SYNTHESIS AND APPLICATION OF MOLECULARLY IMPRINTED SOLID-PHASE EXTRACTION FOR THE DETERMINATION OF TERBUTALINE IN BIOLOGICAL MATRICES

by

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TABLE OF CONTENTS

	Page
ACKNOWLEDGEMENTS	ii
TABLE OF CONTENTS	iii
LIST OF TABLES	viii
LIST OF FIGURES	xii
LIST OF PLATES	xix
LIST OF ABBREVIATION	xx
LIST OF SYMBOLS	xxiii
ABSTRAK	xxiv
ABSTRACT	xxvi

CHAPTER ONE: INTRODUCTION TO MOLECULARLY IMPRINTED POLYMER (MIP)

1.1 Introduction	1
1.2 General Principle of Molecular Imprinting	2
1.3 Molecular Imprinting Approaches	4
1.3.1. Covalent Imprinting	4
1.3.2. Non-covalent Imprinting	5
1.3.3. Comparison Between Covalent and Non-covalent Imprinting	6
1.4 Synthesis of Molecularly Imprinted Polymer	7
1.4.1. Polymerisation Method	9
1.4.1.1. Bulk Polymerisation	9
1.4.1.2. Suspension Polymerisation	9
1.4.1.3. Precipitation Polymerisation	10
1.4.1.4. Two-step Swelling Polymerisation	10
1.4.1.5. Emulsion Core-shell Polymerisation	11
1.4.2. Polymerisation Reagents	12
1.4.2.1. Template Molecule	13
1.4.2.2. Functional Monomer	14
1.4.2.3. Cross-linking Agent	14
1.4.2.4. Porogen	15
1.4.2.5. Initiator	15

1.4.3. Factors to Consider in the Synthesis of Selective MIP 16
1.4.3.1. Molar Ratio of Template: Monomer: Cross-linker (T: M: X) 16
1.4.3.2. Stability of Monomer-template Assemblies 17
1.4.3.3. Polymerisation Temperature 19
1.5 Polymer Structure and Morphology 20
1.5.1. Types of Pores 20
1.5.2. Adsorption / Binding Isotherms 21
1.6 Application of Molecularly Imprinted Polymers 23
1.6.1. Affinity Based Solid-phase Extraction 23
1.6.2. Sensors and Membranes 25
1.6.3. Others 25
1.7 Aims and Objectives of the Present Investigation 26

CHAPTER TWO: PRODUCTION OF SALBUTAMOL IMPRINTED POLY(METHACRYLIC ACID-CO-ETHYLENE GLYCOL DIMETHACRYLATE) AND NON-IMPRINTED POLYMER

2.1 Introduction 28
2.1.1. Proton Nuclear Magnetic Resonance Spectroscopy (^{1}H NMR) 29
2.1.2. Infrared Spectroscopy (IR) 30
2.2 Aim of Experiment 30
2.3 Materials 31
2.4 Methods 32
2.4.1. Stock and Working Standard Solutions 32
2.4.2. Preparation of Chemical Solutions 33
2.4.3. Extraction of Salbutamol Free Base from Salbutamol Sulphate Aqueous Solution 34
2.4.4. Preparation of Salbutamol Imprinted Poly(methacrylic acid-co-ethylene glycol dimethacrylate) and Non-imprinted Polymer 34
2.4.5. Soxhlet Extraction of Salbutamol Template from the Imprinted Poly(methacrylic acid-co-ethylene glycol dimethacrylate) 36

2.4.6. Preparation of Molecularly Imprinted Polymer as Solid-phase Extraction (MIPSPE) Column 37
2.4.7. Selectivity Test of Imprinted and Non-imprinted Polymers 37
2.4.8. Preparation of Samples for ^1H NMR Analysis in Deuterated CD_3CN-CD_3OD (75: 25 % v/v) 38
2.4.9. Preparation of Samples for FTIR Analysis 39
2.4.10. Instrumentation 39
2.5 Results and Discussion 40
2.5.1. Synthesis of Imprinted and Non-imprinted Polymers 40
2.5.2. Removal of Template Molecule from Imprinted Polymer 45
2.5.3. Selectivity Test of Imprinted and Non-imprinted Polymers 46
2.5.4. Spectroscopic Analysis of Pre-organised Monomer-template Assemblies 52
2.5.4.1. Infrared Spectroscopy (FTIR) 52
2.5.4.2. Proton Nuclear Magnetic Resonance Spectroscopy (^1H NMR) 55
2.6 Conclusion 61

CHAPTER THREE: EVALUATION OF MIP FOR THE APPLICATION OF SOLID-PHASE EXTRACTION AND MATRIX INTERFERENCE STUDY

3.1 Introduction 62
3.1.1. General Overview of Solid-phase Extraction 62
3.1.2. Types of Sorbents and Modes of Interaction 65
3.1.3. Limitations of Conventional SPE Method 66
3.2 Aim of Experiment 67
3.3 Materials 67
3.4 Methods 68
3.4.1. Stock and Working Standard Solutions 68
3.4.2. Preparation of Chemical Solutions 69
3.4.3. Binding Study 70
3.4.4. Elution Study 72
3.4.5. Sample Application / Loading Study 73
3.4.5.1. Stage 1 73
3.4.5.2. Stage 2: Method Optimisation 74

4.5.1.3. Between Day Validation 143

4.5.1.4. Within Day Validation 144

4.5.2. Method Comparison Study: SPE Mixed Mode as Reference Method 146

4.5.2.1 Pearson's Correlation between Proposed Method (MISPE) and Reference Method (SPE Mixed Mode) 146

4.5.2.2 Advantages of the MIPSPE Method 155

4.5.3 Confirmatory Analysis (Qualitative Study) 157

4.6 Conclusion 164

CHAPTER FIVE: GENERAL CONCLUSIONS

5.1 General Conclusions 165

5.2 Limitations of Developed Method 170

5.3 Recommendations for Future Research 171

BIBLIOGRAPHY 173

APPENDICES

Appendix A: Signal to Noise Report by Chemstation Software 182

Appendix B: Analytical Method Validation 184

LIST OF TABLES

Tables		Page
1.1	Summary of advantages and disadvantages of MIPs for analytical applications (Mahony *et al.*, 2005).	2
1.2	Advantages and disadvantages of covalent and non-covalent imprinting (Komiyama *et al.*, 2003).	7
1.3	Comparison of different polymerisation methods (Mayes *et al.*, 1997).	8
1.4	Choice of reagents and molar ratios of T: M: X for the syntheses of MIPs by bulk polymerisation technique.	18
1.5	Summary of studies in which MIPs have been applied to SPE.	24
2.1	Amount of glacial acetic acid added into ACN to produce various mixtures of ACN- glacial acetic acid.	33
2.2	Fractions collected for analysis in selectivity test from each individual cartridge.	38
2.3	Summary of the reagents and amount employed for the production of MIP by bulk polymerisation method.	41
2.4	Percent breakthrough of terbutaline in each fraction collected during the loading and elution stage. 10 mL of ACN spiked with 100 ng of terbutaline was loaded into the MIP and blank polymer (n = 4). Elution was performed with 1 mL of ACN containing increasing amount of acetic acid (HAc). Terbutaline was detected in the form of terbutaline tris trimethylsily after derivatization by MSTFA at 70 °C for 30 minutes. (a) Polymer Batch # 1; (b) Polymer Batch # 2; (c) Polymer Batch # 3 and (d) Polymer Batch # 4.	48
2.5	Chemical shift of salbutamol protons in salbutamol solution and pre-polymerisation mixture of CD_3CN-CD_3OD (75: 25 % v/v).	57
2.6	Chemical shift of protons in methacrylic acid solution of CD_3CN-CD_3OD (75: 25 % v/v).	57
3.1	Common sorbents available for SPE application (Thurman *et al.*, 1998).	66
3.2	Type of solvents applied as loading medium.	71
3.3	Fractions collected for analysis in binding study.	72
3.4	Fractions collected for analysis in elution study.	73

3.5	Fractions collected for analysis in loading study (Stage 1).	74
3.6	Fractions collected for analysis in loading study (Stage 2).	75
3.7	MIPSPE extraction procedure for urine and plasma samples. Samples were diluted with 50 mM ammonium acetate pH 7.	76
3.8	Fractions collected for analysis in cross-specificity study.	78
3.9	Diagnostic ions and retention times for the analysis of terbutaline, salbutamol and nalorphine under SIM mode. All the analytes were derivatised to form TMS derivatives using MSTFA at 70 °C for 30 minutes. Chromatographic conditions were as described in Section 2.4.10.	79
3.10	Binding of terbutaline to imprinted and blank polymers in different solvents (n = 3). Ten mL of spiked solvent at 10 ng/mL of terbutaline was loaded into each cartridge (total amount loaded per column was 100 ng).	83
3.11	Amount of terbutaline measured in various stages of SPE extraction (n = 3). Ten mL of spiked ACN at 10 ng/mL was loaded into each column. Different eluting solvents were applied as a) ACN-glacial acetic acid (95: 5 % v/v); b) ACN-glacial acetic acid (90: 10 % v/v); c) ACN-glacial acetic acid (80: 20 % v/v); d) ACN-trifluoroacetic acid (99: 1 % v/v) and e) ACN-triethylamine (99: 1 % v/v).	87
3.12	Amount of terbutaline measured in various stages of SPE extraction (n = 3). Five mL of spiked solution at 20 ng/mL terbutaline was loaded into each cartridge. Different loading solutions (Stage 1) were applied as a) Distilled water; b) 50 mM ammonium acetate at pH 5; c) 50 mM ammonium acetate at pH 7 and d) 50 mM ammonium acetate at pH 9.	95
3.13	Amount of terbutaline measured in various stages of SPE extraction (n = 3). Five mL of spiked solution at 20 ng/mL of terbutaline was loaded into each cartridge. Different loading solutions (Stage 2) were applied as a) 50 mM ammonium acetate at pH 7; b) 50 mM ammonium acetate at pH 9 and c) 50 mM ammonium acetate at pH 11.	99
3.14	Recoveries of terbutaline spiked into diluted urine samples at various concentration levels. Dilution was conducted at ratio 1: 1	103

	v/v. Extraction procedure was as described in Section 3.4.6. Urine samples were from human, bovine and equine.	
3.15	Recoveries of terbutaline spiked into diluted human plasma samples at various concentrations. Extraction procedure was as described in Section 3.4.6.	105
3.16	Optimisation of MIPSPE extraction procedure for the determination of terbutaline in plasma and urine samples.	106
3.17	Diagnostic ions and the retention times for GC-MS analysis under SIM mode. All the analytes were derivatised to form TMS derivatives using MSTFA or enol solution (for clenbuterol and metoprolol only) at 70 °C for 30 minutes. Chromatographic conditions were as described in Section 2.4.10.	108
3.18	Percent breakthrough of analytes at various SPE stages from the imprinted and non-imprinted polymers (n = 4). Loading solutions were in a) 50 mM ammonium acetate at pH 7 and b) ACN.	124
3.19	Log *P* and pKa value for each of the substance evaluated in cross-specificity test (Hansch *et al.*, 1990 and Moffat *et al., 1986*).	126
4.1	Accuracy and precision data for the determination of terbutaline in spiked plasma samples at the lower limit of quantification level (LLOQ at 1 ng/mL) (n = 6).	141
4.2	Equation of calibration curves and correlation of determination (r^2) values for six consecutive days of validation.	143
4.3	Precision for between day validation (n = 6) at low, medium and high QC samples.	143
4.4	Within day validation, accuracy and extraction recoveries of terbutaline in spiked plasma samples (n = 7) at the concentrations of a) 2 ng/mL; b) 5 ng/mL and c) 8 ng/mL.	145
4.5	Diagnostic ions and retention times for the analysis of terbutaline and salbutamol under SIM mode. All the analytes were derivatised to form *t*-BDMS derivatives using *N-tert*-butyldimethylsilyl-*N*-methyl trifluoroacetamide – pyridine (2:1 v/v) at 60 °C for 1 hour. Chromatographic conditions were as described in Section 4.4.6.	147

4.6	The concentrations of terbutaline measured by MIPSPE and SPE mixed mode procedures following the extraction from spiked plasma samples (n = 20).	152
4.6	The concentrations of terbutaline measured by MIPSPE and SPE mixed mode procedures following the extraction from spiked plasma samples (n = 20) (continued).	153
4.7	Summary output of Excel spreadsheet used to analyse the regression line in Figure 4.8.	154
4.8	Comparison between the retention times and maximum permitted difference in relative abundance expressed in term of absolute and relative of the diagnostic ions of terbutaline extracted from spiked urine samples and pure standards.	162

2.5	Infrared spectrums of a) pre-polymerisation mixture; b) methacrylic acid and c) salbutamol free base (solid).	54
2.6	Protons position in a) salbutamol and b) methacrylic acid	57
2.7	^{1}H NMR spectrum of salbutamol free base in CD_3CN-CD_3OD (75: 25 % v/v).	58
2.8	^{1}H NMR spectrum of methacrylic acid in CD_3CN-CD_3OD (75: 25 % v/v).	59
2.9	^{1}H NMR spectrum of pre-polymerisation mixture in CD_3CN-CD_3OD (75: 25 % v/v).	60
3.1	General process of solid phase extraction. Step 1: Conditioning of sorbent; Step 2: Sample application; Step 3: Interference elution or washing and Step 4: Analyte elution (Adapted from: Thurman *et al.*, 1998).	64
3.2	The mass spectrum for terbutaline tris TMS and postulated fragmentation pathways (Source: NIST mass spectral database).	80
3.3	The mass spectrum for salbutamol tris TMS and postulated fragmentation pathways (Source: NIST mass spectral database).	80
3.4	The mass spectrum for nalorphine *O, O'*-bis TMS and postulated fragmentation pathways (Source: NIST mass spectral database).	81
3.5	Typical calibration curve (from pure standard) prepared for the quantification of terbutaline as terbutaline tris TMS derivative (range from 10 to 120 ng).	81
3.6	Typical calibration curve (from pure standard) prepared for the quantification of terbutaline as terbutaline tris TMS derivative at lower amount (range from 2.50 to 30 ng).	82
3.7	Plot of cumulative recovery of terbutaline breakthrough versus the volume of solvent loaded into the SPE cartridge. The loading solutions were ACN-glacial acetic acid at a) 99: 1 % v/v; b) 98: 2 % v/v and c) 97: 3 % v/v.	85

3.8	Plot of percentage breakthrough and cumulative recovery of terbutaline versus the SPE stages. Different eluting solvents were applied as a) ACN-glacial acetic acid (95: 5 % v/v); b) ACN-glacial acetic acid (90: 10 % v/v); c) ACN-glacial acetic acid (80: 20 % v/v); d) ACN-trifluoroacetic acid (99: 1 % v/v) and e) ACN-triethylamine (99: 1 % v/v).	89
3.9	Plot of percentage recovery of terbutaline eluted out during elution step versus the different types of eluting solvents. Terbutaline measured was from fraction E1 to E4.	91
3.10	Plot of percentage breakthrough and cumulative recovery of terbutaline versus the SPE stages. Different loading solutions (Stage 1) were applied as a) Distilled water; b) 50 mM ammonium acetate at pH 5; c) 50 mM ammonium acetate at pH 7 and d) 50 mM ammonium acetate at pH 9.	97
3.11	Plot of percentage breakthrough and cumulative recovery of terbutaline versus the SPE stages. Different loading solutions (Stage 2) were applied as a) 50 mM ammonium acetate at pH 7; b) 50 mM ammonium acetate at pH 9 and c) 50 mM ammonium acetate at pH 11.	100
3.12	Plot of percentage recovery of terbutaline versus the pH values of 50 mM ammonium acetate buffers. 4 mL of eluting solvent was applied into each cartridge.	101
3.13	Chemical structures of substances tested in the cross-specificity study.	107
3.14	The mass spectrum for clenbuterol *N*, *O*-bis TMS and postulated fragmentation pathways (Source: NIST mass spectral database).	109
3.15	The mass spectrum for fenoterol tetrakis TMS and postulated fragmentation pathways (Source: NIST mass spectral database).	109
3.16	The mass spectrum for isoxsuprine di TMS and postulated fragmentation pathways (Source: NIST mass spectral database).	110
3.17	The mass spectrum for metoprolol TMS and postulated fragmentation pathways (Source: NIST mass spectral database).	110

3.18	The mass spectrum for ractopamine tris TMS and postulated fragmentation pathways. Mass spectrum was obtained from the analysis of pure ractopamine tris TMS standard at 4 ng/μL under full scan mode.	111
3.19	The mass spectrum for ibuprofen TMS and postulated fragmentation pathways (Source: NIST mass spectral database).	111
3.20	The mass spectrum for boldenone *O*-TMS and postulated fragmentation pathways (Source: NIST mass spectral database).	112
3.21	Typical example of the extracted ion chromatogram of fenoterol tetrakis TMS obtained from the selective wash fraction of MIP in which ammonium acetate pH 7 was the loading medium. Retention times peak 1: nalorphine *O*, *O'* bis TMS, 12.16 min; 2: fenoterol tetrakis TMS, 12.25 min.	112
3.22	Typical example of the extracted ion chromatogram of isoxsuprine di TMS obtained from the selective wash fraction of MIP in which ammonium acetate pH 7 was the loading medium. Retention times peak 1: isoxsuprine di TMS, 11.44 min; 2: nalorphine *O, O'* bis TMS, 12.16 min.	113
3.23	Typical example of the extracted ion chromatogram of clenbuterol N, *O*-bis TMS obtained from the elution (E1+E2) fraction of MIP in which ammonium acetate pH 7 was the loading medium. Retention times peak 1: clenbuterol N, *O*-bis TMS, 9.52 min; 2: nalorphine *O, O'* bis TMS, 12.23 min.	113
3.24	Typical example of the extracted ion chromatogram of ractopamine tris TMS obtained from the elution (E1+E2) fraction of MIP in which ammonium acetate pH 7 was the loading medium. Retention times peak 1: nalorphine *O, O'* bis TMS, 12.20 min; 2: ractopamine tris TMS, 12.39 min.	114
3.25	Typical example of the extracted ion chromatogram of metoprolol TMS obtained from the elution (E1+E2) fraction of MIP in which ammonium acetate pH 7 was the loading medium. Retention times peak 1: metoprolol TMS, 9.56 min; 2: nalorphine *O, O'* bis TMS, 12.16 min.	114

3.26	Typical example of the extracted ion chromatogram of ibuprofen TMS obtained from the selective wash fraction of MIP in which ammonium acetate pH 7 was the loading medium. Retention times peak 1: ibuprofen TMS, 6.37 min; 2: nalorphine *O, O'* bis TMS, 12.16 min.	115
3.27	Typical example of the extracted ion chromatogram of boldenone *O*- TMS obtained from the selective wash fraction of MIP in which ammonium acetate pH 7 was the loading medium. Retention times peak 1: nalorphine *O, O'* bis TMS, 12.16 min; 2: boldenone *O*-TMS, 12.52 min.	115
3.28	Typical calibration curves (from pure standard) for the quantification of a) clenbuterol; b) fenoterol; c) isoxsuprine; d) metoprolol; e) ractopamine; f) ibuprofen and g) boldenone as TMS derivative (range from 10 to 120 ng).	116
3.29	Percent breakthrough of analyte at various SPE stages from imprinted and non-imprinted polymers (n = 4). Loading medium were as in a) 50 mM ammonium acetate at pH 7 and b) ACN. Selective washing was conducted by applying 1 mL of ACN-glacial acetic acid (99: 1 % v/v) followed by 1 mL x 4 of ACN-glacial acetic acid (90: 10 % v/v) for the elution step.	122
4.1	Extracted ion chromatogram of terbutaline as terbutaline tris TMS at m/z = 356 from the blank human plasma and spiked plasma at 1 ng/mL. Blank and spiked samples were extracted using the proposed MIPSPE method as described in Table 3.16 but at the dilution ratio of 1: 4 v/v with 62.5 mM ammonium acetate pH 7 as sample diluent.	140
4.2	Typical calibration curve prepared for the quantification of terbutaline as terbutaline tris TMS derivative following the extraction of the drug from spiked plasma.	142
4.3	The mass spectrum for terbutaline tris *t*-BDMS. Mass spectrum was obtained from the analysis of pure terbutaline tris *t*-BDMS standard at 4 ng/μL under full scan mode.	148
4.4	The mass spectrum for salbutamol tris *t*-BDMS. Mass spectrum was obtained from the analysis of pure salbutamol tris *t*-BDMS standard at 4 ng/μL under full scan mode.	148

4.5	The chemical structures of terbutaline and salbutamol as *t*-BDMS derivatives and the postulated fragmentation pathways.	149
4.6	Typical example of the extracted ion chromatogram of terbutaline as *t*- BDMS derivative following the extraction from spiked plasma sample at 9 ng/mL. Salbutamol was added as internal standard. Retention times peak 1: terbutaline tris *t*-BDMS, 9.46 min; 2: salbutamol tris *t*-BDMS , 9.80 min.	149
4.7	Typical calibration curve prepared for the quantification of terbutaline as terbutaline tris *t*-BDMS derivative following the extraction of the drug from spiked plasma by SPE mixed mode method.	150
4.8	Correlation between terbutaline concentrations in human plasma (n = 20) measured by the SPE mixed mode method (*x* axis) and the proposed MIPSPE method (*y* axis).	153
4.9	Extracted ion chromatograms at *m/z* = 356 of pure terbutaline standard, blank and spiked plasma samples. Extraction procedures were MIPSPE and SPE mixed mode. Terbutaline was measured as terbutaline tris TMS derivative by using MSTFA as the derivatisation reagent for extracts obtained from both methods.	156
4.10	Full scan mass spectrum of terbutaline tris TMS from spiked urine sample at 20 ng/mL of terbutaline. Five mL of urine was diluted at the ratio of 1: 1 v/v before loading into two separate cartridges. The extracts collected were combined as a single sample.	158
4.11	Extracted ion chromatogram of terbutaline tris TMS from spiked urine sample at *m/z* = 86, 356 and 426. Retention time was at 8.48 min.	158
4.12	Extracted ion chromatogram of terbutaline tris TMS from pure standard at *m/z* = 86, 356 and 426. Concentration of the injected sample was at 1 ng/μL. Retention time was at 8.48 min.	159
4.13	Full scan mass spectrum of terbutaline tris *t*-BDMS from spiked urine sample at 20 ng/mL of terbutaline. Five mL of urine was diluted at the ratio of 1: 1 v/v before loading into two separate cartridges. The extracts collected were combined as a single sample.	159

4.14	Extracted ion chromatogram of terbutaline tris *t*-BDMS from spiked urine sample at m/z = 86 and 482. Retention time was at 9.23 min.	160
4.15	Extracted ion chromatogram of terbutaline tris *t*-BDMS from pure standard at m/z = 86 and 482. Concentration of the injected sample was 2 ng/µL. Retention time was at 9.23 min.	160

4.14	Extracted ion chromatogram of terbutaline tris *t*-BDMS from spiked urine sample at m/z = 86 and 482. Retention time was at 9.23 min.	160
4.15	Extracted ion chromatogram of terbutaline tris *t*-BDMS from pure standard at m/z = 86 and 482. Concentration of the injected sample was 2 ng/μL. Retention time was at 9.23 min.	160

LIST OF PLATES

Plates		Page
2.1a	Opaque monolith macroporous polymer.	45
2.1b	Fine irregular particles within the size of 40 to 75 μm. The particles were obtained by manually grinding using pestle and mortar.	45

LIST OF ABBREVIATION

%	Percent
% v/v	Percent volume per volume
μg/mL	Microgramme per millilitre
μL	Microlitre
μm	Micron, micrometre
^{1}H NMR	Proton nuclear magnetic resonance
Å	Angstrom
ABDV	Azobisdimethylvaleronitrile
ACN	Acetonitrile
AIBN	Azobisisobutyronitrile
AORC	Association of Official Racing Chemist
Blk	Blank polymer
C=O	Carbonyl group
CD_3CN	Deuterated acetonitrile
CD_3OD	Deuterated methanol
CH_3COONH_4	Ammonium acetate
cm/sec	Centimetre per second
cm^{-1}	Wave length in centimetre unit
-COOH	Carboxylic acid
CV	Coefficient of variation
DVB	Divinylbenzene
EC	European Council
EGDMA	Ethylene glycol dimethacrylate
EIC	Extracted ion chromatogram
FDA	Food and Drug Administration
FTIR	Fourier transform infrared
g	Gramme
GC-MS	Gas chromatography coupled to mass spectrometry
H_2O	Water
HAc	Glacial acetic acid
HBr	Hydrobromide
HCl	Hydrochloride
HPLC, LC	High performance liquid chromatography
ICH	International Conference on Harmonisation

IOC	International Olympic Committee
ISO	International Organisation for Standardisation
KBr	Potassium bromide
LC-MS	Liquid chromatography coupled to mass spectrometry
LLOQ	Lower limit of quantification
LOD	Limit of detection
Log *P*	Log of octanol-water partition coefficient
M	Molar
m/z	Mass to charge ratio
MAA	Methacrylic acid
MeOH	Methanol
mg	Milligramme
MIP(s)	Molecularly imprinted polymer(s)
MIPCE	Capillary electrophoresis based on molecularly imprinted polymer
MIPCEC	Capillary electrochromatography based on molecularly imprinted polymer
MIPSPE	Solid-phase extraction based on molecularly imprinted polymer
mM	Millimolar
mmHg	Millimetre of mercury
MSTFA	*N*-methyl-*N*-trimethylsilyltrifluoroacetamide
n	Number of replicate
NaOH	Sodium hydroxide
ng/mL	Nanogramme per millilitre
-NH	Amino group
NH_4I	Ammonium iodide
NIP	Non-imprinted polymer
NIST	National Institute of Standards and Technology
°C	Degrees celcius
-OH	Hydroxyl group
pKa	Log of acidity constants
ppm	Parts per million
psi	Pounds per square inch
QC	Quality control
r^2	Correlation of determination
rpm	Revolutions per minute
Rt	Retention time

SD	Standard deviation
SIM	Selected ion monitoring
SPE	Solid-phase extraction
T: M: X	Ratio of template molecule: functional monomer: cross-linker
t-BDMS	*Tert*-butyl dimethylsilyl
TEA	Triethylamine
TFA	Trifluoroacetic acid
TMS	Trimethylsilyl
UV	Ultraviolet

LIST OF SYMBOLS

δ	Chemical shift
$\Delta\delta$	Difference in chemical shift
π	Pi
α	Alpha
β	Beta
$\sim$	Approximately
$\geq$	Equal or greater than
$\leq$	Equal or less than
$\pm$	Plus minus

SINTESIS DAN APLIKASI PENGEKSTRAKAN FASA PEPEJAL BERASASKAN "MOLECULAR IMPRINTING" UNTUK PENENTUAN TERBUTALINA DI DALAM SAMPEL BIOLOGI

ABSTRAK

Kajian ini tertumpu kepada penilaian keberkesanan penggunaan MIPs sebagai bahan padatan dalam SPE untuk pengekstrakan terbutalina di dalam sampel plasma dan urin. Polimer ini dihasilkan dengan menggunakan asid metakrilik (MAA) sebagai monomer dan etilena glikol dimetakrilat (EGDMA) sebagai bahan silang manakala salbutamol dengan struktur yang hampir serupa dengan analit dipilih sebagai molekul templat. Campuran asetonitril-metanol (75: 25 % v/v) digunakan sebagai pelarut untuk pempolimeran secara terma pada suhu 50 °C selama 24 jam. Nisbah molar untuk molekul templat: monomer: bahan silang ialah pada 1: 4: 20. Polimer kawalan tanpa molekul templat turut dihasilkan melalui teknik yang serupa. Polimer perlu ditumbuk dan ditapis untuk mendapatkan partikel halus bersaiz 40 hingga 75 μm. Kemudian, ia diekstrak menggunakan kaedah pengekstrakan pelarut separa-selanjar untuk menyingkirkan molekul templat.

Kajian terhadap penjerapan terbutalina dengan pelbagai jenis pelarut telah dijalankan untuk mengenalpasti pelarut-pelarut yang sesuai untuk digunakan semasa penambahan sampel, pembilasan selektif dan pengelusian analit. Kajian tentang selektiviti MIP berbanding polimer kawalan mendapati kedua-dua polimer menunjukkan ciri pengenalpastian yang berbeza terhadap terbutalina. Graf pengelusian yang amat berlainan menandakan kehadiran ikatan spesifik dalam MIP manakala pada polimer kawalan pula hanya wujud ikatan yang lemah dan tidak spesifik. Kajian spesifikasi silang pula menunjukkan MIP yang dihasilkan boleh mengenalpasti molekul-molekul lain yang mempunyai struktur dan ciri-ciri yang hampir seiras dengan molekul templat. Sampel urin dan plasma dicairkan dengan menggunakan 100 mM ammonium acetat

pH 7 pada nisbah 1: 1 v/v sebelum ia dialirkan melalui turus padatan yang diisi dengan 50 mg polimer. Kemudian turus padatan dibilas dengan 2 mL 50 mM ammonium acetat pH 7 dan 1 mL asetonitril-asid asetik pekat (99: 1 % v/v). Pengelusian dilakukan dengan menggunakan 4 mL asetonitril-asid asetik pekat (90: 10 % v/v). Proses penerbitan dijalankan sebelum sampel dianalisis menggunakan GC-MS.

Hasil analisis sampel plasma memberikan 60 hingga 70 % pemerolehan semula. Kajian pengesahan terhadap teknik yang digunakan mendapati ia memberikan kepersisan dan ketepatan yang baik pada kepekatan antara 1 ng/mL hingga 10 ng/mL. Teknik ini juga menunjukkan korelasi yang baik (r = 0.9860) dengan kaedah rujukan yang berasaskan SPE fasa campuran. Ekstrak yang diperolehi adalah lebih bersih. Kromatogram dan spektrum jisim yang dihasilkan juga kurang kompleks. Untuk kajian pengenalpastian urin pula, kriteria yang ditetapkan oleh Jawatankuasa Olimpik Antarabangsa (IOC) telah dipatuhi sepenuhnya. Perbezaan masa retensi bagi sampel adalah kurang daripada 1 % berbanding masa retensi piawai manakala nisbah ion yang dikaji menunjukkan had perbezaan maksimum kurang daripada 5 % (nilai mutlak) dan 20 % (nilai relatif). Oleh itu, MIP merupakan di antara alternatif sebagai bahan padatan SPE untuk pembersihan sampel biologi.

SYNTHESIS AND APPLICATION OF MOLECULARLY IMPRINTED SOLID-PHASE EXTRACTION FOR THE DETERMINATION OF TERBUTALINE IN BIOLOGICAL MATRICES

ABSTRACT

A study was performed to evaluate the feasibility of applying MIPs as sorbent material in SPE for clean up of terbutaline from urine and plasma samples. The imprinted polymer was prepared by using methacrylic acid (MAA) as functional monomer, ethylene glycol dimethacrylate (EGDMA) as cross-linker and salbutamol, a closely structural analogue to the targeted analyte as template molecule. Porogen used was a mixture of acetonitrile-methanol (75: 25 % v/v). Free radical polymerisation was conducted at 50 °C for 24 hours. Molar ratio of template molecule: functional monomer: cross-linker applied was fixed at 1: 4: 20. The output was a monolith macroporous polymer which required grinding and sieving to obtain fine particles between 40 to 75 μm. Soxhlet extraction method was conducted to remove the imprint molecule in order to create the recognition sites. A blank/non-imprinted polymer was produced simultaneously using the same procedure except in the absent of template molecule.

A binding study of terbutaline in several solvents was performed to determine suitable solvent for loading, selective washing and elution steps. Selectivity test on the MIP against blank polymer demonstrated that both polymers exhibited different recognition properties towards terbutaline. Significant differences in elution curves between both polymers were observed, indicating the presence of specific binding in imprinted polymer. In blank polymer, only weak non-specific interactions occurred. Cross-specificity studies showed the MIP also exhibited molecular recognition properties towards other structurally related compounds. Spiked urine and plasma samples were diluted in 100 mM ammonium acetate buffer pH 7 (ratio = 1: 1 v/v) prior to direct loading (5 mL) into a cartridge filled with 50 mg of MIP. Thereafter, the column

CHAPTER ONE

INTRODUCTION TO MOLECULARLY IMPRINTED POLYMER (MIP)

1.1. Introduction

Molecular imprinting is an established method for the production of polymeric artificial receptors for specific molecular recognition. The imprinted polymers have the ability of precise recognition of the original imprinting molecules and can distinguish the minor structural differences of substrates in the interaction sites (Lu *et al.*, 2002). Thus, much of the literature available on the subject frequently underlines the "biomimetic" properties by these imprinted polymers with the substrate-selective mechanisms being analogous to that of natural entities such as antibodies and enzymes (Haupt *et al.*, 1998). Because of the high selectivity and stability of molecularly imprinted polymers (MIPs), this technology has developed rapidly in recent years and is documented in several comprehensive reviews (Mahony *et al.*, 2005; Masque *et al.*, 2001; Mosbach, 1994; Takeuchi *et al.*, 1999b; Xu *et al.*, 2004; Ye *et al.*, 2004 and Komiyama *et al.*, 2003). In all the examples, MIPs have been used as substitutes for antibodies, showing strong binding to the targeted analytes in affinity separations, assay systems and as biosensors. Among the advantages of MIPs for analytical applications compared to the conventional antibodies are easy preparation, chemical and thermal stability (Svenson *et al.*, 2001) and cost effectiveness, as the materials used are inexpensive and readily available (Mahony *et al.*, 2005). A summary of advantages and disadvantages of MIPs is given in Table 1.1.

Table 1.1: Summary of advantages and disadvantages of MIPs for analytical applications (Mahony *et al.*, 2005).

Advantages	Disadvantages
• Cost effective alternative to biomolecule-based recognition • Ease of preparation, enhanced thermal and chemical stability versus natural antibodies • Can be prepared in different formats (bead, block or thin film) following the need of application • Can be stored for a long period without loss of affinity for target analyte	• Lower catalytic capabilities than biological counterparts • Unfavorable adsorption isotherm and slow mass transfer in the polymer matrix • Template bleeding requires suitable template analogue for the imprinting step and this will affects the quantitative applications • Grinding and sieving of bulk polymer for SPE/LC application is labor intensive and inefficient in material yield (high losses).

1.2. General Principle of Molecular Imprinting

The concept of MIP involves three main steps which are as follows (Komiyama *et al.*, 2003 and Sellergren, 2001):

- First step: Complex formation of a given template molecule with polymerisable monomers bearing functional groups capable of interacting to each other by covalent or non-covalent bonding. For non-covalent bonding, the functional monomer and template are placed nearby through hydrogen bond, electrostatic, hydrophobic, apolar or other non-covalent interactions.

- Second step: Polymerisation in order to maintain the alignment of the functional groups which are optimally set for binding the template molecule. Structures of the conjugates or adducts are frozen in a three dimensional network of polymer.

- Third step: Removal of the template molecule from the resulting polymer matrices, allowing "tailor made" binding sites for the targeted analyte to be generated. Hence, the space in the polymer originally occupied by the template molecule is left as a cavity. Under appropriate conditions, these cavities satisfactorily remember the size, structure and other physicochemical properties of the template and bind this molecule or other structurally analogue molecule efficiently and selectively.

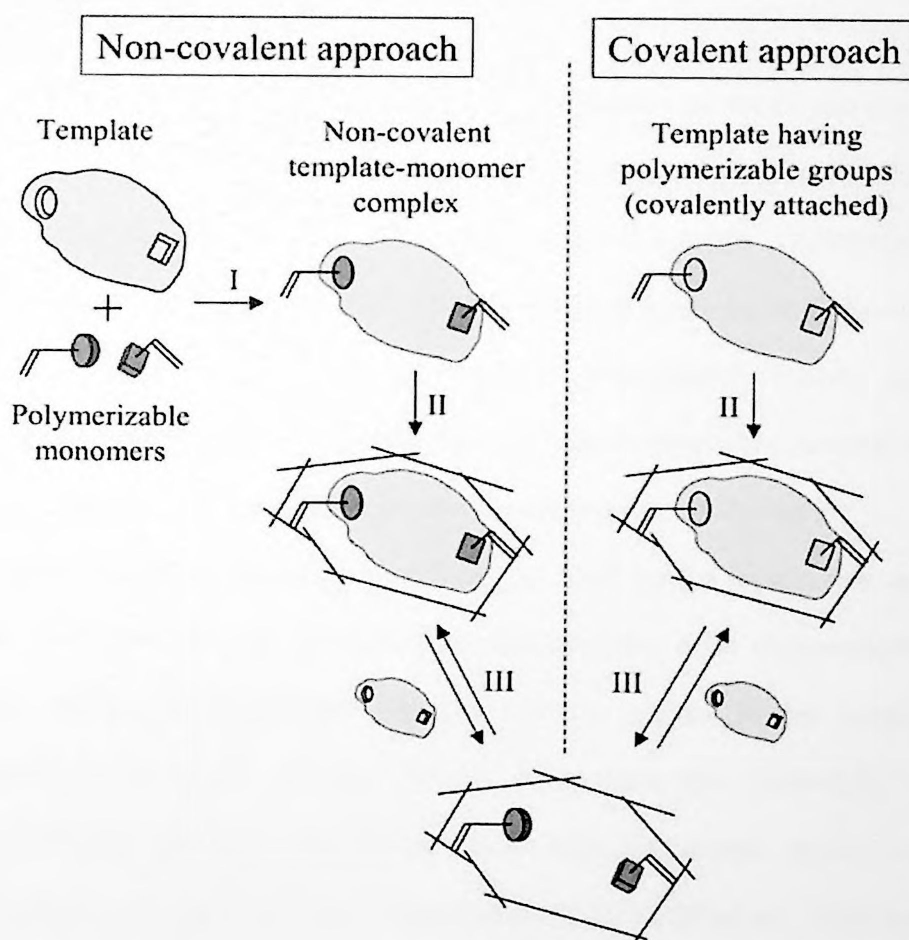


Figure 1.1: Schematic diagram of molecularly imprinted polymer. I: self assembly of template and functional monomers (non-covalent approach); II: polymerisation process; III: extraction of template and rebinding of analyte (Adapted from: Turiel *et al.*, 2004).

1.3. Molecular Imprinting Approaches

Depending to the chemical bonding involved in molecular imprinting, the technique can be classified into two systems, whether they are covalent bonding based or non-covalent bonding based (Takeuchi *et al.,* 1999b; Turiel *et al.*, 2004 and Komiyama *et al.*, 2003).

1.3.1. Covalent Imprinting

The idea of covalent imprinting was first introduced by Wulff and co-workers in 1972 from Germany (Wulff *et al.*, 1973 and Wulff, 1995) and followed by Shea and co-workers from California (Mosbach, 1994). In the covalent system, a template-monomer complex is formed through reversible covalent binding such as boronic acid esters, acetals, ketals, Schiff bases, disulfide bonds, coordination bonds and others (Komiyama *et al.*, 2003). These linkages must be stable, reversible and for binding the target guest promptly, both the formation and dissociation must be fast.

A typical covalent imprinting was boronic acid esters which are synthesised from boronic acid and cis-1,2- or cis-1,3-diol compounds. After polymerisation, these linkages are cleaved by hydrolysis and the boronic groups in the conjugates are arranged suitably for guest binding. These conjugates are especially useful for molecular imprinting towards carbohydrates and their derivatives which have cis-diol moieties. Examples of this imprinting were discussed by Wulff *et al.*, 1991 and Wulff *et al.,* 1997. For acetals and ketals bonding, ketone and aldehyde compounds are reacted with 1,3-diol compounds and the resultant ketals and acetals products are used as functional monomers. Imprinting with Schiff bases involved the reaction of aldehyde with amino compounds to yield Schiff bases compounds. Coordination bonds involved the interaction of metals ion with functional monomer to produce polymerisable metal complexes, in which it acts as the functional monomers in the presence of an

appropriate ligand (template). Example of this type of imprinting was discussed by Matsui *et al.*, 1996.

1.3.2. Non-covalent Imprinting

Mosbach and co-workers have introduced this system to molecular imprinting (Mosbach, 1994 and Komiyama *et al.*, 2003). Compared to covalent imprinting, typical interactions in non-covalent imprinting are hydrogen bonding, ionic, electrostatic, π-π interactions, etc. Functional monomers are simply combined with template in the polymerisation mixture and copolymerised with cross-linking agent. Procedure is simple and easy to perform because it does not need to synthesise covalent conjugates prior to polymerisation. Furthermore, the template can easily be removed under mild conditions by simple extraction. However, non-covalent bonding may not be strong enough to maintain template-functional monomer complexes. Thus, excess of functional monomers are usually added to the reaction mixture in order to complete the template-monomer complexation and to maintain stability under polymerisation conditions. This results in a heterogeneous property of the binding sites in term of affinity.

Many of the particular important molecules, example in pharmaceutical, herbicides, biologically active substances and environmental contaminants possess polar groups such as hydroxyl, carbonyl, amino and amide which are suitable groups for non-covalent interactions. Of these, hydrogen bonding is the most appropriate for precise molecular recognition since the bonding is highly dependent on both the distance and direction between monomers and templates. However, for electrostatic interactions, strong acids and bases are unfavorable as it is less dependent on distance and direction. A proton is completely transferred to the base from acid. If the combination consists of an intermediate strength of acid and base, hydrogen bonding will be dominant and efficient imprinting should be achieved. Weak acids and bases

are also inappropriate for imprinting as the interactions are too weak. Because of its simplicity and versatility, this technique has been widely attempted. For example, Zurutuza *et al.*, 2005 prepared a non-covalent molecularly imprinted solid-phase material for the extraction of cocaine metabolites from aqueous samples. Vallano *et al.*, 2000 have successfully prepared a highly selective MIP column for capillary electrochromatography. Other breakthroughs include the work from Zander *et al.*, 1998; Sellergren, 2001 and Kempe *et al.*, 1994.

1.3.3. Comparison Between Covalent and Non-covalent Imprinting

There are significant differences between the covalent and non-covalent imprinting. Table 1.2 shows the summary of advantages and disadvantages of these two techniques (Komiyama *et al.*, 2003 and Remcho *et al.*, 1999). Among the advantages of covalent imprinting are the monomer-template conjugates are stable and stoichiometric. A wide variety of polymerisation conditions, example by high temperature, at extreme pH and highly polar solvent can be employed since the linkages are stable. For non-covalent technique, the main advantages are easy removal of template molecule and the rate of guest binding and guest release is much faster. Experimental work carried out throughout this study will focus on the non-covalent imprinting technique as it is widely applied in various analytical fields.

Table 1.2: Advantages and disadvantages of covalent and non-covalent imprinting (Komiyama *et al.*, 2003).

Factors	Covalent	Non-covalent
Synthesis of monomer-template conjugate	Necessary	Unnecessary
Polymerisation condition	Wide variety	Restricted
Removal of template after polymerisation	Difficult	Easy
Guest binding and guest release	Slow	Fast
Structure of guest binding site	Clearer	Less clear

1.4. Synthesis of Molecularly Imprinted Polymer

To date, imprinted polymers in the form of particles are reportedly made by various polymerisation methodologies, each of them developed to suit specific target and application. So far, most of the MIPs have been prepared by bulk, suspension, two-step swelling, precipitation and emulsion core-shell polymerisation (Perez-Moral *et al.*, 2004). Other less common methods include film synthesis, aerosol polymerisation and polymerisation on silica particles. Each of these procedures involves the control of different parameters during the synthesis and it produces polymers with different properties and characteristics.

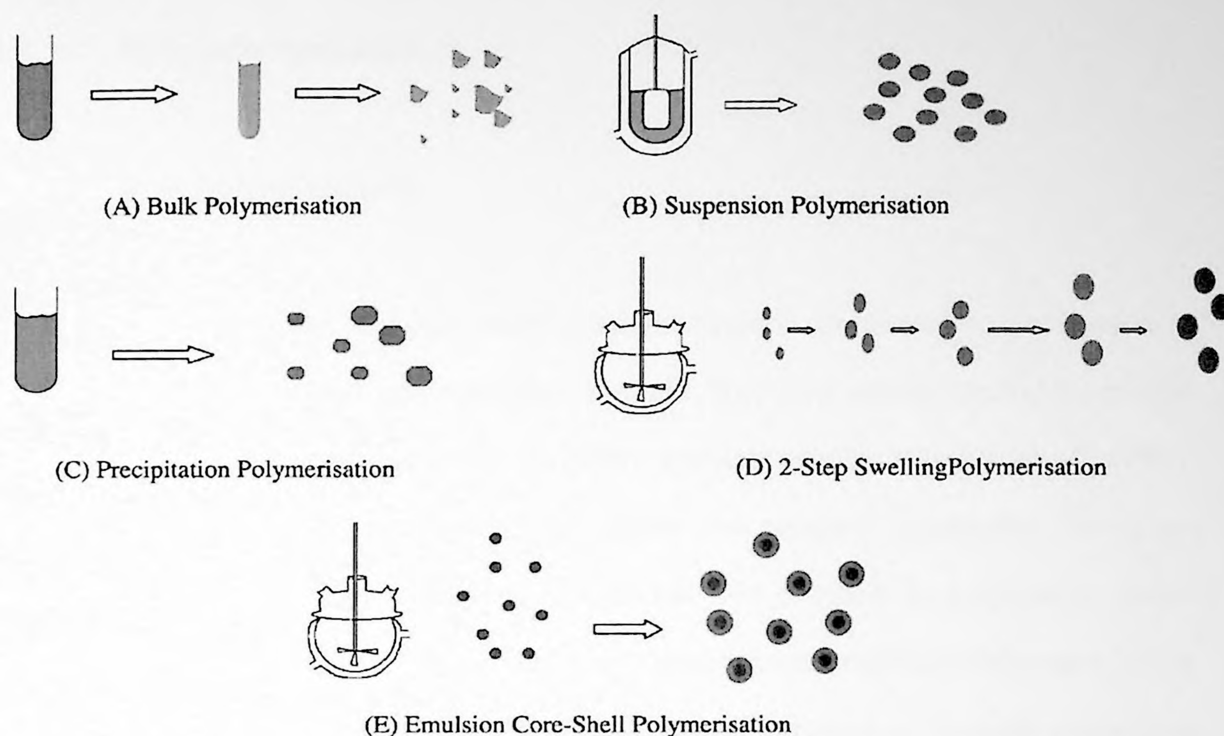


Figure 1.2: Schematical representation of the synthetic steps in the different polymerisation procedures (Adapted from: Perez-Moral *et al.*, 2004).

Table 1.3: Comparison of different polymerisation methods (Mayes *et al.*, 1997).

Polymerisation Methods	Complexity	Product	Advantages	Disadvantages
Bulk- in block	Very straight forward	Random fragments after grinding	Simple, imprinting not affected by method derived factors	Tedious processing, wasteful, poor particle shape for HPLC
Bulk- in columns (in situ)	Moderate	Solid block filling column	Simple, no column packing required	High back pressure, poor peak shape
Suspension – in water	Complex	Spherical beads, polydisperse	Highly reproducible results, large scale possible, high quality beads	Water is incompatible with most imprinting procedures, only possible for some covalent and metal chelate based processes
Two-step swelling	Most complex	Monodisperse beads	Monodisperse beads, excellent packing for HPLC	Need for aqueous emulsion, rules out many imprinting processes

1.4.1. Polymerisation Method

1.4.1.1. Bulk Polymerisation

Chronologically, the first polymerisation method employed to synthesise a MIP was based on the bulk polymerisation method. It is most widely applied by groups working on imprinting because of its simplicity and universality (Mayes *et al.*, 1997). Basically all the components, which are mainly the template molecule, monomer, cross-linker, initiator and porogen are mixed well and proceed to polymerise under heating or ultra violet radiation. The result is a macroporous monolith polymeric block that needs to be crushed and ground in order to obtain particles of irregular shape and size. This process is time consuming and wasteful since a lot of the polymer is lost in the process of grinding and sedimentation to eliminate fine particles. It may also produce areas of heterogeneity in the polymeric matrix resulting from the lack of control during polymerisation process, particularly when UV initiation is used.

1.4.1.2. Suspension Polymerisation

Suspension polymerisation in fluorocarbon solvent was first described by Mayes and Mosbach (Mayes *et al.*, 1997). It is a fast and reliable methodology that synthesizes particles by ultra violet irradiation in less than 2 hour. The beads obtained have a diameter that can vary between 5 to 50 μm depending on the stirring speed and amount of surfactant added. It uses a perfluorocarbon or known as perfluoro-(1,3-dimethylcyclohexane) solvent in the continuous phase which allows the same interactions that occur in bulk polymerisation. The fluorocarbon suspending medium can be easily recycled by distillation. This method offers a simple one step route to high quality beads polymers with quantitatively yield of product and offers a very attractive alternative to grinding and sieving, especially to chromatographic applications

(Mayes *et al.*, 1997). When coupled with the use of trimethylpropane trimethacrylate (TRIM) as cross linker, Mayes and group produced beads with high load capacities and good separation.

1.4.1.3. Precipitation Polymerisation

Precipitation polymerisation is another method that can provides particles in the submicron scale (0.3 to 10 μm). It is based on the precipitation of the polymeric chains out of the solvent in the form of particles as they grow more and more insoluble in an organic condition medium. There is no need of extra stabilizer because the particles are prevented from coalescence by the rigidity obtained from the cross linking of the polymer. The group of Cacho *et al.*, 2004 successfully produced a polymer with more homogeneous binding sites distribution and high affinity constants than those obtained by bulk polymerisation using two different propazine imprinted methacrylic based polymers as models. A non-covalent ferunon imprinted polymer which leaded to the synthesis of spherical particles (~ 1 μm) with homogeneous binding sites distribution have been developed by Tamayo *et al.*, 2003. They have successfully evaluated the polymers for trace-enrichment and clean up of fenuron from plant sample extracts.

1.4.1.4. Two-step Swelling Polymerisation

Two-step swelling polymerisation was developed with MIP by Hosoya and Haginaka teams (Haginaka *et al.*, 2002; Haginaka *et al.*, 2004b; Haginaka *et al.*, 2004a and Hosoya *et al.*, 1996). This technique requires several swelling steps on the initial particles with the imprinting mixture before polymerisation proceeds. In this case, the polymerisation medium is water. This method produces monodisperse particles in the micron size range (2 to 50 μm) with good control of the final size and number of the

particles. It could be easily prepared, *in-situ* modification could be performed, and the obtained MIPs are suitable for HPLC packing materials or SPE materials (Haginaka, 2004). A molecularly imprinted uniform sized polymer based stationary phase for naproxen has been successfully developed by Haginaka *et al., 1*997. The imprinted polymer materials showed enantioselectivity towards naproxen whereas the blank polymer showed no chiral recognition ability. In another work of Haginaka *et al.*, 2002, they have obtained a uniformed enantioselective MIP for *d*-chlorpheniramine using methacrylic acid and ethylene glycol dimethacrylate as functional monomer and cross-linker respectively.

1.4.1.5. Emulsion Core-shell Polymerisation

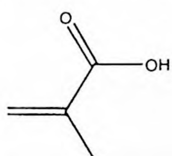
This method produced core-shell particles in which they have a structured morphology that allows the incorporation of any added property into the core of the particles without interfering the imprinted shell (Perez-Moral *et al.*, 2004). The continuous medium during polymerisation is water. Particles obtained by this method are monodisperse and can be used for surface imprinting. Generally, the seed was first prepared using a standard batch emulsion polymerisation in a three-necked jacketed reactor connected to a water bath to control the temperature. The system was equipped with a condenser, a mechanical stirrer and a gas inlet to maintain an inert argon atmosphere. A solution of sodium hydrogen carbonate and sodium dodecyl sulphate in distilled water was added to the reactor and purged with argon to remove oxygen under gentle stirring, while increasing the temperature to 90 °C. Once the temperature was reached, the monomer mixture (methacrylic acid and ethylene glycol dimethacrylate) was introduced into the reactor and the stirring speed increased to 600 rpm. Then, the initiator was added to initiate the polymerisation. The temperature was maintained at 90 °C for 24 hours and the final latex was filtered through a fine nylon mesh. Next, the core-shell particles were synthesised using the similar method as for

preparing the seed. Solution containing water and sodium dodecyl sulphate was added into the reactor and purged with argon. Gentle stirring was applied and solution of monomer, cross-linker, template molecule and seed that have been previously mixed was charged into the reactor followed by an aqueous solution of ammonium peroxodisulphate. The stirring speed was increased and the reaction is allowed to proceed for 6 hours before cooling to room temperature. Non-imprinted polymer was prepared following the same procedure but excluding the template from the formulation.

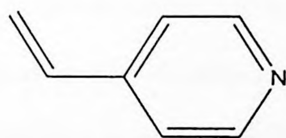
1.4.2. Polymerisation Reagents

The essential chemicals required to produce a MIP are the functional monomer, template molecule, cross-linker, initiator and polymerisation solvent or known as porogen. Chemical structures of the typical reagents used are as presented in Figure 1.3.

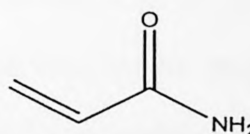
Functional Monomer



Methacrylic Acid

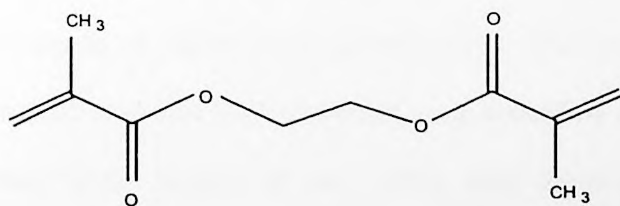


4-Vinylpyridine



Acrylamide

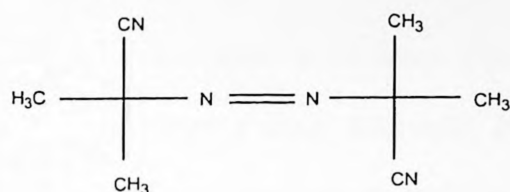
Cross-linking Agent



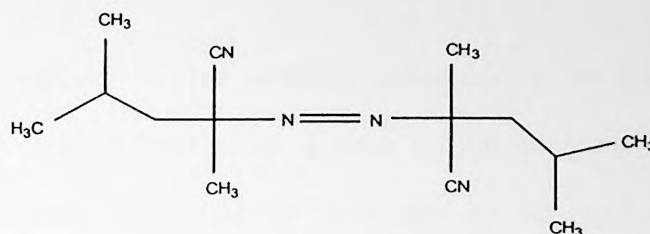
Ethylene Glycol Dimethacrylate (EGDMA)

Figure 1.3: Typical reagents for polymerisation.

Radical Initiator



Azobisisobutyronitrile (AIBN)



Azobisdimethylvaleronitrile (ABDV)

Figure 1.3: Typical reagents for polymerisation (continued).

1.4.2.1. Template Molecule

In all imprinting process, template is one of the most important components. The template chosen must be chemically inert and stable under polymerisation conditions since all polymerisations are based on the free radical interactions. The template molecule must not participate in the radical reaction and stable upon exposure to UV or high polymerisation temperature (Cormack *et al.*, 2004). Usually, a closely structural analogue to the targeted analyte was chosen as template molecule. This is to prevent the template leaching or bleeding problem during analysis especially for quantitative analysis at trace level as not the entire template molecules are successfully extracted out from the imprinted polymer even after extensive washing (Martin *et al.*, 2004). Blomgren *et al.*, 2002 used brombuterol as template for the analysis of clenbuterol in calf urine. The structures of these two molecules are very similar and the MIP obtained was selective and sensitive for analysis of clenbuterol at low level. Martin *et al.*, 2000 also employed the similar approach by preparing propanolol imprinted polymer to extract five compounds structurally related to propanolol.

1.4.2.2. Functional Monomer

Functional monomers are responsible for the binding interactions in the imprinted binding sites. Normally, in non-covalent imprinting, it was added in excess relative to the number of template molecule. The ratio of template to functional monomer of 1: 4 and upwards are rather common (Cormack *et al.,* 2004). It is very important to match the functionality of the template with the functionality of the monomer in a complementary fashion in order to obtain maximum complex formation. Basically, functional monomers can be divided into three different groups which are mainly the acidic, basic and neutral monomer. Methacrylic acid is the most common acidic monomer and are widely selected by various groups of researchers (Dong *et al.*, 2004; Zurutuza *et al.*, 2005 and Theodoridis *et al.*, 2002). For acidic template molecules, vinylpyridine and acrylamide can be selected as functional monomer (Zhou, *et al.*, 1999; Simon *et al.*, 2004 and Bastide *et al.*, 2005).

1.4.2.3. Cross-linking Agent

A cross-linker is added to fulfill three major functions in imprinting. The fundamental role is to fix and control the morphology of the polymer matrix besides from stabilise the imprinted binding sites. They also make the imprinted polymer insoluble in solvents and impart the mechanical stability to the polymer matrix. High cross-link ratios are generally preferred in order to access permanently porous materials and to generate materials with adequate mechanical stability. Thus, polymers with a high degree of cross-linking (70 to 90 %) are required (Masque *et al.*, 2001; Cai, *et al.*, 2004 and Komiyama *et al.*, 2003). For effective imprinting, the reactivity of cross-linker should be similar to that of the functional monomer in a cocktail polymerisation to ensure smooth incorporation of the co-monomer. Several of well-known, commercially

available cross-linking agents such as ethylene glycol dimethacrylate (EGDMA) and divinylbenzene (DVB) are compatible with molecular imprinting.

1.4.2.4. Porogen

The function of polymerisation solvent is to bring all the components into one phase. Besides that, it also responsible for creating pores in macroporous polymer. The nature and level of porogen added will determine the morphology and total pore volume. Thermodynamically good solvents tend to lead to polymers with well developed pore structures and high specific surface areas whereas thermodynamically poor solvents lead to poorly developed pore structures and low specific surface areas. Selection of the porogen is mainly dependent on the type of imprinting. For covalent imprinting, many kind of solvents are employed as long as they are able to dissolve all components. However in non-covalent imprinting, it is critical to the formation of conjugates between the functional monomer and template. Normally, this implies that apolar and non-protic solvents such as acetonitrile, toluene and chloroform are preferred as such solvents stabilise the hydrogen bonds (Chassaing *et al.*, 2004 and Mena *et al.*, 2002). However, in certain application, polar protic porogen was chosen even though polar solvent will disrupt the hydrogen bonding (Baggiani *et al.*, 2001 and Caro *et al.*, 2004).

1.4.2.5. Initiator

The initiator is added into the system to initiate free radical polymerisation. When the initiator is trigged either by heat, UV radiation or chemicals, carbon centered free radicals will formed and these unpair electrons are capable to react with other monomer /cross-linker in order to propagate into longer chains. Polymerisation process stopped when two free radicals reacted with each other (Young *et al.*, 1992). The

amount of initiator added is relatively at low level compared to the monomer. Azo initiator such as azobisisobutyronitrile can be conveniently decomposed by photolysis (UV) or thermolysis.

1.4.3. Factors to Consider in the Synthesis of Selective MIP

1.4.3.1. Molar Ratio of Template: Monomer: Cross-linker (T: M: X)

Number and quality of the MIP recognition sites are highly dependable on the molar relationship between template and functional monomer. The common optimum mole ratio of template molecule, monomer and cross-linker for production of MIP is 1: 3-5: 20-30 (Komiyama *et al.*, 2003). Theoretically, high molar ratio of T: M affords less than optimal complexation on account of insufficient functional monomer and too low of T: M causes non-selective binding (Andersson *et al.*, 1999). Results by Andersson *et al.*, 1999 clearly indicate that an excess of either template or functional monomer during polymerisation is unfavourable regard to selectivity. The group prepared a series of polymers with different T: M ratios for selectivity test. Polymer with the ratio of T: M = 1: 4 has the best selectivity properties as compared to the others. Baggiani *et al.*, 2004 research supported the finding as polymers prepared at T: M = 1: 15 and 1: 20 exhibited poor recognition effect as it is difficult to clearly discriminate them from the corresponding blank polymers. Experiments carried out by Theodoridis *et al.*, 2004 showed that despite high molar ratio of T: M, high affinity recognition sites would be limited as the agglomeration of template in organic solvent environment could occur. Thus, polymers prepared at the ratio of 1: 2.7: 13.4 exhibited poor recognition properties compared to polymers synthesised at ratio 1: 46: 230 and 1: 4.6: 23. Other groups such as Caro *et al.*, 2004; Mena *et al.*, 2002; Spivak *et al.*, 2001 and Zander *et al.*, 1998 also synthesised their imprinted polymers according to the ratio of T: M: X = 1: 4: 20. However, the work done by Davies *et al.*, 2004 showed that their optimum

predicted ratio for T: M: X were 1: 10: 55 and 1: 10: 10 according to the chemometrics approach. Several examples of reagents selection for polymerisation process and the molar ratio of T: M: X employed by various research groups are described in Table 1.4.

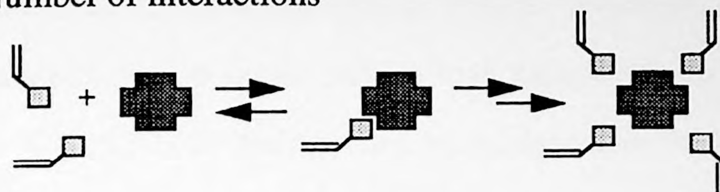
1.4.3.2. Stability of Monomer-template Assemblies

A stable monomer-template assembly is also important for achieving a larger number of imprinting sites and at the same time, the number of non-specific binding sites will be minimised (Sellergren, 1999). Therefore, the type of functional monomer selected and porogen choices are very important to produce a stable monomer-template conjugate. As mentioned previously in Section 1.4.2.2 and 1.4.2.4, monomer selected should be able to serve as a hydrogen bond, proton donor and as a hydrogen bond acceptor. Best porogen will be the aprotic and non polar solvents as these solvents have poor hydrogen binding capacity and low dielectric constant. Thus, they lead to large interaction energy between the template and the functional monomer, resulting in a better affinity and selectivity. Work from Schmidt *et al.*, 2005 and Wu *et al.*, 2005 have proven that the influences of porogens are essential on the affinity and selectivity of MIPs. Figure 1.4 shows the factors affecting the recognition properties of MIPs related to the monomer-template assemblies.

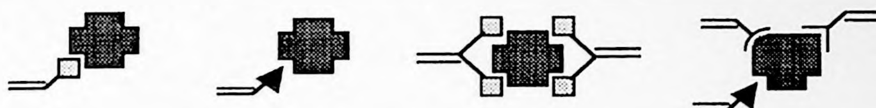
Table 1.4: Choice of reagents and molar ratios of T: M: X for the syntheses of MIPs by bulk polymerisation technique.

Description	References				
	Ersoz *et al.*, 2004	Lu *et al.*, 2004	Svenson *et al.*, 2001	Cacho *et al.*, 2004	Zhou *et al.*, 1999
Template molecule (T)	4- nitrophenol	3-*L*-phenylalanyl-aminopyridine	Theophylline	Propazine	5,5-diphenylhydantoin
Monomer (M)	Methacrylamido-antipyridine	Methacrylic acid	Methacrylic acid	Methacrylic acid	Acrylamide
Cross-linker (X)	EGDMA	EGDMA	EGDMA	EGDMA	EGDMA
Porogen	Acetonitrile	Chloroform	Chloroform	Toluene	Tetrahydrofuran
Initiator	Azobisisobutyronitrile	Azobisdimethyl-valeronitrile	Azobisisobutyronitrile	Azobismethylpropionitrile	Azobisisobutyronitrile
Ratio of T: M: X	~ 1: 6: 29	~ 1: 4: 20	~ 1: 4: 18	~ 1: 4: 20	~ 1: 4: 20
Initiation method	UV radiation	Thermal	Thermal	Thermal	Thermal
Polymerisation process	25 °C for 24 hours	40 °C for 24 hours (highest enantioselectivity)	65 °C for 20 hours	60 °C for 24 hours	60 °C for 24 hours

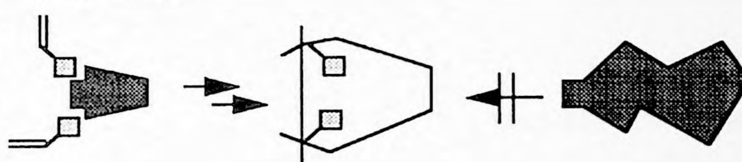
• Number of interactions



• Nature and position of the interactions



• Template shape



• Monomer template rigidity

Figure 1.4: Factors affecting the recognition properties of MIPs related to the monomer-template assemblies (Adapted from: Sellergren, 1999).

1.4.3.3. Polymerisation Temperature

Temperature is an important factor influencing the recognition properties of MIPs as it affects the polymerisation process and polymer structure. The polymer's affinity and specificity can be improved significantly by optimising the polymerisation temperature (Lu *et al.*, 2004). According to the research done by Lu and co-workers, lower polymerisation temperature is advantageous to the stability of the template-functional monomer assemblies in the pre-polymerisation mixture. However, higher polymerisation temperature is favorable for completeness of the polymerisation reaction, which improves the quality and quantity of MIPs recognition sites. They prepared three types of polymers imprinted with 3-L-phenylalanylaminopyridine at 10

°C, 40 °C and 60 °C for 24 hours respectively. Polymer prepared at 40 °C has both the highest enantioselectivity and largest sample load capacity as compared to polymers prepared at 10 °C and 60 °C. Besides that, study conducted by Piletsky *et al.*, 2002 suggested that the polymer is able to memorise the temperature used in the polymerisation process in a manner similar to previously documented MIP memory effects for the template and polymerisation solvent.

1.5. Polymer Structure and Morphology

The structure integrity of the monomer-template assemblies must be preserved during the polymerisation process to allow the functional groups to be confined in space in a stable arrangement complementary to the template. However, the role of the polymer matrix is not only to contain the binding sites in a stable form but also to provide porosity allowing easy access for the guest to all sites. This can be achieved by applying a high level of cross-linking agent and sufficient porogen during polymerisation. Thus, most of the cross-linked network polymers used for imprinting have a wide distribution of pore sizes associated with various degrees of diffusional mass transfer limitations and a different degree of swelling.

1.5.1. Types of Pores

Pores sites in an imprinted polymer can be classified according to different types based on the site accessibility, integrity and stability criteria. Nitrogen adsorption-desorption and mercury porosimetry are techniques for the determination of polymer pore structures in a dry state (Sellergren, 1993). There are typically three types of pores which are the mesopores, macropores and micropores (Sellergren, 1999). Meso and macropores with pore size larger than 20 Å are expected to be easily accessible compared to sites located in the smaller micropores (pore sizes smaller than 20 Å)

where the diffusion is slow. The number of the latter may be higher since the surface area for a given pore volume of micropores is higher than that of macropores. For most applications in liquid media, permanent porosity and a large surface area of accessible meso and macropores are preferred. Referring to Figure 1.5, this gives materials containing mainly accessible sites of type A and B although a significant number of non-specific sites (type F) may present. One of the undesirable effects of adding an excess of template is the loss of site integrity due to coalescence of the binding sites (type D), which is related to the extent of template self-association. Site G contributes to problem of extracting template molecule as the template is remain strongly bound to the polymer even after careful extraction.

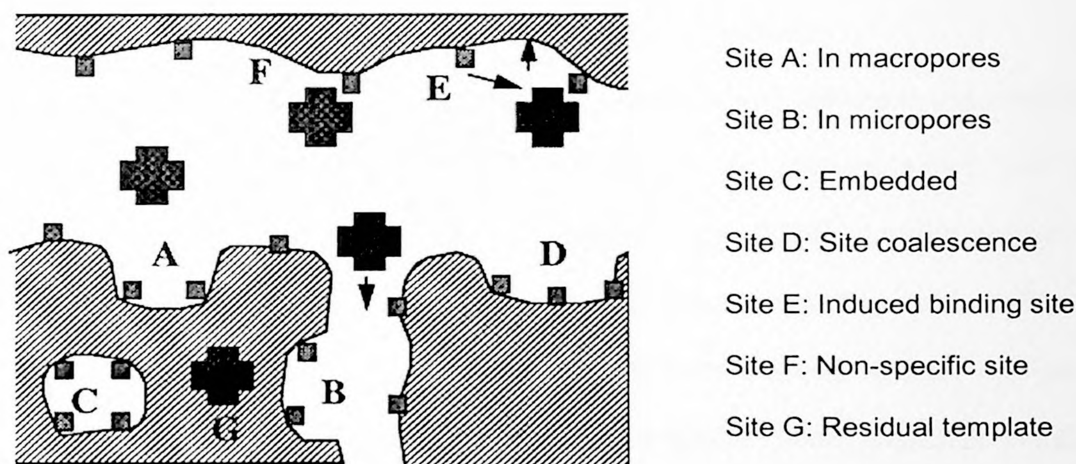


Figure 1.5: Types of binding sites in MIPs (Adapted from: Sellergren, 1999).

1.5.2. Adsorption / Binding Isotherms

Adsorption or binding isotherms can yield important information concerning the binding energies, modes of binding and sites distributions in the interaction of small molecule ligands with receptor as happened in MIPs. In MIPs, a soluble ligand interacts with binding sites in a solid adsorbent. The adsorption isotherms are plots of

equilibrium concentrations of bound ligand (adsorbate) versus concentration of free ligand. This isotherm helps to characterize the MIPs and calculate the corresponding binding parameters and affinity distributions. Adsorption or binding isotherms for MIPs can be obtained from batch rebinding studies in which a constant weight of polymer is equilibrated with a known concentration of analyte (Umpleby *et al.*, 2004). This is then measured over a range of analyte concentrations. The concentration of the analyte remaining free in solution is measured by HPLC, UV spectroscopy or radio-ligand assay. The corresponding concentration of bound analyte is calculated as the difference between the total and free concentrations. Selection of binding model is primarily based on its ability to accurately reproduce the experimental isotherm. The physical basis for the model should also reflect the distribution of sites found in the measured system in order to generate realistic binding parameters.

The isotherm can be fitted using various models where different assumptions are made. Generally, the models can be grouped into two classes which are the discrete and continuous distribution models (Umpleby *et al.*, 2004). Langmuir and bi-Langmuir isotherms are the most commonly applied of discrete binding models. These models simplify a distribution into a finite number of different classes of sites, with each class of site having a different binding affinity. The Langmuir model assumes there is only a single class of sites and the bi-Langmuir assumes there are only two classes of sites. The Freundlich and Langmuir-Freundlich are both examples of continuous distribution models in which a continuous function containing an infinite number of different types of binding sites is used to model the distribution. These models provide more accurate approximations for the heterogeneity present in most MIPs and also provide quantitative measures of heterogeneity. Studies conducted by Umpleby and co-workers suggested that MIPs contain a broad unimodal distribution that exponentially tails into the high affinity region. This exponentially decaying region appears to be the most important with respect to the enhanced affinity and selectivity of MIPs.

1.6. Application of Molecularly Imprinted Polymers

In analytical separation science, molecularly imprinted polymers have been applied in several analytical techniques such as in liquid chromatography, capillary electrochromatography and capillary electrophoresis, solid-phase extraction, immunoassay and as a selective sorbent in chemical sensors. The improved selectivity of imprinted polymers compared to conventional sorbents may lead to cleaner chromatographic traces in the subsequent analytical separation.

1.6.1. Affinity Based Solid-phase Extraction

Out of all the MIPs applications, the most widely acceptance and close to practical realisation is probably that of solid-phase extraction (Andersson, 2000). A number of groups have presented SPE applications based on MIP for various types of analytes in various types of samples matrices. The matrices include biological fluids, tissues, food, aqueous environment samples and pharmaceutical compounds. As shown by Zander *et al.,* 1998, analysis of nicotine and its oxidation products in nicotine chewing gum was possible by applying MIPSPE technique. Besides from obtaining high and reproducible recoveries of less polar analytes, this MIP method eliminates liquid-liquid extraction steps which consume lots of organic solvents. Research by Mena *et al.*, 2002; Caro *et al.*, 2004; Gallego-Gallegos *et al.*, 2005 and Zurutuza *et al.*, 2005 have successfully synthesised and evaluated MIP as selective SPE sorbent for the analysis of aqueous samples. Other examples are extracts of various analytes from plasma and serum samples (Andersson *et al.*, 2004; Bereczki *et al.*, 2001; Theodoridis *et al.*, 2002; Wu *et al.*, 2004 and Mullett *et al.*, 1998). Chassaing *et al.*, 2004 have developed MIP packed into 96-well blocks enabling high throughput analysis and good accuracy and precision. Their research showed that analysis with MIP produced much cleaner baseline, leading to lower background noise and higher sensitivity as

compared to the conventional C18 SPE method. Table 1.5 summarises the application of MIP as SPE sorbent. As the technology is becoming well known, there are commercially available MIPSPE in the market. One of the leading companies is MIP Technologies AB from Lund, Sweden (www.miptechnologies.se). This company offers various ranges of chromatography products from SPE to HPLC. Studies conducted by Kootstra *et al.*, 2005 and Widstrand *et al.*, 2004 using MIPSPE columns from MIP Technologies for multi-residues clean-up of beta-agonists in bovine muscle and calves urine have proven that the method meets the requirements for quantitative determination.

Table 1.5: Summary of studies in which MIPs have been applied to SPE.

Analyte	Sample	Application	Reference
Cocaine metabolites (Benzoylecgonine)	Aqueous samples	Selectively extracted and quantified at clinical relevant concentration.	Zurutuza *et al.*, 2005
Naphthalene sulfonates	Environment samples	Analysed water from Ebro river.	Caro *et al.*, 2004
Tributyltin	Seawater	Quantitation limit of 0.04 μg/L for 1 L.	Gallego-Gallegos *et al.*, 2005
Pirimicarb	Water samples	On-line pre-concentration.	Mena *et al* 2002
Phenytoin	Plasma	Good linearity at 2.5 – 40 μg/mL	Bereczki *et al.*, 2001
Local anesthetics (bupivacaine, ropivacaine and mepivacaine)	Human plasma	Determination of bupivacaine: 3.9 – 500 nmol/L Deternmination of ropivacaine: 7.8 – 500 nmol/L	Andersson *et al.*, 2004
Esculetin	Ash bark of traditional chinese medicine	-	Hu *et al.*, 2005b
Atrazine	Beef liver extracts	Tolerance level in meat products: 0.02 ppm	Muldoon *et al.*, 1997
Theophylline	Serum	-	Mullett *et al.*, 1998
Monosulfuron	Soil	Recoveries (MIPSPE-HPLC): > 93 %, CV < 3.2 %	Dong *et al.*, 2004
Cephalexin and α-aminocephalosporin antibiotics	Human serum	Linearity of Cephalexin: 0.3 – 25 μg/mL Analysis using MIPSPE-PE-MS	Wu et al., 2004

1.6.2. Sensors and Membranes

In biosensor application, a chemical or physical signal is generated upon the binding of the analyte to a biological recognition element such as antibody, a receptor or an enzyme. A transducer then translates the signal into a quantifiable output signal. The same general principle applies if a molecularly imprinted polymer is used as the recognition element instead of the biomolecule (Ye *et al.*, 2004). If the targeted analyte exhibits special properties such as fluorescent or electrochemically activity, it can be exploited for the design of MIP based sensors. An example of MIP based sensor was developed by Piletska *et al.*, 2005. This group has successfully developed a synthetic receptor based on imprinted polymers to detect cocaine, deoxyephedrine, methadone and morphine. Suedee *et al.*, 2004 developed a conductometric sensor for the screening of complex mixture of haloacetic acids (HAAs) in drinking water. This sensor showed good linearity ($r^2 > 0.97$) over the concentration range of 25-1000 [mu]g/L and detection limit in the range of 0.2-5.0 [mu]g/L.

For the application of MIP in membranes, Sergeyeva and co-workers (Sergeyeva *et al.*, 1999) have developed a type of polymeric membranes having artificial recognition sites for atrazine by polymerisation of triethylene glycol dimethacrylate, methacrylic acid and oligourethane acrylate in the presence of atrazine as template molecule. The MIP membrane when analysed in aqueous environment generated the highest sensors responses.

1.6.3. Others

Other application of MIP in chemical analysis is *in-situ* imprinting technique in a liquid chromatography column. In this technique, polymerisation was carried out inside a liquid chromatography column and samples were injected for analysis. Evaluation was carried out based on the capacity factor and separation factor. One of the