer at 50 mM, pH 7.5 isolated an ideal candidate. The "NH₂" cartridge (weak anionic) released over 70% of the bound material in the first 3 ml wash. This was confirmed and chosen for use within the protocol. In order to complete the study of the "NH₂" material an extract of maize was loaded onto the cartridge as previously described. The results showed that although a fair part of the interfering extract is bound to the cartridge, very little fluorescent interference could be released in the same conditions which were used to elute OTA.

The weak anionic material appeared to have useful properties in model solutions required for extraction of OTA, therefore in a next step the extraction of OTA in a maize extract was studied. Initial experiments led to recovery of OTA in quite low concentrations. The recovery was increased by removal intermediate acetonitrile washing step and by adding SDS, a surfactant to aid elution. This set of experiments resulted in the development of a practical working OTA extraction protocol from maize extract. Recoveries of ~70%, were considered suitable for integration of recovery protocol with MIP sensor. To validate this opportunity the sample of extract with OTA acquired from an extraction with the IEX cartridge was loaded onto the MIP made of polymer A to produce a sample suitable for measurement by fluorescence. If successful a protocol for extraction, clean-up by two cartridges and fluorescent measurement were to be optimised and a sensor designed which exploits this protocol for OTA detection.

However it was found that the presence of certain components in the extract, even after IEX clean up, severely affected the binding of OTA to the MIP. It was theorised that part of the extract (undetected by fluorescence) was interacting with the OTA and preventing its binding with the MIP. It was suggested that a lipid might form a complex with OTA due to its hydrophobic nature and prevent its binding to MIP. A model sample of OTA in buffer was spiked with a phospholipid and loaded onto the polymer. It exhibited the same lack of binding seen in the extract, supporting this theory. Attempts were made to remove the lipid from the extract, however these were unsuccessful as they resulted also in reducing the recovery level of OTA from IEX cartridge.

It was demonstrated also in experiments with the membrane made of polymer B that the presence of extract interferes with the basic binding between template and polymer. The addition of surfactants or chemicals to remove interfering materials also reduced polymer affinity, in turn affecting binding. Thus although the IEX extraction protocol might be useful for OTA measurement by HPLC, the integration of IEX with MIP polymer as a further clean-up material, or as the part of a sensor device (piezoelectric, optical membrane, or conductometric) proved to be too difficult with a current experimental set-up.

In conclusion, the partial success achieved with developing ion-exchange cartridge capable to extract OTA from organic solvent and transfer it to water

can be further exploited in developing new protocols for toxins analysis by HPLC.

Chapter 5

Conclusions and Further Work

4.1 Introduction

In this section I summarise the work presented in the previous four chapters and conclusions, based on the experimental evidence are presented. Also presented are recommendations for further study in and around this work, with explanations to the reasoning behind the need for this work.

4.2 Conclusions

- Two polymers for ochratoxin A were synthesised by using molecular imprinting technique. The composition of the two polymers (monomers/template ratio) was optimised by computer modelling in different conditions representing a vacuum (the standard modelling scenario) and in the presence of acetonitrile.
- NMR was used to confirm the modelling results in acetonitrile. It was found that methacrylic acid and acrylamide did not interact with OTA under these conditions. DEAEM was found to interact with OTA, with the site of interaction at the NH residue.
- The final results of the computational design, and NMR analysis led to two recipes, which were used to produce the polymers. Polymer A was made using a ratio of one acrylamide molecule, one methacrylic acid molecule to one molecule of OTA. Polymer B was made from a ratio of one DEAEM molecule to one molecule of OTA.

- Polymer A was found to have a novel and previously undescribed binding mechanism, in which, the recognition of template by the polymer depended on the presence of an aqueous buffer.
- The presence of organic solvents severely affects the binding capabilities of this polymer. Bound template can be eluted with de-ionised water and organic solvent.
- Cross reactivity studies demonstrated the specificity of this polymer with respect to recovery of template in water.
- Analysis of polymer conformation was performed using a novel image analysis technique. It was demonstrated that the polymer structure is affected by solvent change, and this corresponds to the binding data.
- A theoretical binding mechanism is presented and discussed.
- Both polymers were used in attempts to develop a sensor system for OTA. Imprinted membranes were produced of polymer A and polymer B. Washing of these proved difficult, and a technique known as “burning” was utilised to reduce the background fluorescent signature of OTA.
- The membrane of Polymer A demonstrated uptake of OTA from grape juice and wine samples, and preliminary results suggest the possible use of this material in a “dipstick” sensor.

- The membrane of polymer B demonstrated uptake of OTA from acetonitrile, however any attempt to use this in conjunction with a maize extract proved unsuccessful.
- Attempts to develop a protocol to isolate OTA from a maize extraction produced mixed results. A method was developed, using a commercially available ion exchange material in which, OTA could be recovered with removal of a high percentage of interfering extract.
- Any attempt to provide further clean-up by a polymer A SPE cartridge was hindered by the presence of lipids in the extraction, which interfered with the binding of template to polymer.

5.3 Further work

The novel binding mechanism of polymer A requires further study. The role of changes in pH and buffer concentration on particle size and on the binding of the template needed to be studied to find a way to maximise the changes in polymer conformation. Further cross-reactivity studies can be performed in an attempt to characterise the shape and requirements of the binding pockets.

Other techniques such as surface potential measurement, measurements of electrokinetic effects, solid state NMR, and neutron diffraction could be used

to understand the mechanisms of selective recognition and exploiting them for the development of new type of sensors, assays and SPE materials.

The removal of the leaching, seen in polymer B needs to be addressed, as this polymer demonstrates the possibility of use with a direct extraction of OTA from organics. Once this has been performed the binding and elution studies of this material need to be characterised.

With respect to the membrane sensor system, the requirements of producing more homogenous polymers is a clear priority. Study into the manufacture of these, and obtaining the “perfect” membrane needs to be addressed. The washing step, and reduction/removal of any background signal needs to be studied, as this will increase the sensitivity of any sensor system.

The measurement system, based on a fibre-optic probe requires the optimisation of the polymer support, both with fixing the membrane in a holder and positioning it in the exact position each time. A simple locking slide mechanism would suffice.

On a side note, the use of polymer membranes with fluorophoric monomers (those that emit a fluorescent signal upon binding), would provide the basis of a generic membrane sensor technology based on molecular imprinting.

The possibility of integrating the polymer onto the fibre-optic probe would remove the requirements for a membrane.

The extraction protocols, based around SPE, can be developed further. The work developed here clearly shows the potential of this type of clean-up. Study into the removal of the interfering materials, perhaps by liquid/liquid extraction, must be a priority as with these materials gone, the market potential of a sensor system linked with specific properties of the imprinted polymers developed in this project is high.

Chapter 6

References

- Akiyama, H., Goda, Y., Tanaka, T. & Toyoda, M. (2001).** Determination of aflatoxins B1, B2, G1 and G2 in spices using a multifunctional column clean-up. *Journal of Chromatography A* **932**, 153-157.
- Alberty, R. A. & Silbey, R. J. (1997).** *Physical Chemistry* 2nd edn. New York: John Wiley and Sons Inc.
- Allen, T. (1990).** *Particle size measurement*. New York: Chapman and Hall.
- Allender, C. J., Brain, K. R. & Heard, C. M. (1997).** Mobile phase effects on enantiomer resolution using molecularly imprinted polymers. *Chirality* **9**, 238-242.
- Andersson, H. & Nicholls, I. (1999).** A historical perspective of the development of Molecular imprinting. In *Molecularly Imprinted Polymers. Man-made mimics of antibodies and their applications in analytical chemistry*, pp. 1-17. Edited by B. Sellegren. Amsterdam: Elsevier.
- Andersson, L. I. (2000).** Molecular imprinting for drug bioanalysis: A review on the application of imprinted polymers to solid-phase extraction and binding assay. *Journal of Chromatography B: Biomedical Sciences and Applications* **739**, 163-173.
- Ansell, R. J., Kriz, D. & Mosbach, K. (1996).** Molecularly imprinted polymers for bioanalysis: chromatography, binding assays and 'biomimetic sensors'. *Current Opinions in Biotechnology* **7**, 89-94.
- AOAC. (1997).** *Official methods of analysis of AOAC International*. 16th Ed. 3rd Rev. Gaithersburg: AOAC Int.
- Arshady, R. & Mosbach, K. (1981).** Synthesis of substrate-selective polymers by host-guest polymerisation. *Makromolekulare Chemie* **182**, 687-692.
- Baggiani, C., Giovannoli, C., Anfossi, L. & Tozzi, C. (2001).** Molecularly imprinted solid-phase extraction sorbent for the clean-up of chlorinated phenoxyacids from aqueous samples. *Journal of Chromatography A* **938**, 35-44.
- Baggiani, C., Girauldi, G. & Vanni, A. (2002).** A molecular imprinted polymer with recognition properties towards the carcinogenic mycotoxin ochratoxin A. *Bioseparation* **10**, 389-394.
- Bartels, H. (1967).** On the order in silica gel with specific adsorption power. *Journal of Chromatography* **30**, 113-116.
- Bauer, J. & Gareis, M. (1987).** Ochratoxin A in der Nahrungsmittelkette. *Journal of Veterinary Medicine Series B* **34**, 613-627.

Becker, M., Degelmann, P., Herderich, M., Schreier, P. & Humpf, H. (1998). Column liquid chromatography–electrospray ionisation–tandem mass spectrometry for the analysis of ochratoxin. *Journal of Chromatography A* **818**, 260-264.

Beckett, A. & Youssef, H. (1963). Active sites in stereoselective adsorbents as models of drug receptors and enzyme active sites. *Journal of Pharmacy and Pharmacology* **15**, 253T-266T.

Betina, V. (1989). Mycotoxins: chemical, biological and environmental aspects. Amsterdam: Elsevier.

Biró, K., Barna-Vetró, I., Pécsi, T., Szabó E., Winkler, G., Fink-Gremmels, J. & Solti, L. (2003). Evaluation of spermatological parameter in ochratoxin A-challenged boars. *Theriogenology* **8847**, 1-9.

Bjarnason, B., Chimuka, L. & Ramstrom, O. (1999). On-line solid-phase extraction of triazine herbicides using a molecularly imprinted polymer for selective sample enrichment. *Analytical Chemistry* **71**, 2152-2156.

Brera, C., Caputi, R., Miraglia, M., Iavicoli, I., Salerno, A. & Carelli, G. (2002). Exposure assessment to mycotoxins in workplaces: aflatoxins and ochratoxin A occurrence in airborne dusts and human sera. *Microchemical Journal* **73**, 167-173.

Bullerman, L. B., Schroeder, L. L. & Park, K-Y. (1984). Formation and control of mycotoxins in food. *Journal of Food Protection* **47**, 637-646.

Chianella, I., Lotierzo, M., Piletsky, S. A., Tothill, I. E., Chen, B., Karim, K. & Turner, A. P. F. (2002). Rational design of a polymer specific for microcystin-LR using computational approach. *Analytical Chemistry* **74**, 1288-1293.

Chianella, I., Piletsky, S. A., Tothill, I. E., Chen, B. & Turner, A. P. F. (2003). MIP-based solid phase extraction cartridges combined with MIP-based sensors for the detection of microcystin-LR. *Biosensors and Bioelectronics* **18**, 119-127.

Chianella, I. (2003). Development of affinity sensor for microcystin L-R based on a computational designed molecularly imprinted polymer. Ph.D. Thesis. Cranfield Press.

Chiavaro, E., Dall'Asta, C., Galaverna, G., Biancardi, A., Gambarelli, E. & Dossena, A. (2001). New reversed phase liquid chromatographic method to detect aflatoxins in food and feed with cyclodextrins as fluorescence enhancers added to the eluent. *Journal of Chromatography A* **937**, 31-40.

- Chow, C., Lam, M. H. M. & Leung, M. K. P. (2002).** Fluorescent sensing of homocysteine by molecular imprinting. *Analytica Chimica Acta* **466**, 17-30.
- Creppy, E. E. (2002).** Update of survey, regulation and toxic effects of mycotoxins in Europe. *Toxicology Letters* **127**, 19-28.
- Dawlatana, M., Coker, R. D., Nagler, M. J. & Blunden, G. (1996).** A normal phase HPTLC method for the quantitative determination of ochratoxin A in rice. *Chromatographia* **42**, 25-28.
- Delgado, T., Gómez-Cordovés, C. & Scott, P. M. (1996).** Determination of alternariol and alternariol methyl ether in apple juice using solid-phase extraction and high-performance liquid chromatography. *Journal of Chromatography A* **731**, 109-114.
- De Saeger, S., Sibanda, L., Desmet, A. & Van Peteghem, C. (2002).** A collaborative study to validate novel field immunoassay kits for rapid mycotoxin detection. *International Journal of Food Microbiology* **75**, 135-142.
- Dickey, F. (1949).** The preparation of specific adsorbents. *Proceedings of the National Academy of Science of the USA* **35**, 227-229.
- D'Mello, J. P. F., Placinta, C. M. & Macdonald, A. M. C. (1999).** *Fusarium* mycotoxins: a review of global implications for animal health, welfare and productivity. *Animal Feed Science and Technology* **80**, 183-205.
- Dutton, M. F. (1996).** Fumonisin, mycotoxins of increasing importance: their nature and their effects. *Pharmacology and Therapeutics* **70**, 137-161.
- Ekberg, B. & Mosbach, K. (1989).** Molecular Imprinting: a technique for producing specific separation materials. *Trends in Biotechnology* **7**, 92-96.
- Elizalde-Gonzalez, M. P., Mattusch, J. & Wennrich, R. (1998).** Stability and determination of aflatoxins by high-performance liquid chromatography with amperometric detection. *Journal of Chromatography A* **828**, 439-444.
- Ellis, W. O., Smith, J. P., Simpson, B. K. & Oldham, J. (1991).** Aflatoxins in food. Occurrence, biosynthesis, effects on organisms, detection and methods of control. *Critical Reviews of Food Science and Nutrition* **30**, 403-409.
- F.A.O. (1997).** Worldwide regulations for mycotoxins 1995. A compendium. *Food and Nutrition Paper* **64**. pp43. Rome: Food and Agriculture Organisation.
- Ferreira, N. P. & Pitout, M. J. (1969).** The biosynthesis of ochratoxin. *Journal of the South African Chemical Institute* **22**, S1.

Frisvad, J. C. & Lund, F. (1993). Toxin and secondary metabolite production by *Penicillium* species growing on stored cereals. In *Proceedings of the United Kingdom workshop on occurrence and significance of mycotoxins*. pp. 146-171. Edited by K. A. Scudamore. Slough, England: MAFF.

Frisvad, J. & Thrane, U. (1987). Standardized high-performance liquid chromatography of 182 mycotoxins and other fungal metabolites based on alkylphenone retention indices and UV—VIS spectra (diode array detection). *Journal of Chromatography A* **404**, 195-214.

Frisvad, J. C. (1994). Identification and characterization of pest organisms. Edited by D. L. Hawksworth. Wallingford: CAB International.

Garden, S. R. & Strachan, N. J. C. (2001). Novel colorimetric immunoassay for the detection of aflatoxin B1. *Analytica Chimica Acta* **444**, 187-191.

Gharbi, A., Trillon, O., Betbeder, A. M., Counord, J., Gauret, M. F., Pfohl-Leszkiwicz, A., Dirheimer G. & Creppy, E. E. (1993). Some effects of ochratoxin A, a mycotoxin contaminating feeds and food, on rat testis. *Toxicology* **83**, 9-18.

Gelderbolm, W. C. A., Kriek, N. P. J., Marasas, W. F. O. & Thiel, P. G. (1991). Toxicity and carcinogenicity of the *Fusarium moniliforme* metabolite, fumonisin B1, in rats. *Carcinogenesis* **12**, 1247-1251.

Giacomelli, L., Boggetti, H., Agnelli, H., Anunziata, J., Silber, J. J. & Cattana, R. (1998). Relevant physicochemical factors in chromatographic separation of *Alternaria alternata* mycotoxins. *Analytica Chimica Acta* **370**, 79-89.

de Groene, E. M., Jahn, A., Horbach, G. J. & Fink-Gremmels, J. (1996). Mutagenicity and genotoxicity of the mycotoxin ochratoxin A. *Environmental Toxicology and Pharmacology* **1**, 21-26.

Grove, J.F. (1993). Macrocyclic trichothecenes. *Natural Products Report* **10**, 429-448.

Haginaka, J. & Sanbe, H. J. (2001). Uniformly sized molecularly imprinted polymer for (S)-naproxen: Retention and molecular recognition properties in aqueous mobile phase. *Journal of Chromatography A* **913**, 141-146.

Harris, J. P. & Mantle, P. G. (2001). Biosynthesis of ochratoxins by *Aspergillus ochraceus*. *Phytochemistry* **58**, 709-716.

Harwig, J., Kuiper-Goodman, T. & Scott, P. M. (1983). Microbial food toxicants: ochratoxins. In *Handbook of foodborne diseases of biological origin*. pp. 193-238. Edited by M. Reichgel. Boca Raton, FL: CRC Press.

- Hattori, K., Yoshimi, Y. & Sakai, K. (2001).** Gate effect of cellulosic dialysis membrane grafted with molecularly imprinted polymer. *Journal of Chemical Engineering of Japan* **34**, 1766-1469.
- Haupt, K. (1999).** Molecularly imprinted sorbent assays and the use of non-related probes. *Reactive and Functional Polymers* **41**, 125-131.
- Hilal, N., Kochlodan, V., Busca, G., Kochkodan, O. & Atkin, B. P. (2003).** Thin layer composite molecularly imprinted membranes for selective separation of cAMP. *Separation and Purification Technology* **31**, 281-289.
- Holappa, S., Karesoja, M., Shan, J. & Tenhu, H. (2002).** Solution properties of linear and branched block copolymers consisting of acidic and PEO blocks. *Macromolecules* **35**, 4733-4738.
- Holcomb, M., Wilson, D. M., Trucksess, M. W. & Thompson-Jr, H. C. (1992).** Determination of aflatoxins in food products by chromatography. *Journal of Chromatography* **624**, 341-352.
- Holcomb, M., Thompson, H. C. & Hankins, L. J. (1993).** Analysis of fumonisin B1 in rodent feed by gradient elution HPLC using precolumn derivatization with FMOC and fluorescence detection. *Journal of Agricultural Food Chemistry* **41**, 764-767.
- Holcomb, M., Thompson-Jr, H. C., Cooper, W. M. & Hopper, M. L. (1996).** SFE extraction of aflatoxins (B₁, B₂, G₁, & G₂) from corn and analysis by HPLC. *Journal of Supercritical Fluids* **9**, 118-121.
- Holroyd, S. E., Groves, P., Searle, M. S., Gerhard, U. & Williams, D. H. (1993).** Rational design and binding of modified cell-wall peptides to vancomycin-group antibiotics: Factorising free energy contributions to binding. *Tetrahedron* **49**, 9171-9182.
- Huff, W. E. & Hamilton, P. B. (1979).** Mycotoxins- their biosynthesis in fungi: ochratoxins- metabolites of combined pathways. *Journal of Food Protection* **42**, 815-820.
- Hurst, W. J. & Martin-Jr., R. A. (1998).** High-performance liquid chromatographic determination of ochratoxin A in artificially contaminated cocoa beans using automated sample clean-up. *Journal of Chromatography A* **810**, 89-94.
- IARC. (1993).** IARC monographs on the evaluation of carcinogenic risks to humans: some naturally occurring substances: food items and constituents, heterocyclic aromatic amines and mycotoxins. **56**, pp. 489-521. Geneva: International Agency for Research on Cancer.

Idziak, I., Benrebouh A. & Deschamps, F. (2001). Simple NMR experiments as a means to predict the performance of an anti-17-ethynylestradiol molecularly imprinted polymer. *Analytica Chimica Acta* **435**, 137-140.

Jenkins, A. L., Uy, O. M. & Murray, G. M. (1999). Polymer-based lanthanide luminescent sensor for detection of the hydrolysis product of the nerve agent soman in water. *Analytical Chemistry* **71**, 373 –378.

Jiao, Y., Blaas, W., Rühl, C. & Weber, R. (1992). Identification of ochratoxin A in food samples by chemical derivatization and gas chromatography-mass spectrometry. *Journal of Chromatography A* **595**, 364-367.

Jie, Z. & Xiwen, H. (1999). Study of the nature of recognition in molecularly imprinted polymer selective for 2-aminopyridine. *Analytica Chimica Acta* **381**, 85-91.

Jimenez, M., Mateo, J. J. & Mateo, R. (2000). Determination of type A trichothecenes by high performance liquid chromatography with coumarin-3-carbonyl chloride derivitisation and fluorescence detection. *Journal of Chromatography A* **870**, 473-481.

Jodlbauer, J., Maier, N. & Lindner, W. (2002). Towards ochratoxin A selective molecularly imprinted polymers for solid-phase extraction. *Journal of Chromatography A* **945**, 45-63.

Jorgensen, K. (1998). Survey of pork, poultry, coffee, beer and pulses for ochratoxin A. *Food Additives and Contaminants* **15**, 550-554.

Jornet, D., Busto, O. & Guasch J. (2000). Solid-phase extraction applied to the determination of ochratoxin A in wines by reversed-phase high-performance liquid chromatography. *Journal of Chromatography A* **882**, 29-35.

Karim, K., Piletsky, S., Piletska, E., Subrahmanyam, S., Chianella, I., Lotierzo, M. & Turner, A. P. F. (2000). *Proc. 1st International Workshop on Molecular Imprinting*. Cardiff. 13.

Kellerman, T. S., Marasas, W. F. O., Thiel, P. G., Gelderbolm, W. C. A., Cawood, M. E. & Coetzer, J. A. W. (1990). Leukoencephalomalacia in two horses induced by oral dosing of fumonisin B1. *Onderstepoort Journal of Veterinary Research* **57**, 269-275.

Kempe, M. (1996). Antibody-mimicking polymers as chiral stationary phases in HPLC. *Analytical Chemistry* **68**, 1948-1953.

Kochkodan, V., Weigel, W. & Ulbricht, M. (2002). Molecularly imprinted composite membranes for selective binding of desmetryn from aqueous solutions. *Desalination* **149**, 323-328.

Kuiper-Goodman, T. (1996). Risk assesment of ochratoxin A: an update. *Food Additives and Contaminants* **13**, (Suppl.), 53-57.

Kussak, A., Andersson, B., Andersson, K. & Andersson, J. (1995). Immunoaffinity column clean-up for the high-performance liquid chromatographic determination of aflatoxins B₁, B₂, G₁, G₂, M₁ and Q₁ in urine. *Journal of Chromatography B* **672**, 253-259.

Lacey, J., Ramakrishna, N., Hamer, A., Magan, N. & Marfleet, C. (1991). Grain fungi. In: *Handbook of Applied Mycology: foods and feeds*. Edited by D.K Arora, K.G. Mukerji, and E.H. Marth. New York: Marcel Dekker..

Lakowicz, J. R. (1983). *Principles of Fluorescence Spectroscopy*. New York: Plenum Press.

Lanza, F. & Sellergren, B. (1999). Method for synthesis and screening of large groups of molecularly imprinted polymers. *Analytical Chemistry* **71**, 2092-2096.

Lee, H. B. & Magan, N. (2000). Impact of environment and interspecific interactions between spoilage fungi and *Aspergillus ochraceus* on growth and ochratoxin production in maize grain. *International Journal of Food Microbiology* **61**, 11-16.

Leitner, A., Zöllner, P., Paolillo, A., Stroka, J., Papadopoulou-Bouraoui, A., Jaborek, S., Anklam, E. & Lindner, W. (2002). Comparison of methods for the determination of ochratoxin A in wine. *Analytica Chimica Acta* **453**, 33-41.

Lin, L., Zhang, J., Wang, P., Wang, Y. & Chen J. (1998). Thin-layer chromatography of mycotoxins and comparison with other chromatographic methods. *Journal of Chromatography A* **815**, 3-20.

Lu, Y., Li, C., Zhang, H. & Liu, X. (2003). Study on the mechanism of chiral recognition with molecularly imprinted polymers. *Analytica Chimica Acta* **489**, 33-43.

MAFF. (1993). In: *The 36th report of the steering group on chemical aspects of food surveillance. Mycotoxins: Third Report*. pp. 40. London: HMSO.

Mantle, P. G. (2002). Risk assessment and the importance of ochratoxins. *International Biodeterioration & Biodegradation* **50**, 143-146.

Marquardt, R. R. & Frolich, A. A. (1992). A review of recent advances in understanding ochratoxicosis. *Journal of Animal Science* **70**, 2968-3988.

Masqué, N., Marcé, R. M. & Borrull, F. (2001). Molecularly imprinted polymers: new tailor-made materials for selective solid-phase extraction. *TrAC Trends in Analytical Chemistry* **20**, 477-486.

- Mateo, J. J., Mateo, R., Hinojo, M. J., Llorens, A. & Jimenez, M. (2002).** Liquid chromatographic determination of toxigenic secondary metabolites produced by *Fusarium* strains. *Journal of Chromatography A* **955**, 245-256.
- Matsui, J., Fujiwara, K. & Takeuchi, T. (2000a).** Atrazine-selective polymers prepared by molecular imprinting of trialkylmelamines as dummy template species of atrazine. *Analytical Chemistry* **72**, 1810-1813.
- Matsui, J., Fujiwara, K., Ugata, S. & Takeuchi, T. (2000b).** Solid-phase extraction with a dibutylmelamine-imprinted polymer as triazine herbicide-selective sorbent. *Journal of Chromatography A* **889**, 25-31.
- McKinley, E. K. & Carlton, W. W. (1991).** In: *Mycotoxins and phytoalexins*. pp. 191-236. Edited by R.P Sharma and D.K Salunkhe. Boca Raton, FL. CRC Press.
- McCormick, C. L. & Brent Johnson, C. (1989).** Synthetically structured water-soluble copolymers: Associations by hydrophobic or ionic mechanisms. In: *Polymers in Aqueous Media*, pp. 437-454. Edited by J. Glass. Washington DC: American Chemical Society.
- Mena, M. L., Martínez-Ruiz, P., Reviejo, A. J. & Pingarrón, J. M. (2002).** Molecularly imprinted polymers for on-line preconcentration by solid phase extraction of primicarb in water samples. *Analytica Chimica Acta* **451**, 297-304.
- Van der Meurve, K. J., Steyn, P. S. & Fourie, L. (1965).** Mycotoxins. Part II. The constitution of ochratoxins A, B and C, metabolites of *Aspergillus ochraceus*. *Wilh. Journal of Chemical Society* 7038-7088.
- Montreal Protocol. (1998).**
<http://www.uneptie.org/ozonation/compliance/protocol/main.html>.
Accessed 09 June 2003
- Morgan, M. R. A. (1988).** Mycotoxin immunoassays (with special reference to ELISAs). *Tetrahedron* **45**, 2237-2239.
- Mullet, W., Lai, E. P. C. & Yeung, J. M. (1998).** Immunoassay of fumonisins by a surface plasmon resonance biosensor. *Analytical Biochemistry* **258**, 161-167.
- Nawaz, S., Coker, R. D. & Haswell, S. J. (1992).** Development and evaluation of analytical methodology for determination of aflatoxins in palm kernels. *Analyst* **117**, 67-74.
- Neely, F. L. & Emerson, C. S. (1990).** Determination of sterigmatocystin in fermentation broths by reversed-phase high-performance liquid chromatography using post-column fluorescence enhancement. *Journal of Chromatography A* **523**, 305-311.

- Nicholls, I. A. (1995).** Thermodynamic considerations for the design of and ligand recognition by molecularly imprinted polymers. *Chemical Letters* 1035-1036.
- Nielsen, K. F. & Thrane, U. (2001).** Fast methods for screening of trichothecenes in fungal cultures using gas chromatography–tandem mass spectrometry. *Journal of Chromatography A* 929, 75-87.
- Norrlöw, O., Glad, M., & Mosbach, K. (1984).** Acrylic polymer preparations containing recognition sites obtained by imprinting with substrates. *Journal of Chromatography* 299, 29-41.
- Olsson, J., Borjesson, T., Lundstedt, T. & Schnurer, J. (2002).** Detection and quantification of ochratoxin A and deoxynivalenol in barley grains by GC-MS and electronic nose. *International Journal of Food Microbiology* 72, 203-214.
- Onji, Y., Aoki, Y., Tani, N., Umebayashi, K., Kitada, Y. & Dohi, Y. (1998).** Direct analysis of several *Fusarium* mycotoxins in cereals by capillary gas chromatography–mass spectrometry. *Journal of Chromatography A* 815, 59-65.
- O'Shannessy, D. J., Ekberg, B. & Mosbach, K. (1989).** Molecular imprinting of amino acid derivatives at low temperature (0 °C) using photolytic homolysis of azobisnitriles. *Analytical Biochemistry* 177, 144-149.
- Panasyuk-Delaney, T., Mirsky, V. M., Ulbricht, M. & Wolfbeis, O. S. (2001).** Impedometric herbicide chemosensors based on molecularly imprinted polymers. *Analytica Chimica Acta* 435, 157-162.
- Pap, T., Horváth, V., Tolokán, A., Horvai, G. & Sellergren, B. (2002).** Effect of solvents on the selectivity of terbutylazine imprinted polymer sorbents used in solid-phase extraction. *Journal of Chromatography A* 973, 1-12.
- Pelegri, J. M., Velazquez, C., Sanchis, V. & Canela, R. (1997).** Solid phase extraction on SAX columns as an alternative for ochratoxin A analysis in maize. *Revista Iberoamericana de Micología* 14, 194-196.
- Pena, R., Alcaraz, M. C., Arce, L., Rios, A. & Valcarel, M. (2002).** Screening of aflatoxins in feed samples using a flow system coupled to capillary electrophoresis. *Journal of Chromatography A* 967, 303-314.
- Pestka, J. J., Abouzied, M. M. & Sutikno. (1995).** Immunologically-based assays for mycotoxin detection. *Food Technology* 120-128.
- Petkova-Bocharova, T., Chernozemsky, I. N. & Castegnaro M. (1988).** Ochratoxin A in human blood in relation to Balkan Endemic Nephropathy and urinary system tumours in Bulgaria. *Food Additives and Contaminants* 5, 299-301.

Petzinger, E. & Weidenbach, A. (2002). Mycotoxins in the food chain: the role of ochratoxins. *Livestock Production Science* **76**, 245-250.

Piletsky, S. A., Butovich, I. A. & Kukhar, V. P. (1992). Design of molecular sensors on the basis of substrate-selective polymer membranes. *Zhurnal Analiticheskoy Khimii* **47**, 1681-1684.

Piletsky, S. A., Piletskaya, E. V., Panasyuk, T. L., El'skaya, A.V., Levi, R., Karube, I. & Wulff, G. (1998). Imprinted membranes for sensor technology: opposite behaviour of covalently and non-covalently imprinted membranes. *Macromolecules* **31**, 2137-2140

Piletsky, S. A., Panasyuk, T. L., Piletskaya, E. V., Nicholls, I. A. & Ulbricht, M. (1999). Receptor and transport properties of imprinted polymer membranes – a review. *Journal of Membrane Science* **157**, 263-278.

Piletsky, S. A., Alcock, S. & Turner, A. P. F. (2001). Molecular imprinting: at the edge of third millennium. *Trends in Biotechnology* **19**, 9-12.

Piletsky, S. A., Karim, K., Piletska, E. V., Day, C. J., Freebairn, K. W., Legge, C. H. & Turner, A. P. F. (2001). Recognition of ephedrine enantiomers by MIPs designed using a computational approach. *Analyst* **126**, 1826-1830.

Piletsky, S. A., Piletska, E. V., Karim, K., Freebairn, K. W., Legge, C. H. & Turner, A. P. F. (2002). Polymer cookery: influence of polymerisation conditions on the performance of molecularly imprinted polymers. *Macromolecules* **35**, 7499-7504.

Plattner, R. D. & Branham, B. E. (1994). Labelled fumonisins: production and use of fumonisin B₁ containing stable isotopes. *Journal of AOAC International* **77**, 525-532.

Pohland, A. E., Schuller, P. L., Steyn, P. S. & van Egmond, H. P. (1982). Physicochemical data for some selected mycotoxins. *Pure and Applied Chemistry* **54**, 2219-2284.

Polyakov, M. (1931). Adsorption properties and structure of silica gel. *Zhurnal Fizicheskoy Khimii* **2**, 799-806.

Polyakov, M. (1933). On the structure of silica. *Zhurnal.Fizicheskoy Khimii* **4**, 454-459.

Polyakov, M., Kuleshina, L. & Neimark, I. (1937). On the dependence of silica gel adsorption properties on the character of its porosity. *Zhurnal.Fizicheskoy Khimii* **10**, 100-112.

- Porter, R., van der Logt, P., Howell, S., Kyröläinen-Reay, M. & Badley, A. (2001).** An electro-active system of immuno-assay (EASI assay) utilising self assembled monolayer modified electrodes. *Biosensors and Bioelectronics* **16**, 875-885.
- Qiu, Q., Lou, A., Somasundaran, P. & Pethica, B. A. (2002).** Intramolecular association of poly(maleic acid/octyl vinyl ether) in aqueous solution. *Langmuir* **18**, 5921-5926.
- Radová, Z., Holadová, K. & Hajlová, J. (1998).** Comparison of two clean-up principles for determination of trichothecenes in grain extract. *Journal of Chromatography A* **829**, 259-267.
- Ramos, A. J., Labernia, N., Marín, S., Sanchis, V. & Magan, N. (1998).** Effect of water activity and temperature on growth and ochratoxin production by three strains of *Aspergillus ochraceus* on a barley extract medium and on barley grains. *International Journal of Food Microbiology* **44**, 133-140.
- Reddy, C. S. & Berndt, W. O. (1991).** In: *Mycotoxins and phytoalexins*. pp. 237-250. Edited by R.P. Sharma, and D.K. Salunkhe. Boca Raton, FL: CRC Press.
- Reinhard, H. & Zimmerli, B. (1999).** Reversed-phase liquid chromatographic behavior of the mycotoxins citrinin and ochratoxin A. *Journal of Chromatography A* **862**, 147-159.
- Rheeder, J. P., Marasas, W. F. O., Thiel, P. G., Sydenham, E. W., Shephard, G. S. & van Schalkwyk, D. J. (1992).** *Fusarium moniliforme* and fumonisins in corn in relation to human esophageal cancer in Transkei. *Phytopathology* **82**, 353-357.
- Roch, O. G., Blunden, G., Coker, R. D. & Nawaz, S. (1995).** The validation of a solid phase clean-up procedure for the analysis of aflatoxins in groundnut cake using HPLC. *Food Chemistry* **52**, 93-98.
- Rottinghaus, G. E., Coatney, C. E. & Minor, H. C. (1992).** A rapid, sensitive thin layer chromatography procedure for the detection of fumonisin B1 and B2. *Journal of Veterinary Diagnostic Investigation* **4**, 326-329.
- Schlatter, C., Studer-Rohr, J. & Rasonyi, T. (1996).** Carcinogenic and kinetic aspects of ochratoxin A. *Food Additives and Contaminants* **13**, (Suppl.) 43-44.
- Schothorst, R. C. & Jekel, A. A. (2001).** Determination of trichothecenes in wheat by capillary gas chromatography with flame ionisation detection. *Food Chemistry* **73**, 111-117.
- Scott, P. M. (1993).** In. *Chromatography of Mycotoxins: Techniques and Applications*. pp. 373-425. Edited by V. Betina. Amsterdam: Elsevier.

- Scott, P. M. (1995).** Mycotoxin methodology. *Food Additives and Contaminants* **12**, 395-403.
- Scott, P. M., Weber, D. & Kanhere, S. R. (1997).** Gas chromatography-mass spectrometry of *Alternaria* mycotoxins. *Journal of Chromatography A* **765**, 255-263.
- Scudamore, K. A. & MacDonald, S. J. (1998).** A collaborative study of an HPLC method for determination of ochratoxin A in wheat using immunoaffinity column clean-up. *Food Additives and Contaminants* **15**, 401-410.
- Searcy, J. W., Davis, N. D. & Diener, U. L. (1969).** Biosynthesis of ochratoxin A. *Applied Microbiology* **18**, 622-627.
- Sellergren, B., Lepistoe, M. & Mosbach, K. (1988).** Highly enantioselective and substrate-selective polymers obtained by molecular imprinting utilising non-covalent interactions: NMR and chromatographic studies on the nature of recognition. *Journal of the American Chemical Society* **110**, 5853-5860.
- Sellergren, B. (1989).** Molecular imprinting by noncovalent interactions: tailor-made chiral stationary phases of high selectivity and sample load capacity. *Chirality* **1**, 63-68.
- Sellergren, B. & Andersson, L. (1990).** Molecular recognition in macroporous polymers prepared by molecular imprinting strategy. *Journal of Organic Chemistry* **55**, 3381-3383.
- Sellergren, B. & Shea, K. (1990).** Enantioselective ester hydrolysis catalysed by imprinted polymers. *Tetrahedron Asymmetry* **5**, 1403-1406.
- Sellergren, B. & Shea, K. (1993).** Influence of polymer morphology on the ability of imprinted network polymers to resolve enantiomers. *Journal of Chromatography* **635**, 31-49.
- Sellergren, B., Dauwe, C. & Schneider, T. (1997).** Pressure-induced binding sites in molecularly imprinted network polymers. *Macromolecules* **30**, 2454-2459.
- Sellergren, B. (2003).** Making the most of MIPs: Report on the MIP2002 (the second international workshop on Molecularly Imprinted Polymers and Related Technologies), Held at La Grande Motte, France, 16-19 September 2002). *Trends in Analytical Chemistry* **22**, xii – xv.
- Sergeyeva, T. A., Piletsky, S. A., Brovko, A. A., Slinchenko, E. A., Sergeeva, L. M. & El'skaya, A. V. (1999a).** Selective recognition of atrazine by molecularly imprinted polymer membranes. Development of conductometric sensor for herbicides detection. *Analytica Chimica Acta* **392**, 105-111.

Sergeyeva, T. A., Piletsky, S. A., Brovko, A. A., Slinchenko, E. A., Sergeeva, L. M., Panasyuk, T. L. & El'skaya, A. V. (1999b). Conductometric sensor for atrazine detection based on molecularly imprinted polymer membranes. *Analyst* **124**, 331-334.

Sharma, M. & Márquez, C. (2001). Determination of aflatoxins in domestic pet foods (dog and cat) using immunoaffinity column and HPLC. *Animal Feed Science and Technology* **93**, 109-114.

Shay, G. D. (1989). Alkali-swellaable and alkali-soluble thickener technology: A review. In: *Polymers in Aqueous Media*. pp 457-494. Edited by J. Glass. Washington DC: American Chemical Society.

Shea, K. & Dougherty, T. (1986). Molecular recognition on synthetic amorphous surfaces. The influence of functional group positioning on the effectiveness of molecular recognition. *Journal of American Chemical Society* **108**, 1091-1093.

Shephard, G. S., Sydenham, E. W., Thiel, P. G. & Gelderblom, C. A. (1990). Quantitative determination of fumonisins B1 and B2 by high performance liquid chromatography and fluorescence detection. *Journal of Liquid Chromatography* **13**, 2207-2208.

Shephard, G. S. (1998). Chromatographic determination of the fumonisin mycotoxins. *Journal of Chromatography A* **815**, 31-39.

Solfrizzo, M., Avantaggiato, G. & Visconti A. (1998). Use of various clean-up procedures for the analysis of ochratoxin A in cereals. *Journal of Chromatography A* **815**, 67-73.

SMI. Society for Molecular Imprinting. (2003). <http://www.smi.tu-berlin.de/> Accessed 10 Jan 2004.

Stevenson, D. (1999). Molecular imprinted polymers for solid-phase extraction. *TrAC Trends in Analytical Chemistry* **18**, 154-158.

Steyn, P. S., Holzapfel, C. W. & Ferreira, N. P. (1970). The biosynthesis of ochratoxins, metabolites of *Aspergillus ochraceus*. *Phytochemistry* **9**, 1977-1983.

Stormer, F. C. & Lea, T. (1995). Effects of ochratoxin upon early and late events in T-cell proliferation. *Toxicology* **95**, 45-50.

Studer-Rohr, I., Dietrich, D. R., Schlatter, J. & Schlatter, C. (1995). The occurrence of Ochratoxin A in coffee. *Food and Chemical Toxicology* **5**, 341-355.

Suárez-Rodríguez, J. L. & Díaz-García, M. E. (2000). Flavonol fluorescent flow-through sensing based on a molecular imprinted polymer. *Analytica Chimica Acta* **405**, 67-76.

- Suárez-Rodríguez, J. L. & Díaz-García, M. E. (2001).** Fluorescent competitive flow-through assay for chloramphenicol using molecular imprinted polymers. *Biosensors and Bioelectronics* **16**, 955-961.
- Subrahmanyam, S., Piletsky, S. A., Piletska, E. V., Chen, B., Karim, K. & Turner, A. P. F. (2001).** "Bite-and-Switch" approach using computationally designed molecularly imprinted polymers for sensing of creatinine. *Biosensors & Bioelectronics* **16**, 631-637.
- Subrahmanyam, S. (2002).** Design of molecularly imprinted polymer for sensors and solid phase extraction. Ph.D. Thesis. Cranfield Press.
- Supelco. (1998).** Guide to solid phase extraction. *Supelco Bulletin*. **910**, 1-12.
- Svenson, J. & Nicholls, I. A. (2001).** On the thermal and chemical stability of molecular imprinted polymers. *Analytica Chimica Acta* **435**, 19-24.
- Takagishi, T. & Klotz, I. (1972).** Macromolecule-small interactions; introduction of additional binding sites in polyethyleneimine by disulfide cross-linkages. *Biopolymers* **11**, 483-491.
- Takeuchi, T., Fukuma, D. & Matsui, J. (1999).** Combinatorial molecular imprinting: an approach to synthetic polymer receptors. *Analytical Chemistry* **71**, 285-290.
- Thrane, U. (1989).** In: *Fusarium. Mycotoxins, taxonomy and pathogenicity*. pp. 199-225..Edited by J. Chelowski. Amsterdam: Elsevier.
- Toribio, F., Puignou, L. & Galceran, M. T. (1999).** Evaluation of different clean-up procedures for the analysis of heterocyclic aromatic amines in a lyophilized meat extract. *Journal of Chromatography A* **836**, 223-233.
- Torres, M. R., Sanchis V. & Ramos, A. J. (1998).** Occurrence of fumonisins in Spanish beers analysed by an ELISA method. *International Journal of Food Microbiology* **39**, 139-143.
- Tripos Inc. (2003).** *Sybyl 6.9*. Tripos. Inc., 1699 South Hanley Rd., St. Louis, Missouri, 63144, USA.
- Vainio, J., Heseltine, E. & Wilbourn, J. (1993).** Report on an IARC working group meeting on some naturally occurring substances. *International Journal of Cancer* **53**, 535-537.
- Valenta, H. (1998).** Chromatographic methods for the determination of ochratoxin A in animal and human tissues and fluids. *Journal of Chromatography A* **815**, 75-92.
- Vasquez, B. I., Fente, C., Franco, C., Cepeda, A., Prognon, P. & Mahuzier, G. (1996).** Simultaneous high-performance liquid chromatographic

determination of ochratoxin A and citrinin in cheese by time-resolved luminescence using terbium. *Journal of Chromatography A* **727**, 185-193.

Visconti, A. & Pascale, M. (1998). Determination of zearalenone in corn by means of immunoaffinity clean-up and high-performance liquid chromatography with fluorescence detection. *Journal of Chromatography A* **815**, 133-140

Visconti, A., Pascale, M. & Centonze, G. (1999). Determination of ochratoxin A in wine by means of immunoaffinity column clean-up and high-performance liquid chromatography. *Journal of Chromatography A* **864**, 89-101.

Visconti, A., Pascale, M. & Centonze, G. (2000). Determination of ochratoxin A in domestic and imported beers in Italy by immunoaffinity clean-up and liquid chromatography. *Journal of Chromatography A* **888**, 321-326.

Wandelt, B., Turkewitsch, P., Wysocki, S. & Darling, G. D. (2002). Fluorescent molecularly imprinted polymer studied by time resolved fluorescence spectroscopy. *Polymer* **43**, 2777-2785.

Weiss, R., Freudenschuss, M., Krska R. & Mizaikoff, B. (2003). Improving methods of analysis for mycotoxins: molecularly imprinted polymers for deoxynivalenol and zearalenone. *Food Additives and Contaminants* **20**, 386-395.

Whitcombe, M. J., Martin, L. & Vulfson, E. N. (1998). Predicting the selectivity of imprinted polymers. *Chromatographia* **47**, 457-464.

Wilkes, J. G. & Sutherland, J. B. (1998). Sample preparation and high-resolution separation of mycotoxins possessing carboxyl groups. *Journal of Chromatography B* **717**, 135-156.

Wulff, G. & Sarhan, A. (1972). Über die Anwendung von enzymanalog gebauten Polymeren zur Racemattrennung. *Angewandte Chemie* **84**, 364.

Wulff, G. & Vesper, W. (1978). Preparation of chromatographic sorbents with chiral cavities for racemic resolution. *Journal of Chromatography* **167**, 171-186.

Wulff, G., Best, W. & Akelah, A. (1984). Enzyme-analogue built polymers, 17) Investigations on the racemic resolution of amino acids. *Reactive Polymers* **2**, 167-174.

Wulff, G., Pol, H-G. & Minarik, M. J. (1986). Enzyme-analogue built polymers. XIX. Racemic resolution on polymers containing chiral cavities. *Journal of Liquid Chromatography* **9**, 385-405.

Wulff, G., Vietmeier, J. & Poll, H-G. (1987). Enzyme-analogue built polymers, 22) Influence of the nature of the crosslinking agent on the performance of imprinted polymers in racemic resolution. *Macromolecular Chemistry and Physics* **188**, 731-740.

Wulff, G. (1995). Molecular imprinting in cross-linked materials with the aid of molecular templates- a way towards artificial antibodies. *Angewandte Chemie - International Edition* **34**, 1812-1832.

Yagen, B., Sintov, A. & Bialer, M. (1986). New, sensitive thin-layer chromatographic-high-performance liquid chromatographic method for detection of trichothecene mycotoxins. *Journal of Chromatography* **356**, 195-201.

Yong, R. K. & Cousin, M. A. (2001). Detection of moulds producing aflatoxins in maize and peanuts by an immunoassay. *International Journal of Food Microbiology* **65**, 27-38.

Yoshikawa, M., Izumi, J., Kitao, T., Koya, S. & Sakamoto S. (1995). Molecularly imprinted polymeric membranes for optical resolution. *Journal of Membrane Science* **108**, 171-175.

Yoshikawa, M., Fujisawa, T., Izumi, J., Kitao, T. & Sakamoto S. (1995). Molecularly imprinted polymeric membranes involving tetrapeptide EQKL derivatives as chiral-recognition sites toward amino acids. *Analytica Chimica Acta* **365**, 59-67.

Yoshimi, Y., Ohdaira, R., Iiyama, C. & Sakai, K. (2001). "Gate effect" of thin layer of molecularly-imprinted poly (methacrylic acid-co-ethyleneglycol dimethacrylate). *Sensors and Actuators B* **73**, 49-53.

Young, J. C. & Games, D. E. (1994). Analysis of *Fusarium* mycotoxins by gas chromatography-Fourier transform infrared spectroscopy. *Journal of Chromatography A* **663**, 211-218.

Yusof, N. A. & Ahmad, M. (2002). A flow cell optosensor for lead based on immobilized gallocynin in chitosan membrane. *Talanta* **58**, 459-466.

Zambonin, C. G., Monaci, L. & Aresta A. (2001). Determination of cyclopiazonic acid in cheese samples using solid-phase microextraction and high performance liquid chromatography. *Food Chemistry* **75**, 249-254.

Zhu, Q., Haupt, K., Knopp, D. & Niessner, R. (2002). Molecularly imprinted polymer for metsulfuron-methyl and its binding characteristics for sulfonylurea herbicides. *Analytica Chimica Acta* **468**, 217-227.

Zi-Hui, M. & Liu Qin, L. (2001). Determination of degradation products of nerve agents in human serum by solid phase extraction using molecularly imprinted polymer. *Analytica Chimica Acta* **435**, 121-127.

Zimmerli, B. & Dick, R. (1995). Determination of ochratoxin A at the ppt level in human blood, serum, milk and some foodstuffs by high-performance liquid chromatography with enhanced fluorescence detection and immunoaffinity column cleanup: methodology and Swiss data. *Journal of Chromatography B* **666**, 85-99.

Appendices

Publications:

I am author/co-author of the following papers:

Piletsky, S.A., Piletska E., Bossi, A., Turner, N. W. & Turner, A. P. F. (2003). Surface functionalization of porous polypropylene membranes with polyaniline for protein immobilization. *Biotechnology and Bioengineering* **82**, 86-92.

Turner, N. W., Subrahmanyam, S. & Piletsky, S. A. (2003). Mycotoxins detection – a review. *In preparation*.

Turner, N. W., Whitcombe, M., Malecha, M., Karim, K., Piletska E., Baggiani, C. & Piletsky, S. A. (2003). Effect of the solvent on recognition properties of molecularly imprinted polymer specific for Ochratoxin A. *Biosensors and Bioelectronics. Under Review*.

Conference proceedings:

Turner, N. W., Piletsky, S. A., Karim, K., Piletska, E. & Turner, A. P. F. (2000). Molecular Imprinted Polymers: Market potential for linked technology. In: *Proceedings of MIP2000*. Cardiff, UK (Invited Lecture).

Turner, N. W., Piletsky, S. A., Subrahmanyam, S., Piletska, E., Chianella, I., Loterizo, M., Karim, K. & Magan, N. (2001). Molecular Imprinted Polymers (MIPs) for toxins: Applications for SPE and sensors. In: *Proceedings of Bioactive Fungal Metabolites- Impact and Exploitation*, Swansea, UK.

Turner, N. W., Piletsky, S. A., Whitcombe, M., Malecha, M. & Magan, N. (2002). Development of molecular scaffold for extraction and detection of ochratoxin A. In: *Proceedings of MIP2002*. La Grande Motte, France.

Turner, N. W., Piletsky, S. A., Piletska, E., Karim, K., Whitcombe, M. & Magan, N. (2003). Effect of the microenvironment conditions on recognition properties of molecularly imprinted polymer specific for ochratoxin. In: *Proceedings of the First World Congress on Synthetic Receptors*, Lisbon, Portugal.