Molecularly Imprinted Solid Phase Extraction – Pulsed Elution for Rapid Screening and Determination of Cephalexin in α-Aminocephalosporin Antibiotics

by

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ABSTRACT

The molecular recognition properties of cephalexin molecularly imprinted polymer (MIP) in various organic solvents were investigated. A molecularly imprinted solid phase extraction (MISPE) method was developed for rapid screening and determination of cephalexin in α-aminocephalosporin antibiotics. Cephalexin MIP particles were packed into a micro-column (i. d. 0.8 mm x 50 mm) for selective solid phase extraction (SPE) of CFL. A total binding capacity of 7.3 μg cephalexin was determined in a saturation study for the ~40 mg of MIP particles. Chloroform proved to be the best solvent among the tested organic solvents for cephalexin binding on this CFL MIP micro-column. A ~94% binding of cephalexin was achieved from one 20-μL injection of sample, with chloroform as the mobile phase at a flow rate of 0.5 ml/min. Other α-aminocephalosporins (cefradine, cefadroxil) and β-lactam antibiotic (ampicillin) only attained 14-80% binding. Methanol + 1% trifluoroacetic acid was good for quantitatively pulsed elution (PE) of cephalexin. However, this micro-column interacted indiscriminately with cefradine and cefadroxil. The separation of cephalexin from cefradine and cefadroxil was ultimately achieved, using differential pulsed elution (DPE) with acetonitrile + 12-14 % acetic acid to desorb cefradine and cefadroxil, before cephalexin was determined in a final pulsed elution (FPE) with methanol + 1% trifluoroacetic acid. This MISPE-DPE-FPE method was verified by human serum and plasma analysis. In human serum analysis, a good linearity ($r^2 \cong 0.9884$) was achieved over the cephalexin concentration range of 0.8-27.0 μg/ml, with detection limit (LOD) of 0.3 μg/ml (or 5.1 ng) of CFL in human serum. In human plasma analysis, the linear dynamic range ($r^2 \cong 0.9987$) was obtained within 1.0-20.0 μg/ml, with LOD of 0.6 μg/ml (or 12.8 ng) of CFL in human plasma. The relative recoveries of cephalexin from human serum and plasma were 105 ± 2% and 95 ± 3%, respectively, indicating the suitability of this method for the quantification of cephalexin in human serum and plasma samples.

The method was further developed by introducing a Quattro triple quadrupole mass spectrometer to substitute the UV detector. Elution of cephalexin (m/z 348) was

quantified using sulindac (m/z 357) as an internal standard to correct for signal variations, by recording the peak intensity ratio of cephalexin / sulindac versus the mass of CFL isolated in MISPE. The mass spectrometer was programmed in the positive electrospray mode, coupled with the selected ion recording (SIR) function, to improve the detection and quantification of the molecular ions of cephalexin and sulindac. This optimized MISPE-PE-MS method proved to be an improvement of the UV detector based MISPE-PE method for serum analysis. A fairly good linear dynamic range ($r^2 \cong 0.9968$) was achieved within 0.3–25 μg/ml (or 5–500 ng) of CFL, with LOD of 0.04 μg/ml (or 0.8 ng) of CFL. The relative recovery of cephalexin from human serum was 93 ± 1 %.

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GLOSSARY

AIBN	2,2'-Azobisisobutyronitrile
AMP	Ampicillin
CFD	Cefadroxil
CFL	Cephalexin
CFR	Cefradine
DPE	Differential pulsed elution
EDMA	Ethylene glycol dimethacrylate
ESI	Electrospray ionization
FIA	Flow injection analysis
FPE	Final pulsed elution
HPLC	High performance liquid chromatography
LC-MS	Liquid chromatography mass spectrometry
LOD	Limit of detection
LOQ	Limit of quantification
MAA	Methylacrylic acid
MIP	Molecularly imprinting polymer
MISPE	Molecularly imprinted solid Phase extraction
PE	Pulsed elution
RSD	Relative standard deviation
SD	Standard deviation
SIR	Selected ion recording

SPE	Solid phase extraction
TFA	Trifluoroacetic acid
TFMAA	2-(Trifluoromethyl)acrylic acid
UV	Ultra-violet

CHAPTER I

INTRODUCTION

1.1 Molecular Imprinting

1.1.1 Historical Development

The concept of molecularly imprinted polymer (MIP) originates from Linus Pauling's early theory regarding the formation of antibodies. Pauling suggested that antibodies were formed when serum proteins assembled around template antigen molecules. The assembled antibodies were found to have specificity-endowing binding pockets complementary to the antigens. Further evidence came from strong energy of antibody-antigen binding, which results from noncovalent binding interactions including hydrogen bonds, ionic bonds, etc. A hypothesis was thereafter made by Pauling and Campbell that artificial antibodies could be assembled using these basic principles.[1] Although Pauling's theory was later disproved, several groups subsequently tried to apply it to some artificial systems. In the 1970s, Wulff, at the University of Düsseldorf (Düsseldorf, Germany), formed covalent bonds between a monomer and the template molecule, followed by polymerization and template cleavage to yield a specific binding site. This method was limited by the synthetic necessities of first preparing a monomer-template molecular conjugate, and later chemically cleaving the template molecule from the polymer.[2]

The further application of Pauling's original concept was the synthesis of artificial antibodies with the development of molecular imprinting by Klaus Mosbach's group at the University of Lund (Sweden).[3] One of the major breakthroughs made by this group was the successful approach of preassembling a noncovalently associated monomer-template complex in solution prior to polymer formation, which enabled molecular imprinting to be used in a variety of applications. From then on, Mosbach's group continued to lead in new developments including studies on various polymer systems, classes of template molecules, aqueous imprinting systems, and novel physical formats to extend the potential usefulness of molecular imprinting. Imprinting has now reached a high level of sophistication, and patent coverage in the field is extensive.[4-7]

1.1.2 General Concept of Making an Imprint

To make MIPs, the molecule to be imprinted is dissolved in solvent together with functional monomer and cross-linker. The monomer is chosen to have a chemical functional group that will interact and associate with the imprint molecule via either hydrogen bond or ionic interaction. Following preassociation, polymerization occurs by addition of an initiator with mild heating or UV irradiation. Once the solid polymer has formed, it is ground in a mortar and pestle and sieved to obtain a desired particle size. The print molecule is extracted by incubation in a solvent, normally involving an acid or base, capable of disrupting the interactions between the imprint molecule and the functional groups inside the MIP binding cavities. (Figure 1)

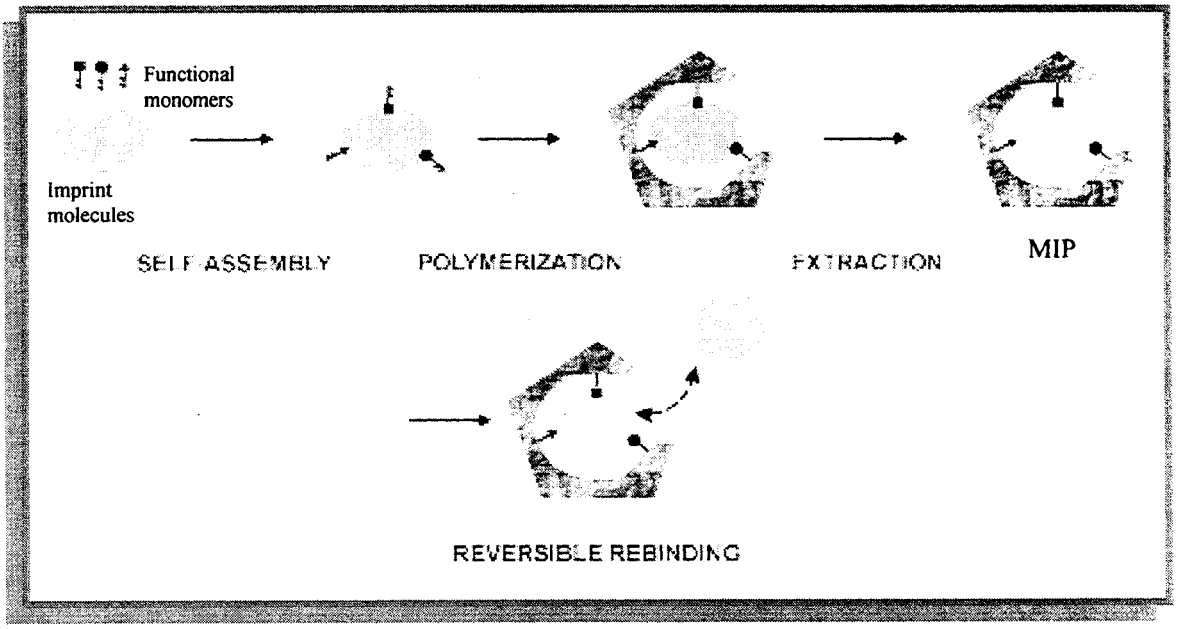


Figure 1. Schematic diagram of molecular imprinted polymerization

1.1.3 Current Development of Molecular Imprinting Technology Combined with Solid Phase Extraction

MIPs can be stored in the dry state at ambient temperatures for several years without loss of recognition capabilities. Generation of molecular imprints does not involve the use of laboratory animals or any material of human origin. MIPs are much more resistant to matrix effects than are biological antibodies, therefore can be used in rigorous experimental conditions. Imprinted polymers can be made against print molecules that are too toxic for immunization in animals to raise antibodies. MIPs have been demonstrated against many classes of molecules, including drugs,[8] hormones,[9] pesticides,[10] proteins,[11] amino acids,[12] peptides, [13] etc.

The technique of molecular imprinting has been applied with success to the preparation of polymers with high affinity and specificity for applications in the area of separations, assays, and sensor science.[14, 15] They have enabled scientists in mimicking the ability of biological hosts to specifically and strongly bind to a target molecular structure.

As mentioned above, the advantages of MIPs over immunoaffinity materials, e.g. antibody, represent on the high affinity of MIPs towards the template and its structural analogues. They are superior to the traditional immunoaffinity methods (immunoaffinity phase, immunoassays, and immunosensors), in terms of the stability, reusability, ease and low cost of preparation. The merits of MIPs also come from the fact that they can tolerate harsh working conditions like acidic environment. One promising area of applications is the development of highly selective solid phase extraction (SPE) methods. After more than fifteen years of experimentation, solid phase extraction is now recognized as the standard tool for sample pretreatment prior to detection, as it is easily automated, flexible and environmentally friendly.

The combination of MIP with SPE (or MISPE) is promising as an alternative to conventional SPE for coupling to both liquid chromatography and gas chromatography.[16] Most of the recently published works have proved that the rebinding of template in polymers, in the form of either ionic or hydrogen bonding, takes place in the presence of

the same solvent as during polymerization. For example, it was reported that the polymers prepared in toluene showed better recognition when the loading solvent was toluene than when it was acetonitrile (more polar than toluene), and analyte retention decrease when the polarity of the solvent increases.[17] These new evidences provide a straightforward guideline for the development of MISPE in selecting the loading solvent. Further optimization of the loading solvent may follow in order to prevent nonspecific interactions. It was reported that the retention of clenbuterol in a control polymer (prepared without template) and in an MIP, both prepared in acetonitrile, was complete when acetonitrile was used as a loading solvent, owing to nonspecific binding between the clenbuterol and the control polymer matrix. By adding 1% acetic acid to the acetonitrile (for the purpose of disrupting hydrogen bonding) the binding decreased to 33% in the control polymer.[18]

MISPE coupled with pulsed elution (PE) was firstly introduced by Mullett and Lai in 1998.[19] MISPE with PE is based on the use of a small amount (less than 50 μl) of polar solvent to elute the analytes that are retained by the MIP particles packed into a micro-column during SPE. In Mullet's work, theophylline in chloroform-extracted serum samples (20 μl) was extracted on a theophylline MIP micro-column, with chloroform as the mobile phase. Any potential interferences and coextractives would pass through the column. A 20-μl aliquot of methanol was injected to elute theophylline for direct determination with an UV detector at 270nm.

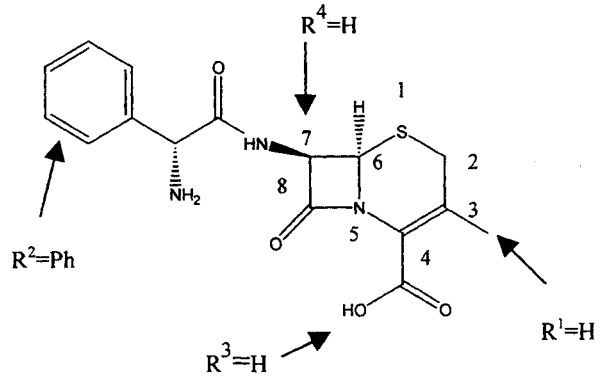
Further improvement to the MISPE-PE methodology was achieved by successive 20-μl aliquot of different solvents to eliminate interferences, which is known as MISPE-DPE.[20] In 2000, Wayne Mullett reported that the effect of aprotic solvent polarity represented a convenient parameter for controlling the binding 4-aminopyridine versus 2-aminopyridine on the MIP micro-column. By adjusting the polarity, the bound 2-aminopyridine (structural interference) could differentially removed from MIP micro-column by an intermediate wash with 20 μl aliquots of dimethyl sulfoxide (DMSO). These previous studies in the area of MISPE-PE laid a good foundation for the present research to develop a MISPE-DPE-FPE method for fast screening and determination of cephalexin.

1.2 Cephalexin

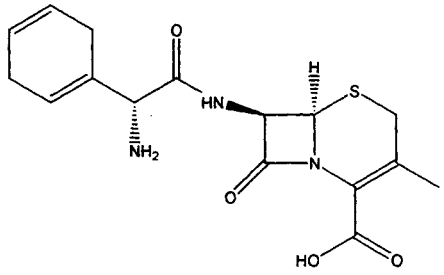
1.2.1 Current Applications

Cephalexin, or 7-[(aminophenylacetyl) amino]-3-methyl-8-oxo-5-thia-1-azabicyclo [4.2.0] oct-2-ene-2-carboxylic acid (Figure 2), is a semi-synthetic drug in a class of antibiotics called cephalosporins. Under the brand names of Ceporex (or Keflex) in the U.S., Novolexin in Canada, and many others outside North America, cephalexin ranks 13th of the top 20 drugs in prescriptions worldwide (Table 1).[21] Tablets, capsules and liquid suspensions are mostly administered for oral administration.

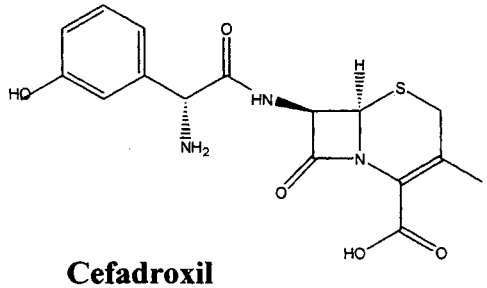
Figure 2 Four typical α-amino cephalosporin antibiotics



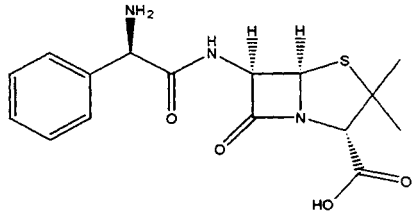
Cephalexin



Cefradine



Cefadroxil



Ampicillin

Table 1. Top 20 drugs in prescriptions in USA (2000)

Ranking	Brand Name	Manufacturer	Generic Name
1	Hydrocodone w/APAP	Various	Hydrocodone w/APAP
2	Lipitor	Parke-Davis	Atorvastatin
3	Premarin	Wyeth-Ayerst	Conjugated Estrogens
4	Synthroid	Knoll	Levothyroxine
5	Atenolol	Various	Atenolol
6	Furosemide (oral)	Various	Furosemide
7	Prilosec	Astra	Omeprazole
8	Albuterol	Various	Albuterol
9	Norvasc	Pfizer	Amlodipine
10	Alprazolam	Various	Alprazolam
11	Propoxyphene N/APAP	Various	Propoxyphene N/APAP
12	Glucophage	B-M Squibb	Metformin
13	**Cephalexin**	**Various**	**Cephalexin**
14	Amoxicillin	Various	Amoxicillin
15	Claritin	Schering	Loratadine
16	Trimox	Apothecon	Amoxicillin
17	Hydrochlorothiazide	Various	Hydrochlorothiazide
18	Zoloft	Pfizer	Sertraline
19	Zithromax (Z-Pack)	Pfizer	Azithromycin
20	Prozac	Lilly	Fluoxetine

1.2.2 Pharmacological Actions

As shown in Figure 2, cephalosporins (R-4 = H) compounds contain a β-lactam ring, which is fused with a six-membered dihydrothiazine ring bearing substituents R-1 and R-2 in the side-chains at C-3 and C-7. They are available either as free acids (R-3 = H) or salts (R-3 = Na or Li). Cephalexin fights bacteria in the human body and is used to treat many different types of bacterial infections such as bronchitis, tonsillitis, ear infections, skin infections, and urinary tract infections. Cephalexin is also used for the treatment of heart diseases due to its enhanced oral activity. Pharmacologically, cephalosporins produce their bactericidal effect by inhibiting peptidoglycan cross-linkage and cell wall synthesis. The site of action for β-lactam antibiotics is the penicillin-binding proteins (PBPs) on the inner surface of the bacterial cell membrane. In actively growing cells, cephalosporins bind to the PBPs within the cell wall and lead to interference in the production of cell wall peptidoglycans and subsequent lysis of the cell in an iso-osmotic environment.

1.2.3 Adverse Reactions

Gastrointestinal: Symptoms of pseudomembranous colitis may appear either during or after antibiotic treatment. The most frequent side effect has been diarrhea. It was very rarely severe enough to warrant cessation of therapy. Dyspepsia, gastritis, and abdominal pain and nausea and vomiting have also occurred. As with some penicillins and some other cephalosporins, transient hepatitis and cholestatic jaundice have been reported rarely.

Hypersensitivity: Allergic reactions may occur in the form of rash, urticaria, angioedema, and erythema multiforme. Stevens-Johnson syndrome, or toxic epidermal necrolysis and anaphylaxis have also been observed. These reactions usually subsided upon discontinuation of the drug. In some of these reactions, supportive therapy may be necessary.

Other reactions may occasionally appear, including genital and anal pruritus, genital moniliasis, vaginitis, dizziness, fatigue, headache, agitation, confusion, hallucinations, arthralgia, arthritis, and joint disorder. Reversible interstitial nephritis,

eosinophilia, neutropenia, thrombocytopenia, and slight elevations in AST and ALT have been reported. [22]

1.2.4 Current Analytical Methods for Cephalexin Determination and Limitations

The standard instrumental method for routine determination of cephalexin in pharmaceutical formulation,[23-27] human plasma [28, 29] and serum [30, 31] is high performance liquid chromatography (HPLC), with either isocratic or gradient elution followed by UV, photodiode array or mass spectrometric detection. This method can be used to evaluate the stability of bulk drugs, process-related impurities, formulation excipients, degradation products and metabolites. The analysis normally requires longer than 7 min.

A high performance liquid thinner layer chromatography (HPTLC) densitometric method was reported for the determination for cephalexin in the 200-1000 ng range, which can run 12 samples simultaneously in less than 15 min with a solvent consumption of 15 ml.[32] The accuracy of the method assessed by spiking cephalexin in blank capsule matrix gave a % average recovery of 106.1% with RSD (n=6) 1.6. Fluorometric method was also reported for the determination of cephalexin and three other α-aminocephalosporins, involving a reaction with fluorescamine at a specific pH.[33] During the last two decades, electroanalytical techniques were developed for sensitive and selective determination of a number of cephalosporin antibiotics. One major advantage of these procedures for analysis of drugs and biological materials is that they often involve little or no pre-separation, which would not only be time-consuming, which would not only be time-consuming but also a possible source of errors.[34] However, all the present analytical methods have certain limitations.

HPLC method, although widely applied in many pharmaceutical industries, usually exhibits overlap with peaks from structural analogues in the chromatogram. The present lowest detection limited reported is 5 μg/ml. In terms of analysis time, HPLC normally takes 7-15 minutes to finish each run. HPTLC suffers a similar problem, especially in screening CFL from α-aminocephalosporin antibiotics.[32] Fluorometric method, due to introduction of the reaction with fluorescamine at pH 9, potentially

involves two problems: (1) result variation caused by fluorescamine reaction, which is dependent on the reaction time, temperature and pH; (2) as β-lactam antibiotics share a common 2-azetidinone four-member ring, fused with a 6-member heterocyclic ring, cephalexin may undergo hydrolysis in either alkaline or acid media solution.[35] The recently reported studies of electroanalytical methods for cephalexin were based on an empirical choice of conditions for recording of current-voltage curves. In the majority of published papers either the question of the nature of the electrode process yielding the measured current was not mentioned or only guesses were made without sufficient experimental evidence. To achieve a proper and most efficient use of electroanalytical methods there is still a lot of work - perhaps even more importantly than for other analytical techniques - to understand at least the nature of the processes involved.[36]

Cephalexin belongs to the group of cephalosporins without an electroactive group in the side chain on C-7 and with an alkyl group on C-3, which normally undergo a two-electron reductive hydrogenation of ethylenic bond in protic media. Such two-electron reduction waves decrease with increasing pH greater than 2.5.[37] However, electrochemical analysis of cephalexin at such low pH environment will simultaneously involve the hydrolysis that may cause degradation of the cephalexin during the analysis.[38]

Many pharmaceutical and biomedical laboratories, therefore, want to perform simpler, faster and highly selective analysis for the screening of cephalexin in α-aminocephalosporins assay.

1.3 Introduction of MISPE-PE for Determination of Cephalexin

MIPs, as a class of smart sorbents for analytical separation, enable a methodology development by mimicking the ability of biological hosts to specifically and strongly bind to a target molecule structure.[39] The application of MIPs in screening of combinatorial library has become an increasing attractive approach. [40] One promising field of challenge in this project is to develop a highly selective solid phase extraction method based on a cephalexin MIP sorbent for a on-line extraction of cephalexin from the clinical human plasma and serum samples. The other application will go into

developing a differential pulsed elution (DPE), following the MISPE step, to eliminate the structural interference of cephalexin, and a final pulsed elution (FPE) for direct quantification of cephalexin.

The CFL MIP to be tested in the present project was synthesized and donated by Dr. Hongsheng Guo and Prof. Xiwen He, Nankai University (Nanjing, China), two years ago. Their recent investigation of this CFL MIP has mainly focused on the batch binding of cephalexin in aqueous solution.[41] Their work partially proved molecular recognition of this MIP for CFL in aqueous media, and also declared two types of binding behaviors coexisting in the MIP particles. However, since their investigation mainly focused on the static binding behavior, the results were still unable to give sufficient support for application of this MIP to MISPE in organic solvents. In terms of selectivity, three structural analogues (cefadroxil, ampicillin, and amoxicillin) were tested, the K_D (distribution coefficient) of CFL did not show any significant difference from the structural analogues, especially cefadroxil.

$$K_D=C_p/C_s \quad (1-1)$$

where

C_p: concentration of substance on the polymer (in μmol/g)

Cs: concentration of substance in the solution (in μmol/ml)

For the present methodology development of MISPE-PE in quantitative determination of cephalexin, three steps must be involved: (1) investigation of molecular recognition of this MIP for cephalexin in organic solvents; (2) test for selectivity of this MIP towards cephalexin versus its structural analogues; (3) development of pulsed elution (PE) and/or differential pulsed elution (DPE) for accurate and quantitative analysis.

1.4 Liquid Chromatography-Mass Spectrometry (LC-MS)

Of the diverse detector technologies available today, mass spectrometry (MS) is the most versatile tool for meeting the analytical demands for drug analysis.[42] After years of research and development in analytical instrumentation, MS no longer is the expensive and specialized tool as it was before; it has rapidly become the detection method of choice in many applications, especially where sensitivity and specificity are important.[43] .Mass spectrometers measure the mass-to-charge (m/z) ratios of gas phase ions. Creating gas phase ions is the role of the ionization method. The typical Ionization methods available on the instruments within the MS facility are electron ionization (EI), chemical ionization (CI), fast atom bombardment (FAB), electrospray ionization (ESI), matrix-assisted laser desorption/ionization (MALDI), and atmospheric pressure chemical ionization (APCI).

ESI and APCI

Electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI) are two examples of atmospheric pressure ionization (API) sources. Such sources ionize the sample at atmospheric pressure and then transfer the ions into the mass spectrometer. Due the sample ionization, which is performed under atmospheric pressure, the ionization efficiency is 103-104 times as great as obtained in a reduced-pressure CI source. Electrospray ionization mass spectrometry (ESI-MS) is a newly established instrumental technique for the analysis of fragile or nonvolatile organic compounds.[44] In summary, the sample solution is sprayed across a high potential difference (a few kilovolts) from a needle into an orifice in the interface, where heat and gas flows are used to desolvate the ions existing in the sample solution. Electrospray ionization can produce multiply charged ions with the number of charges tending to increase as the molecular weight increases. Complementary to APCI, ESI is better for polar or basic compounds. It is being widely used as an on-line detector coupled with liquid chromatography (LC). The separation ability of LC combined with the high sensitivity and selectivity of ESI-

MS has promoted this combination into a standard analytical technique in the areas of biochemistry and environmental chemistry. [45]

Recently, the effective combination of a wide variety of HPLC methods with mass spectrometry plays a vital role in the acceptance of LC-MS. This achievement is significant because HPLC-based methods are universally recognized and utilized throughout every stage of drug discovery and development. The improved performance of LC-MS, in turn, stimulates new requirements for this analytical technology. The recent acceptance of advanced methods for analysis, structural identification and databases allows increased amounts of information to be generated in shorter periods of time. As researchers embrace different approaches for the collection of information on pharmaceutical properties, LC/MS emerges as an advantageous technique for a variety of screening-based approaches. One of those approaches is further optimization of the chromatographic procedure by introducing new materials to replace the conventionally used stationary phase, so as to upgrade the selectivity of analytical separation.

MISPE-PE-MS

In recent years, application of solid phases in either reversed phase type or normal phase type has been employed in HPLC column for on-line separation. Since the stationary phase of the systems only utilize nonspecific interactions, LC separation procedures have to be carefully optimized, especially due to the close chemical characteristics of the target analyte and various coexisting interferences. Another challenge is that the traditional solid phase extraction (SPE) usually have selectivity problems, especially in separating the structural analogues, because those SPE methods can extract a class of compounds with similar polarity, but can not distinguish the analytes from the structural analogues, due to their similar polarity.

Various examples of application of MIPs in SPE cartridges for on-line concentrating and purifying the target molecule or a class of structurally related analytes were reported in the literature. However, application of MIPs as solid phase extraction device coupling with mass spectrometry for on-line screening and separation was not being reported. Comparing with UV detector, mass spectrometer has superior advantages,

especially in terms of high sensitivity and specificity, due to not being affected by the background signal interference.

1.5 Objectives

The project was initialized by the hypothesis that in organic solvents molecular recognition may be achieved, and could be more appropriate for method development of molecularly imprinted solid phase extraction (MISPE). The first objective of the present research work was therefore to investigate the molecular recognition properties of CFL MIP particles (20 to 80 μm in size) in different organic solvents so as to establish dynamic flow conditions for the development of an optimal MISPE method. Once the molecular recognition for CFL under dynamic flow conditions was proven, selectivity would be investigated and optimized for better isolation of CFL from its structural analogues. The second challenge was to develop a differential pulsed elution method for comprehensive elimination of all the interference by structural analogues, which may partially be recognized and retained by the MIP particles, before the final on-line quantification of CFL. Based on the development of MISPE-PE-UV, a quadrupole mass spectrometer programmed in selected ion recording (SIR) mode would next be introduced to replace the UV detector, for a significant improvement of the sensitivity of the method. Serum and plasma analyses would finally be performed for method validation.

CHAPTER II

EXPERIMENTAL PROCEDURES

2.1. MISPE-DPE-FPE

2.1.1 Chemicals

Cephalexin (CFL), cefradine (CFR), cefadroxil (CFD), ampicillin (AMP) were obtained from Sigma-Aldrich (Mississauga, ON). Chloroform ($CHCl_3$), acetonitrile (CH_3CN), and methanol (CH_3OH) were HPLC-grade solvents obtained from Fisher (Fair Lawn, NJ) and Caledon (Georgetown, ON). As cephalexin was available only as a hydrate ($C_{16}H_{17}N_3O_4S.H_2O$) that is not soluble in $CHCl_3$, the white powder (99.3 % assay, no further purification) was first dissolved in CH_3OH before dilution with $CHCl_3$ to make up a standard. Acetic acid (HAc) was purchased from Anachemia (Canada). Trifluoroacetic acid (TFA) was obtained from Aldrich (Milwaukee, WI). Human serum (minus IgG) was purchased from Sigma Immunochemicals. Human plasma was supplied by the Biochemistry Laboratory, Ottawa General Hospital.

2.1.2 Synthesis of CFL MIP

The synthesis of CFL MIP was described in "Study of the binding characteristics of molecular imprinted polymer selective for cefalexin in aqueous media", Hongsheng Guo, Xiwen He, *Fresenius J. Anal. Chem.* (2000) 368, 461-465

To 5 ml of acetonitrile or methanol, 1 mmol of cephalexin and 4 mmol of TFMAA were added. After the cephalexin was dissolved completely, 20 mmol of the cross-linking agent ethylene glycol dimethacrylate (EDMA) and 30-50 mg of the initiator, 2,2'-azobisisobutyronitrile (AIBN), were added. The mixture was poured into a glass ampoule, degassed with ultrasonic wave, and then bubbled with nitrogen for 5 min. Degassing and bubbling were repeated 3-5 times. The ampoule was sealed under vacuum and placed into a thermostatic bath at 60 °C for 24 h. After the tube was crashed, the bulk MIP obtained was ground to obtain a suitable range of cephalexin MIP particles.

2.1.3 Packing CFL MIP micro-column

Before packing CFL MIP micro-column, the polymer particles sizing from 20 to 80 μm were selected by sifting with two different sized screens. Then a slurry solution of these

particles with methanol was manually injected from a syringe through a plastic connection tubing to a stainless steel column (i. d. 0.8 mm x 50 mm) immersed in an ultrasonic water bath. After the column was fully packed in 90 min, sonication was continued for 30 min to attain a more homogeneous packing density. A zero-volume union was put on the newly packed column before acetonitrile was pumped through for 2-3 hours to achieve uniformly tight packing. Approximately 40 mg of MIP particles was contained in the micro-column.

2.1.4 Removal of template molecule from CFL MIP

The packed MIP micro-column was installed by connecting with an Eldex 9600 solvent delivery system (San Carlos, CA). A Rheodyne 7125 switching valve (Cotati, CA) containing a 20-μl sample loop was used for sample injection. A Gilson 115 UV detector was used to monitor the removal of CFL template. The retention time and peak areas were recorded by a Dionex 4270 integrator (Sunnyvale, CA).

Removal of CFL templates was finalized by performing an on-line flushing using 1 % TFA + CH_3OH at flow rate of 0.5 ml/min for 2 hr, followed by flushing with 100 % CH_3CN for 2 hr.

2.2 Investigation of molecular recognition

2.2.1 Instrumental

An Eldex 9600 HPLC pump (San Carlos, CA) or CC-30S micrometer pump was used as solvent delivery system. A Rheodyne 7125 Injector valve or Cheminert VIGI Model C2XL Extended Life Injector valve (Valco Instruments. Co. Inc. TX) installed with a 20μl sample loop was used for sample injection and PE. The breakthrough and elution of the analytes was monitored by Gilson 110 UV detector (Middleton, WI) or Lambda UV 1010 detector (Bischoff, Leonberg, Germany), at the wavelength of 240-275nm. The breakthrough and PE peak area were recorded and integrated by Dionex 4270 integrator (Sunnyvale, CA). (Figure 3)

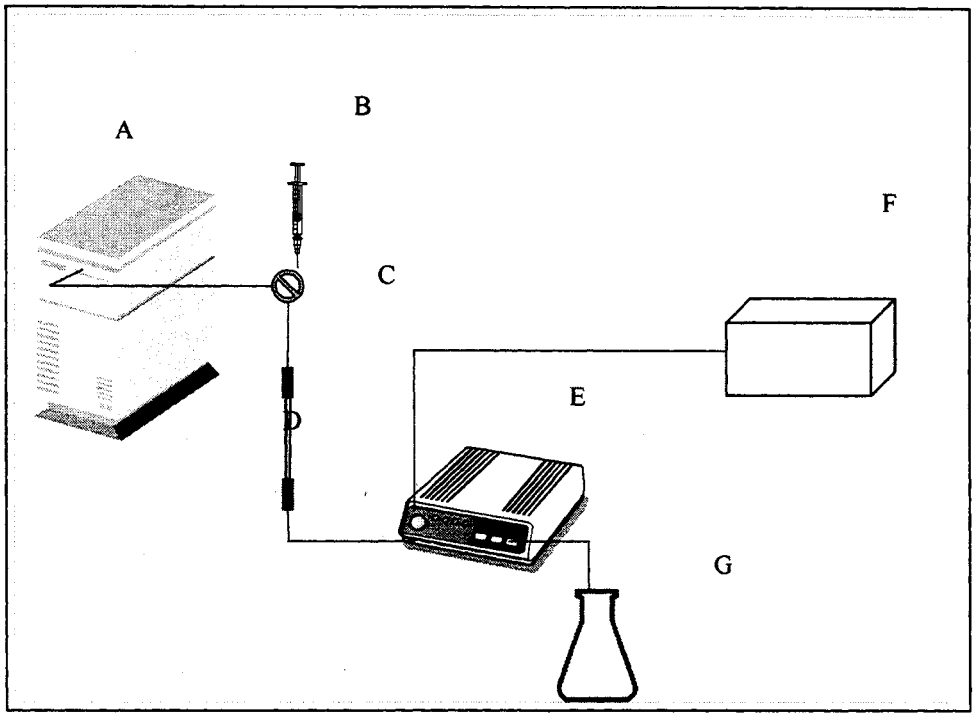


Figure 3 Schematic diagram of MISPE-PE (UV) system

A: Eldex 9600 HPLC pump or CC-30s micrometer pump

B: Syringe (Hamilton)

C: Rheodyne 7125 Injector valve or Cheminert VIGI Model C2XL Extended Life Injector valve, installed with a 20-μl sample loop

D: CFL MIP micro-column (ϕ 0.8 mm x 50 mm)

E: Gilson 110 UV detector or Lambda UV 1010 detector

F: Dionex 4270 integrator

G: Waste collector

2.2.2 Molecular recognition of CFL

2.2.2.1 % Binding evaluation

Different organic solvents including CH_3OH, CH_3CN and CH_3Cl were used as mobile phases for selecting the best solvent for CFL binding. A 20 μg/ml CFL sample solution was prepared with CH_3OH and $CHCl_3$ (CFL was initially dissolved in certain amount of CH_3OH, followed by dilution with $CHCl_3$, $CH_3OH/CHCl_3$=1:80). The experiment initialized by single injection of 20-μl aliquot of 20 μg/ml CFL sample solution bypassing the CFL MIP micro-column, followed by recording CFL peak area, then into the CFL MIP micro-column, with above solvents as mobile phase, individually, at flow rate of 0.5 ml/min. The breakthrough peak areas were measured, and compared. Among the three organic solvents, $CHCl_3$ gave the smallest breakthrough peak area, indicating that CFL in $CHCl_3$ may achieve the highest binding in this MIP micro-column.

2.2.2.2 Binding capacity evaluation

Binding capacity of CFL MIP micro-column towards CFL was investigated by performing multiple injections of a 20 μg/ml CFL sample solution (containing $CH_3OH/CHCl_3 \cong 1/80$) into the CFL MIP micro-column, with $CHCl_3$ as mobile phase, at flow rate of 0.5 ml/min. The binding saturation was finally observed when the breakthrough peak area of each injection became identical to the flow injection analysis (FIA) peak area.

2.2.2.3 Influence of flow rate on % binding

For better understanding the effect of flow rate on binding efficiency, different flow rates of mobile phase over the range from 0.5-1.5 ml/min were tested, with chloroform as mobile phase. 1 % TFA + CH_3OH was used as PE solvent for elution of bound CFL. The % binding was evaluated vs different flow rates.

2.2.2.4 Relationship of concentration and % binding

For further investigation of the relation between CFL concentration and the % binding, CFL standard solutions of different concentrations over the range of 7.0-57 μg/ml (containing $CH_3OH/CHCl_3 \cong 1/80$), were tested. $CHCl_3$ was used as mobile phase at a flow rate 0.5 ml/min. 1 % TFA + CH_3OH was used as PE solvent. MISPE was performed by single injection of individual CFL standard solution, followed by PE with 1 % TFA + CH_3OH. The breakthrough peak area after each single injection was recorded by evaluating and comparing % binding achieved when individual concentration was tested.

2.2.3 Molecular recognition of CFR, CFD and AMP

2.2.3.1 % Binding evaluation

% Binding of CFR, CFD and AMP was investigated by single injection of 20 μl of CFR standard solution (21 μg/ml), CFD standard solution (20 μg/ml) and AMP standard solution (13.3 μg/ml) into the CFL MIP micro-column, respectively, following the same experimental procedure as for CFL. The breakthrough peak areas were recorded, and compared with FIA peak areas (peak area obtained after standard solution was injected by passing the CFL MIP micro-column). After each MISPE, PE by 1 % TFA + CH_3OH was applied to clean the column for the next run.

2.2.3.2 Binding capacity evaluation

The binding capacity of this CFL MIP micro-column towards CFR, CFD and AMP was performed using CFR standard solution (21 μg/ml), CFD standard solution (20 μg/ml) and AMP standard solution (13.3 μg/ml), respectively, following the same procedure as for CFL.

2.2.4 Molecular recognition of different polymer micro-columns towards CFL, CFR and CFD

2.2.4.1 Molecular recognition of control polymer micro-column towards CFL

For further understanding of CFL binding behavior, a control polymer micro-column (packed with Control Polymer particles, i. d. 8 mm x 68 mm) was temporarily used to replace CFL MIP micro-column in the system. MISPE was performed by single and multiple injection of 20 μl of 21 μg/ml CFL standard solution, respectively, with $CHCl_3$ as mobile phase at flow rate of 0.5 ml/min, followed by PE with 1 % TFA + CH_3OH.

2.2.4.2 Binding saturation investigation for CFL on control polymer micro-column

Binding saturation study on this control polymer micro-column towards CFL was conducted by multiple injection of 20 μl each aliquot of 20 μg/ml and 540 μg/ml CFL standard solutions into the control polymer micro-column, respectively, with $CHCl_3$ as mobile phase at a flow rate of 0.5 ml/min. The breakthrough peak area after each injection was recorded until the binding saturation was observed.

2.2.4.3 Investigation of nonspecific binding of CFL on control polymer micro-column

Two different solvents, $CHCl_3$ and CH_3CN, were used as mobile phase, respectively, at a flow rate of 0.5 ml/min. MIPSE was performed by single injection of 9.7 μg/ml CFL in $CHCl_3$ into the control polymer micro-column, followed by PE with 1 % TFA + CH_3OH. The standard calibration curve of MISPE-PE for CFL was constructed by multiple injection of CFL standard solutions in 1 % TFA + CH_3OH, with concentrations ranging from 0.8-160 μg/ml. The breakthrough and PE was monitored and recorded, respectively.

2.2.4.4 Molecular recognition of control polymer micro-column towards CFD

Binding saturation investigation was performed by multiple injection of 20-µl aliquot of 21 µg/ml of CFD standard solution into the control polymer micro-column, with $CHCl_3$ as mobile phase at flow rate of 0.5 ml/min. Breakthrough peak area after each injection was monitored and recorded, respectively.

2.2.4.5 Binding saturation test of CFL on isoproturon MIP micro-column

Investigation of binding saturation test of CFL on Isoproturon MIP micro-column was performed by multiple injection of 20-µl aliquot of 540 µg/ml CFL standard solution in $CHCl_3$ into an Isoproturon MIP micro-column (i. d. 0.8 mm x 55mm), with $CHCl_3$ as mobile phase at a flow rate of 0.5 ml/min. The breakthrough peak area after each injection was monitored and recorded.

2.3 MISPE-PE

2.3.1 PE solvents

0.25 –14 % CH_3COOH + CH_3CN

In the first step, CH_3COOH over the range from 0.25-14 % in CH_3CN were tested as the different DPE solvents individually, after single injection of 20-µl of 20 µg/ml of CFR standard solution prepared with the above solvents, followed by FPE with 1 % TFA + CH_3OH. The ΔFPE peak area was recorded and plotted vs the concentration of CH_3COOH. CFD standard solutions, with concentrations ranging from 18-22 µg/ml, were tested following the same procedure. Based on ~0 ΔFPE peak area, 12-14 % CH_3COOH + CH_3CN was initially located as the DPE solvent.

In the second step CFL standard solutions of 10-20 µg/ml in various organic solvents (CH_3CN, CH_3CN + 0.25-14 % CH_3COOH, CH_3OH, CH_3OH + 10 % H_2O, CH_3OH + 0.025-0.05 % CH_3COOH, and CH_3OH + 0.05-1.0 % TFA) were prepared. 20

μL of each standard solution was injected onto the MIP micro-column for MISPE analysis. From the breakthrough peak area, a % binding result was calculated for each solution of CFL in a different organic solvent. Based on a ~0 % binding, CH_3OH + 1 % TFA was finally chosen for the PE of CFL in all MISPE-PE analyses.

For further confirmation of this experiment, CFR and CFD standard solutions over the concentrations ranging from 10-25 μg/ml, prepared with $CHCl_3$, were investigated by performing MISPE with 12-14 % CH_3COOH + CH_3CN as mobile phase, followed by PE with 1 % TFA + CH_3OH. Based on ~0 ΔPE peak area, 12-14 % CH_3COOH + CH_3CN was finally chosen as the DPE solvent.

Standard calibration curve of MISPE-PE for CFL

MISPE was performed by single injection of 20-μl aliquot of standard solutions over the concentration ranging from 0.3-50 μg/ml in chloroform, followed PE with 1 % TFA + CH_3OH. Standard calibration curve of MISPE-PE for CFL was constructed by recording ΔPE peak area versus concentration (μg/ml) of CFL.

3 % HCl + CH_3OH

For investigation of whether the PE is pH dependent procedure, MISPE was performed by multiple injection (n=3) of 20 μl aliquot 0.4 mg/ml CFL standard solution with $CHCl_3$ as mobile phase at a flow rate of 0.5 ml/min, followed by DPE with 3 % HCl + CH_3OH (~1 mol/L, pH 1.4), and a final pulsed elution (FPE) with 1 % TFA + CH_3OH (~ 0.1 mol/L, pH 2.5).

2.3.2 Elimination of the spectrometric interference from AMP by changing detection wavelength

UV spectrometric analysis was performed using a Cary III UV spectrometer (Varian). AMP standard solution (0.5 mg/ml) prepared with 1 % TFA + CH_3OH was analyzed within the wavelength range 200-330 nm. Verification was conducted by performing MISPE-PE for a mixture solution of 21 μg/ml CFL in the presence 11.2 μg/ml AMP in $CHCl_3$, at λ = 275 nm.

2.3.3 Serum analysis

Human serum (Sigma Immuno Chemicals, S5143) (1 VIAL 051H-4823) was spiked with CFL, followed by treatment with an Octadecyl C_{18} SPE cartridge (T. Baker, 7020-03). Elution was performed with 3 ml of methanol (HPLC grade, Fisher Scientific) afterwards, and the eluent was collected and diluted with chloroform, containing CFL 0.5-50 μg/ml.

The standard calibration curve of MISPE-PE was constructed by performing single injection of MISPE, with 14 % CH_3COOH in CH_3CN as mobile phase, followed by PE with 1 % TFA in CH_3OH. An Eldex CC-30 s micrometer pump was used for delivering mobile phase, giving a flow rate of 0.5 ml/min. A Cheminert VIGI Model C2XL Extended Life Injector valve (Valco Instruments. Co. Inc. TX) containing a 20 μl sample loop was used for injection and PE. The absorbance of micro-column elution was monitored and the output signal was recorded and integrated for retention time and peak area measurement.

CFL serum sample solutions were obtained after being spiked with CFL, CFR and CFD. The sample solutions were treated following the same procedure as for preparation of CFL serum standard solutions, containing CFL 3.4 μg/ml (containing CFR 3.8 μg/ml, CFD 4.1μg/ml) and 7.0 μg/ml (containing CFR 7.9 μg/ml, CFD 8.5 μg/ml), respectively. MISPE-PE was performed following the same procedure in step 2.

2.3.4 Plasma analysis

CFL plasma standard solutions were prepared by spiking human plasma with CFL directly, and treated following the same SPE procedure, as described in **2.3.3**, containing CFL from 0.7-50 μg/ml.

The standard calibration curve of MISPE-DPE-FPE was constructed by performing MISPE with $CHCl_3$ as mobile phase at flow rate of 0.5 ml/min, followed by DPE with 12 % CH_3COOH + CH_3CN and FPE with 1 % TFA + CH_3OH, respectively.

The recovery of the method was investigated by measuring plasma samples (1 ml), containing CFL 0.9-2 μg/ml, CFR 0.9 μg/ml, and CFD 0.9 μg/ml, respectively.

Measurement of the above sample solution was strictly followed the procedure for CFL serum analysis.

2.4 MISPE-PE-MS

2.4.1 Instrumental

MISPE of CFL was performed isocratically on the CFL MIP micro-column (i. d. 8 mm x 50 mm), with chloroform as the mobile phase. A Shimadzu LC-610 pump equipped with a Shimadzu SCL-6B system controller was used to deliver chloroform, at a flow rate of 0.05 ml/min. A Rheodyne 7125 switching valve (Cotati, CA, USA) equipped with a 20-μl sample loop was used for sample injection and PE, where 30 μl of sample or PE solvent was loaded into the injector to ensure complete filling of the loop. 1 % TFA in methanol, containing 20 μg/ml sulindac (internal standard), was used as PE solvent for quantitative elution and determination of the bound CFL. A 50-cm long and 75-μm i.d. fused silica capillary was used to connect the CFL MIP micro-column with the mass spectrometer. It was estimated that the delay time (between the sample injection and elute reaching the mass spectrometer) was 3-4 min. A Quattro triple quadrupole mass spectrometer (Micromass®) programmed at positive ion electrospray ionization (ESI) function was used to monitor the PE of the eluate. Data were processed under the control of Micromass Professional Station, Masslynx version 3.5. Selected ion recording (SIR) mode was programmed as the data collection mode of the MS detector. For each run, the MS detector was set at the m/z value corresponding to the (M+1) of the analytes and the internal standard: CFL (348), CFR (350), CFD (364), and sulindac (357). (Figure 4)

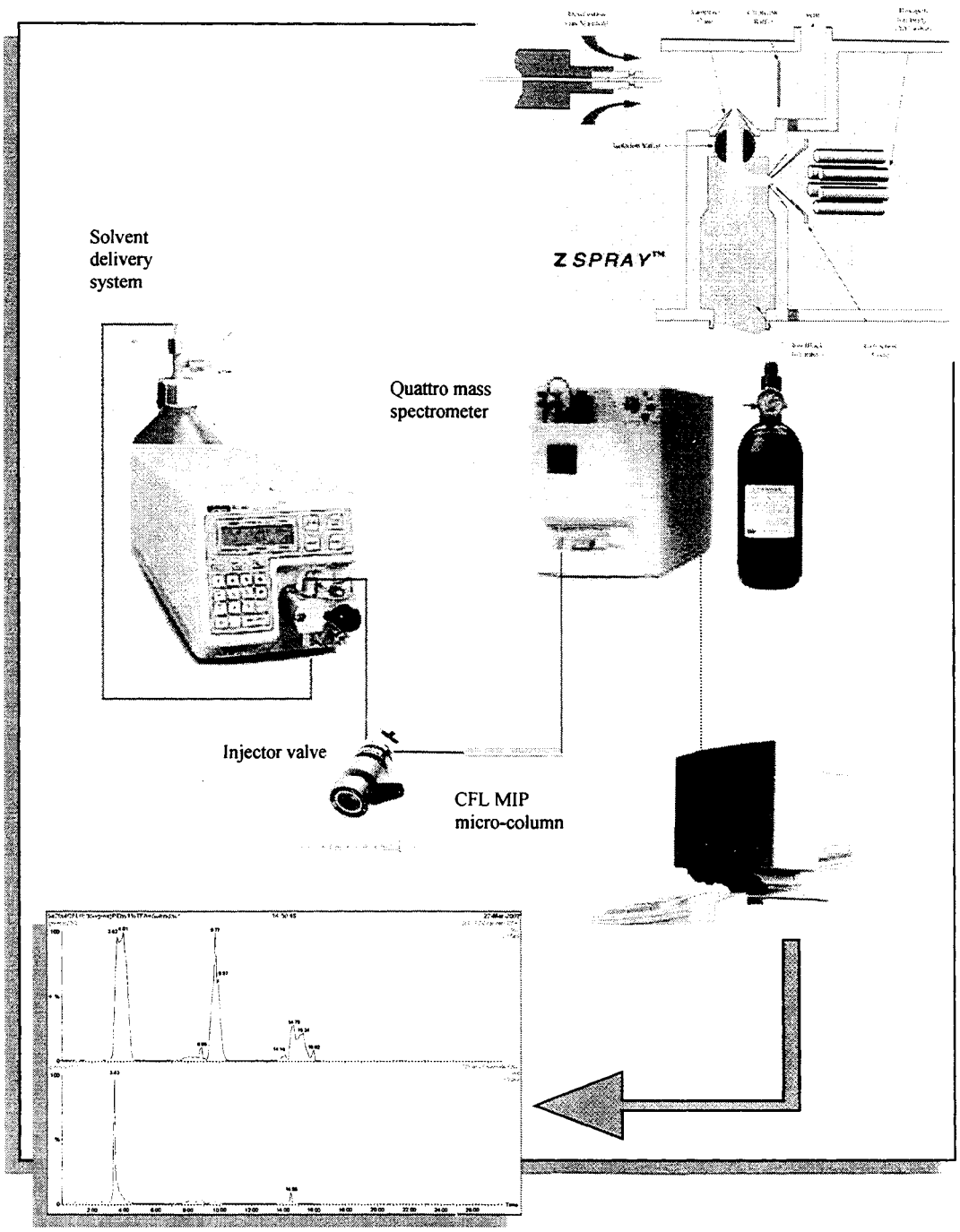


Figure 4. Schematic diagram of MISPE-PE-MS system

2.4.2 Investigation of ionization of CFL, CFR and CFD

Ionization of CFL, CFR and CFD was investigated by injecting a 20-μl aliquot of 0.3 mg/ml of CFL, CFR and CFD in 1 % TFA + CH_3OH, individually, through the CFL MIP micro-column. The mass spectra were recorded.

2.4.3 Verification of binding behavior of CFL and CFR and CFD

MISPE was performed by single injection of 20-μl aliquot of the mixture of CFL (20 μg/ml)+ CFR (20 μg/ml) + CFD (20 μg/ml) into the CFL MIP micro-column, followed by PE with 1 % TFA + CH_3OH. The mass spectra of PE result were recorded.

2.4.4 MISPE-PE-MS for CFL

MISPE of CFL was performed by injecting 20-μl aliquot of CFL standard solutions, containing CFL: 0.1-106 μg/ml, respectively, into the CFL MIP micro-column. PE was performed with 20-μl aliquot of 1 % TFA + CH_3OH. The tune condition was displayed in Table 2. The peak intensity of eluted CFL was recorded.

Table 2 Instrumental Condition of Quattro Quadrupole MS system

Source (ESP^+)	Set		Rdbk	Analyzer		Set	Rdbk
Capillary	4.82		4.83	LM Res 1		15	
Cone	20		20	HM Res 1		15	
Extractor	7		6	I energy		2	
RF lens	0.2			Entrance		43	-43
Source Block Temp	80		79	Collision		0	-1
Desolution Temp	200		200	Exit		42	-42
				LM Res 2		15	
				HM Res 2		15	
				I Energy		2	
				Multiplier		650	-650
Pressure	Rdbk			Gas Flows		Rdbk	
Analyzer Vacuum	6.30E-06			Nebuliser		<20.0	
Gas Cell	2.00E-05			Drying		71.4	
Mobile Phase: $CHCl_3$, Flow rate: 0.05 ml/min							

2.4.5 Method development

2.4.5.1 Selection of internal standards for PE

Sulindac was eventually chosen as the internal standard, mainly due to stable chemical properties and a close m/z (357) as that of CFL (348), which was convenient for comparison. 1 % TFA + CH_3OH, containing 20 μg/ml sulindac, was used as final PE solvent.

2.4.5.2 MISPE-PE-MS for quantification of CFL

Investigation of Ionization Competition between CFL, CFR and CFD

Ionization competition between CFL, CFR and CFD was investigated by injecting a 20 μl aliquot of 20 μg/ml CFL standard solution into the CFL MIP micro-column with 100 % $CHCl_3$ as mobile phase at flow rate of 0.05 ml/min, followed by PE with 1 % TFA + 20 μg/ml sulindac + CH_3OH, containing CFR and CFD with concentrations ranging from 4-75 μg/ml, individually. The mass spectra of different PE results were recorded.

Serum Analysis

CFL serum standard solutions were prepared and treated following the same procedure, as described in **2.3.3**, containing CFL 0.1-50 μg/ml.

Standard calibration curve of MISPE-PE-MS for serum analysis was constructed by single injection of 20-μl aliquot of CFL serum standard series into the CFL MIP micro-column, with $CHCl_3$ as mobile phase at flow rate of 0.05 ml/min. PE was followed by multiple injection of 20-μl aliquot of 1 % TFA + CH_3OH + 20 μg/ml sulindac.

The stock aqueous solution was spiked with CFR and CFD (aqueous solution), followed by Solid Phase Extraction using an Octadecyl C_{18} Cartridge (T. Baker, 7020-03). The elution was performed afterwards using 3 ml of methanol, and the eluent was collected into a 15-ml volumetric flask, and diluted with $CHCl_3$ to the volume, containing CFL 13.5–25 μg/ml, CFR 60.5 μg/ml and CFD 31.6 μg/ml. MISPE-PE was performed following the same experimental condition as for standard calibration curve.

CHAPTER III

RESULTS AND DISCUSSION

3.1 Molecularly imprinted solid phase extraction (MISPE)

3.1.1 Why is a removal of template from CFL MIPs necessary?

Before the CFL MIP can be used in MISPE, the CFL as template molecules must be removed from the polymer. The necessary extent of CFL removal depends on the subsequent application. Thus, in preparative applications incomplete removal may be a marginal problem whereas in analytical applications bleeding of non-extracted template may cause quantification inaccuracies. An additional problem is the legal implications of template bleeding when attempting to procedure for illegal drug use.

It was reported that continuous extraction using a Soxhlet apparatus typically results in the removal of up to 99 % of the template.[46] Several studies, however, showed that a small portion of the template remains unextracted even extensive washing using various organic solvents containing acid or base additives. The remaining template can constitute a problem as it might bleed from the polymer during the elution step of the solid-phase extraction (SPE) procedure, giving erroneous results and an increased limit of quantification (LOQ). This problem hampers the use of MIPs for trace level analysis. To overcome the problem, an often-necessary compromise is to use a close structural analogue of the target analyte as template.[47] Therefore, it is of prime concern to search for methods capable of reducing bleeding to acceptable levels.

The network inside MIP polymers of the type obtained in molecular imprinting is built up of domains with different cross-linking density. The polymerization conditions (temperature, solvent, type and concentration of monomers, cross-linking level, and initiator system) influence the build-up of the porous structure.

Theoretically speaking, the carboxylic groups inside the cavities of CFL MIP particles entrap the CFL molecules by forming multiple hydrogen bonds with four functional groups of CFL molecule, which act as electron-donors. Practically however, single or less than 4 hydrogen bonds may more likely to form due to the steric obstacle, yet the bonding strength would be relatively much weaker, depending on the number hydrogen bonds formed inside the cavities. The working principle of elution is actually a procedure to break the hydrogen bonding between the template and the carboxylic groups

by forming new and stronger hydrogen bonding between the template and the elution solvent. Certain elution solvents may only elute the CFL molecules entrapped by the carboxylic groups with certain number of hydrogen bonds. Besides, the steric obstacles also cause additional difficulty for comprehensive elution of the entrapped template CFL molecules from the particles. Due to distance limitation of the formation of hydrogen bond, one expectable outcome is pulsed elution can only elute the template CFL molecules from the cavities distributed on the surface layer of CFL MIP particles, while the CFL molecules inside the cavities of the deeper layer will prefer staying.

3.1.2 Molecular recognition investigation

3.1.2.1 General knowledge

It is commonly understood that a MIP with maximum selectivity for the template is obtained when prepared with enough functional monomer to form hydrogen bonding with all of the functional groups of the template. For the case of CFL, four molar equivalents of TFMAA (as the functional monomer) were optimally used to form a pre-polymerization complex with CFL, through hydrogen bond interactions with the primary amine, amide, tertiary amine, and carboxylic acid groups. Since TFMAA has a higher acidity than methacrylic acid (MAA), it would afford stronger ionic interaction with CFL. Hence the MIP should be able to recognize CFL molecules in aqueous media. In a recent study, Guo and He performed equilibrium binding experiments to evaluate the recognition characteristics of this CFL-MIP in aqueous media. Scatchard analysis showed that two classes of binding sites were formed in the MIP. Their dissociation constants were estimated to be 0.14 mmol/L and 2.38 mmol/L. The MIP gave much higher binding capacity for CFL than the non-imprinted polymer with the same chemical composition. However, in aqueous environment, H_2O molecules may act as binding competitors against the carboxylic groups inside the MIP cavities to bind CFL molecules, in the form of hydrogen bond. Although hydrogen bond is weaker than the ionic interaction (acid-base) between the amino groups of CFL molecule and the carboxylic group of the fixed functional monomer in terms of bonding energy, yet water may contribute certain negative action to affect MISPE of CFL in aqueous solution. My hypothesis was that in an organic solvent environment this CFL MIP should behave even better, in terms of

molecular recognition, due to much weaker binding competition from the solvent molecules. Two necessary steps must be involved prior to the investigation of molecular recognition properties of CFL MIP particles: (1) removal of the CFL template molecules out of the MIP cavities; (2) selection of a suitable solvent for molecular imprinting.

3.1.2.2 Comprehensive removal of the template entrapped inside the CFL MIP particles

The CFL MIP particles were donated by Dr. He Xiwen, of NanKai University, China two years ago. It was, therefore, essential to verify the molecular recognition of these MIP particles. Before being packed into the micro-column, these polymer particles were not treated with any organic solvent containing acid, and there was no evidence to suggest that certain binding may be achieved due to possible binding saturation of template CFL molecules inside the MIP cavities. The micro-column configuration was the right choice for rapid pulsed elution (PE) of bound CFL, using only 20 μL of an appropriate solvent to minimize both solvent consumption and analyte dilution. Before investigation of molecular recognition of this CFL MIP, a comprehensive elution of the CFL template entrapped inside the MIP particles is very necessary. Since MISPE normally deals with the surface layer of MIP particles, when MISPE is performed, the CFL templates entrapped on the surface of MIP particles will significantly limit the CFL target molecules being absorbed by the binding sites on the surface layer of MIP particles. The second concern is the leaking of the CFL templates from the internal of the CFL MIP particles. The choice of the template can also be decisive for the success of the extraction protocol especially if the analyte is present at trace levels. This is because, despite possible wash during the preparation of the material, traces of the template may remain entrapped in the MIP and slowly leach out during analysis, hindering any accurate determination.[48] The aprotic polar solvent may theoretically be applicable for removal of CFL templates from CFL MIP particles, in the initial step methanol was used as PE solvent to extract the CFL template molecules from the CFL MIP particles, however, the removal of template CFL molecules proved to be incomplete due to a further elution of CFL observed when 1 % TFA + $CHCl_3$ was used pulsed elution solvent. An opposite

investigation was later conducted when CH_3OH was employed as solvent for the preparation of standard CFL solution, where only a 52 % binding only was attained (Table 3). The reason was due to 2-(Trifluoromethyl) acrylic acid (TFMAA), which was used as the monomer in replace of traditionally used methylacrylic acid (MAA) in synthesis of this MIP. TFMAA, exhibiting stronger acidity than MAA, is expected to achieve a more stable complex formation with basic template. The polarity (or dielectric constant) of methanol is not strong enough to disrupt the interactions responsible for the molecular recognition between the imprinted molecules and the binding sites inside the MIP cavities. Trifluoroacetic acid is by far the strongest organic acid, which has a pKa value of –0.25. The removal of imprinted templates was finally optimized by using 1 % TFA in methanol as mobile phase, flushing the CFL MIP micro-column for 2 hours at a constant flow rate of 0.5 ml/min, followed by equilibration with acetonitrile for 2 hours.

1 % TFA + CH_3OH, after being used as mobile phase to flush the CFL MIP micro-column for 2 hours, proved to be very effective in elution of the template CFL molecules entrapped in the CFL MIP particles. The conclusion was confidently supported by the experimental observation that UV detector monitored no eluted CFL after the CFL MIP particles inside the column was allowed to be immersed in CH_3CN for 2-5 hours. This evidence indicated that within the described time interval bleeding of the template CFL molecules was significantly reduced. By comparison with the Soxhlet apparatus, this flushing technique was simple and quite straightforward in terms of operation.

Table 3 % Binding and binding capacity of CFL MIP micro-column for cephalosporin antibiotics prepared in $CHCl_3$ (with $CHCl_3$ as mobile phase, at flow rate of 0.5 ml/min)

Cephalosporin	Concentration (μg/ml)	% Binding	Binding Capacity 40 mg CFL MIP particles (μg)
CFL	23.2-56.8	90-95 % (±3 %, n = 16)	7.3
CFD	20.0	78-80 % (±2 %, n = 10)	3.8
CFR	20.8	68-76 % (±6 %, n = 6)	1.7
AMP	12.3	14-20 % (±1 %, n = 3)	-

3.1.2.3 Choice of appropriate solvent for molecular recognition

Selection of suitable solvent as mobile phase for investigation of molecular recognition was based on the fact that hydrogen bonding prefers environment of weak polar organic solvent. The choice of a good solvent for MISPE of CFL was limited by its poor solubility in both non-polar and polar organic solvents. One notable exception would be methanol, in which CFL (monohydrate) dissolved readily. Theoretically also, MIPSE prefers the same solvent used for MIP synthesis. Therefore, methanol was initially used as solvent for CFL testing solution preparation and mobile phase for MISPE. However, the fact was that CFL could only achieve 52 % binding in the presence of methanol, suggesting that methanol or acetonitrile may still compete with the binding sites (carboxylic acid groups) for entrapping CFL molecules on the surface layer of CFL MIP particles owing to the strong polarity of (ε^{o} =0.95) (Table 4). Structurally speaking, binding competition of these polar organic solvents comes from the hydroxyl group –OH, which in the presence of organic solvent may form hydrogen bond with CFL molecule by donating proton to the amino group. Therefore, selection of appropriate solvent as mobile phase was a very important issue in methodology development. In his Ph. D study, Wayne M. Mullet once mentioned his investigation of the solvent polarity on molecular recognition by adjusting the percentage of polar solvent. His conclusion confirmed that the higher the polarity of the solvent used, the less likely the molecular recognition would occur. [49] The polarity of organic solvent mainly attributes to the ability of dissociating or associating proton. Chloroform was finally chosen, as an appropriate solvent due to the fact that this solvent may not dissociate proton from its trichloromethane structure.

Table 4 Eluent Strength (ε^o) of commonly used organic solvents

Eluent strength (ε^o) is a measure of the solvent adsorption energy, with the value for pentane defined as zero. The greater the eluent strength, the more polar or polarizable the solvent.

Solvent	ε^o	Solvent	ε^o
Fluoroalkanes	-0.25	Dichloromethane	0.42
n-Pentane	0	Tetrahydrofuran	0.45
i-Octane	0.01	1.2-Dichloroethane	0.49
n-Heptane	0.01	2-Butanone	0.51
Cyclohexane	0.04	Acetone	0.56
n-Decane	0.04	Dioxane	0.56
Cyclopentane	0.05	Ethyl Acetate	0.58
Carbon	0.15	Methyl Acetate	0.6
Tetrachloride	0.18	1-Pentanol	0.61
1-Chloropentane	0.26	Dimethyl Sulfoxide	0.62
1-Propyl Ether	0.28	Anline	0.62
1-Propyl Chloride	0.29	Nitromethane	0.64
Toluene	0.29	Acetonitrile	0.65
Chlorobenene	0.30	Pyridine	0.71
1-Chloropropane	0.30	2-Propanol	0.82
Benzene	0.32	Ethanol	0.88
Bromoethane	0.37	**Methanol**	**0.95**
Diethyl Ether	0.38	1,2-Ethanediol	1.11
Chloroform	0.4	Acetic Acid	Large

-- "Quantitative Chemical Analysis", 4th version, Harris, p 673

3.1.2.4 Comparison of % binding of CFL, CFR, CFD and AMP

The previous studies regarding molecular recognition of MIP mainly focused on the investigation of static binding or so-called batch binding. Guo and He in 2000 reported their investigation of CFL batch binding in aqueous solution. Their experiment was performed by emerging the sized and washed CFL MIP polymer particles (20 mg) into a 10 ml of known concentration of selected substrate in water at 25 °C for 16 hr. The concentration of testing solution was determined again by spectrophotometer at appropriate wavelength. The amount of substrates bound to the polymer was calculated by subtracting the concentration of free substrate from the initial substrate concentration. Technically speaking, however, their static binding investigation could not give confidential support in the application of this MIP polymer for the direct on-line MISPE due to lack of dynamical investigation. Comparing with batch (static) binding, the behavior of MISPE relies on the suitable binding sites distributed on the surface of MIP particles. In other words, MISPE only occurs on the surface of MIP particles. With a normal flow rate over the range between 0.1-1.0 ml/min, the molecular recognition occurs within a very short period. By ignoring the dispersion of sample solution in the mobile phase, the total length of sample solution plug was calculated as follows:

$$l = \frac{V}{(d/2)^2 \times \pi} = 3.98 \text{ (cm)} \qquad (3\text{-}1)$$

where

l: length of 20 μl sample solution plug inside the CFL MIP micro-column;

V: volume of sample injection (20×10^{-3} ml);

d: internal diameter of CFL MIP micro-column (0.08 cm).

$$t = \frac{L \times \pi \times (d/2)^2}{c} = 0.05 \text{ min (at flow rate of 0.5 ml/min)} \quad = 0.025 \text{ min (at flow rate of 1.0 ml/min)} \qquad (3\text{-}2)$$

where

L: total length of CFL MIP micro-column (5 cm);
d: internal diameter of CFL MIP micro-column (0.08 cm);
c: flow rate of mobile phase.

It could be estimated that the sample solution plug takes approximately 3 s or less passing through the CFL MIP micro-column, at flow rate of 0.5 ml/min or higher.

% Binding proved to be a very effective and straightforward criterion for evaluating the molecular recognition ability of CFL MIP particles for CFL and its structural analogues. It was calculated as the quotient of the subtraction of the breakthrough peak area from the peak area of sample solution injected bypassing the CFL MIP micro-column (FIA):

$$\% \text{ Binding} = \frac{\text{FIA peak area} - \text{Breakthrough peak area}}{\text{FIA peak area}} \times 100\ \% \qquad (3\text{-}3)$$

When $CHCl_3$ was used as the mobile phase for MISPE, CFL, as shown in Figure 5, 90-95 % binding of CFL could be achieved on this CFL MIP micro-column. In comparison, CFR, CFD and AMP had only 68-76 %, 78-80 %, and 14-20 % binding, respectively.

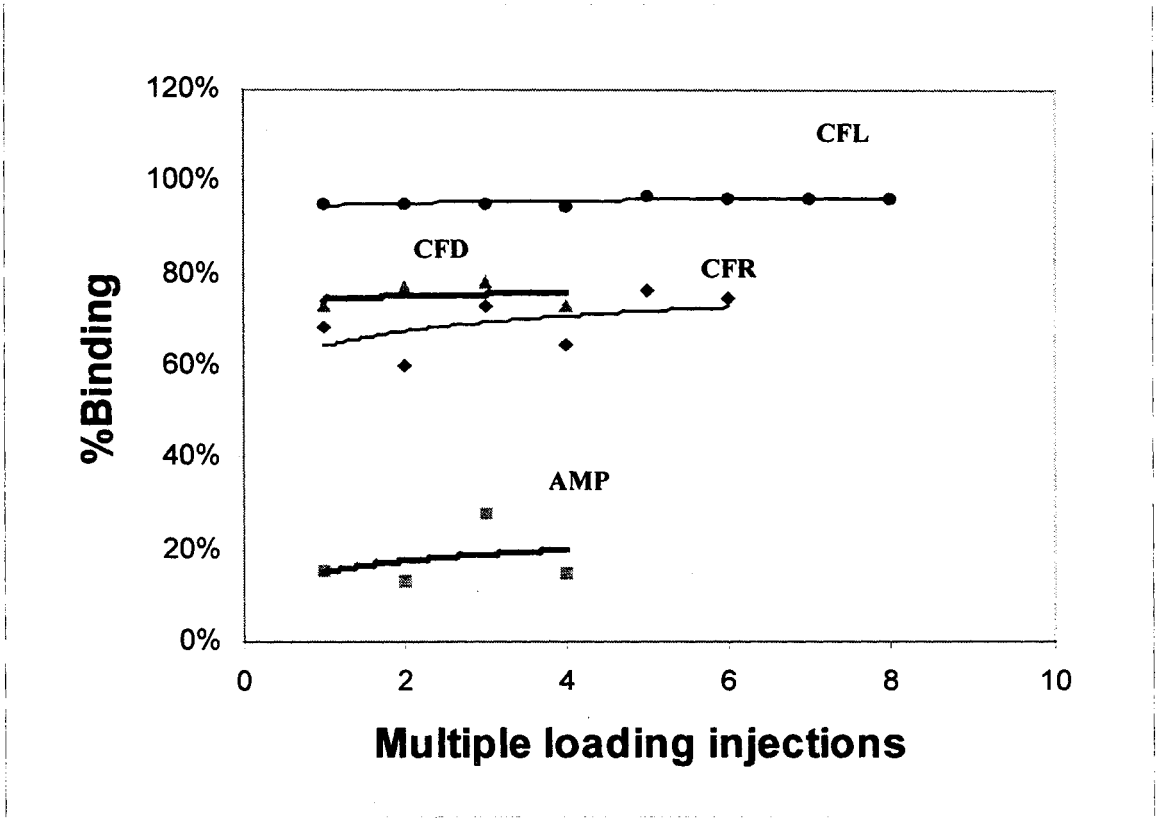


Figure 5 Comparison of % binding of α-aminocephalosporin antibiotics

3.1.2.5 Binding capacity evaluations of CFL, CFR, CFD and AMP

Competitive binding studies using analyte molecules with closely related structures in solvent mixtures of different polarities had previously revealed that, in MIP-based molecular recognition, the entropy-driven hydrophobic effect is significant in polar solvents, whereas enthalpy-driven electrostatic interactions dominate in non-polar media. [50] A key mechanism of the latter is associated with the strength of hydrogen bonding in organic solvents (ranging from chloroform to acetonitrile).

CFL binding onto the MIP micro-column was evaluated in this work by multiple 20-μL loading injections of a 24.7 μg/ml standard CFL solution, with $CHCl_3$ as mobile

phase, at a flow rate of 0.5 ml/min. Each injection would load 0.50 μg of CFL onto the micro-column. As shown in Figure 6, saturation of the MIP recognition sites was reached after approximately 57 loading injections. The final saturation level was comparable with the direct flow injection analysis (FIA) of 24.7 μg/ml CFL with UV detection, bypassing the micro-column entirely. Based on all the break-through peak areas before the micro-column saturation, a total mass of 7.3 μg was determined for the CFL bound to ~40 mg of MIP particles. This loading capacity is comparable with the 20 μg/100 mg for a clenbuterol-MIP recently reported. [51] However, this CFL MIP micro-column did not show high binding capacity for the structural analogues of CFL. As shown in Figure 7, after approximately 10 loading injections of 20.8 μg/ml of CFR standard testing solution, the breakthrough peak area reached the level of the direct flow injection analysis (FIA) of same CFR testing solution. A total mass of 1.7 μg of CFR was achieved in this CFL MIP micro-column, containing ~40 mg of CFL MIP particles. By comparison, CFD could achieve higher binding capacity, approximately 3.8 μg of CFD mass, in this CFL MIP micro-column, suggesting CFD may form stronger hydrogen bonding with the carboxylic groups than CFR (Figure 8). Further confirmation came from the investigation in selecting an optimal DPE solvent to eliminate the interference by structural analogues. Behaving in a different way than CFL, CFR and CFD, the breakthrough peak area AMP did not show an increasing trend during multiple loading of AMP standard solution (12.3 μg/ml, $CHCl_3$). This observation indicates that AMP couldn't effectively bind to the CFL MIP particles. (Figure 9)

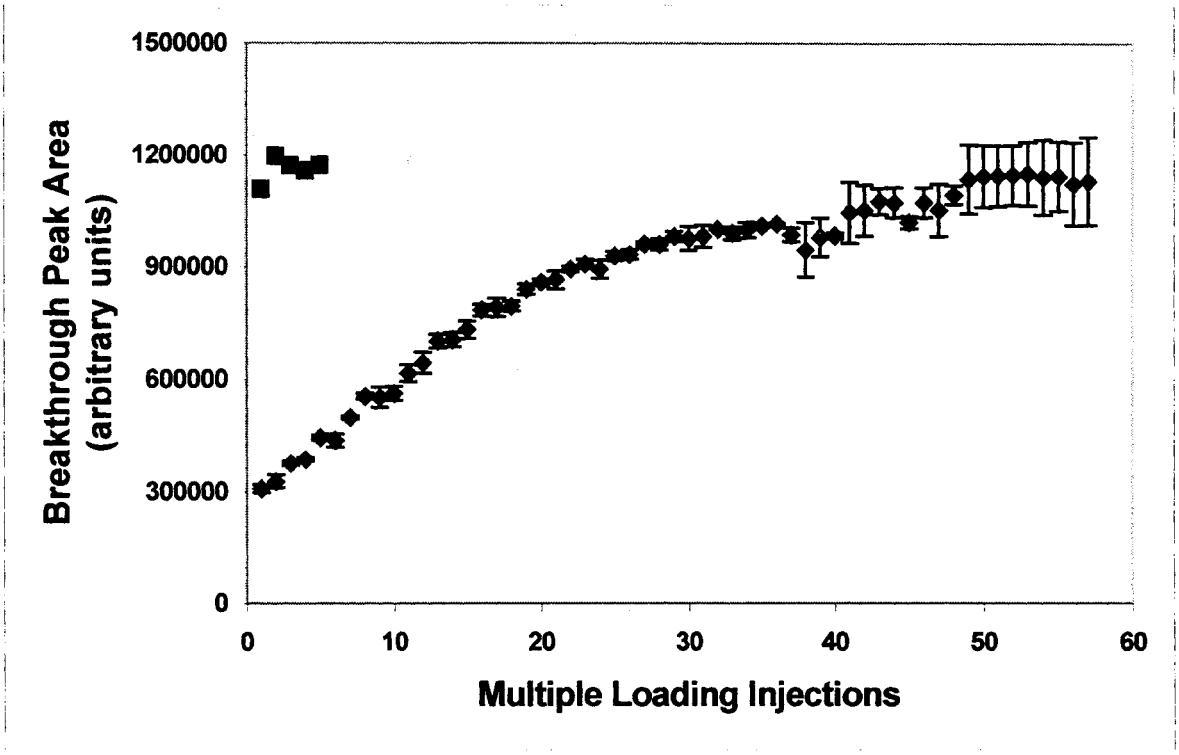


Figure 6 CFL binding saturation study (~ 40 mg of CFL MIP particles) (■ CFL FIA peak area for comparison)

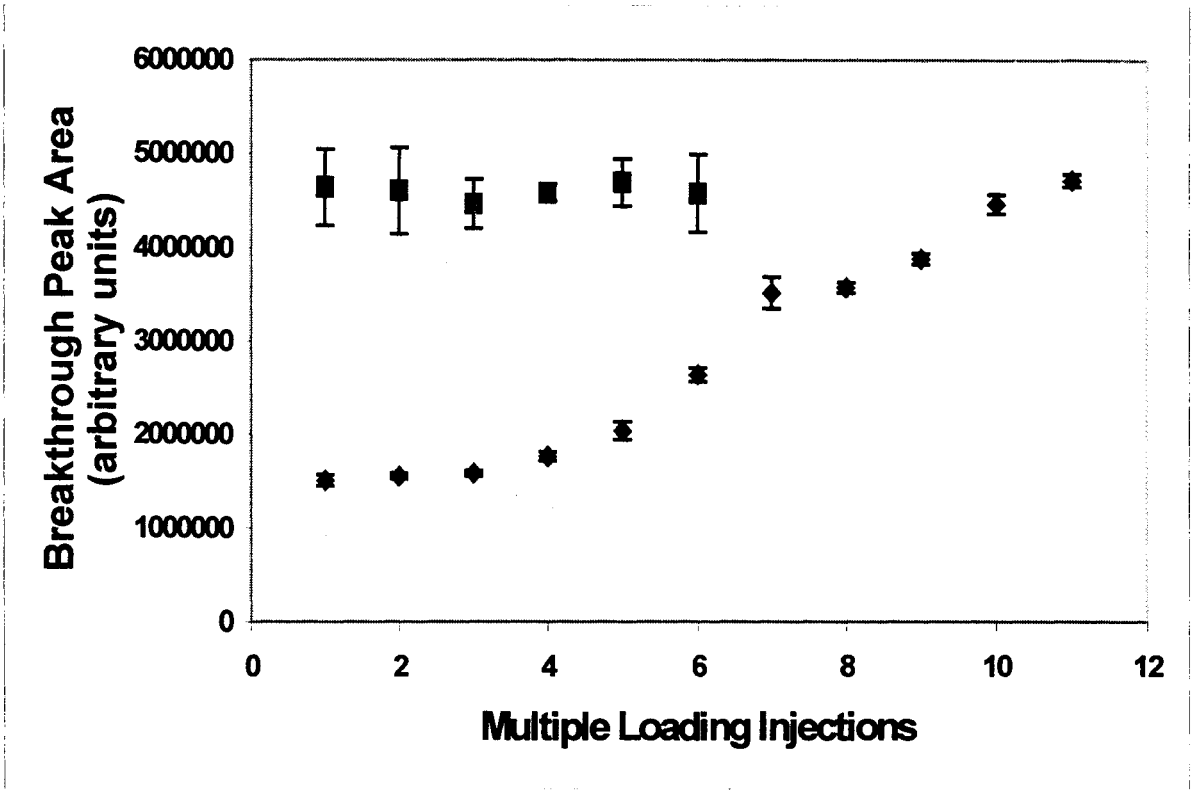


Figure 7. CFR binding saturation study (~ 40 mg of CFL MIP particles)
(■ CFR FIA peak area for comparison)

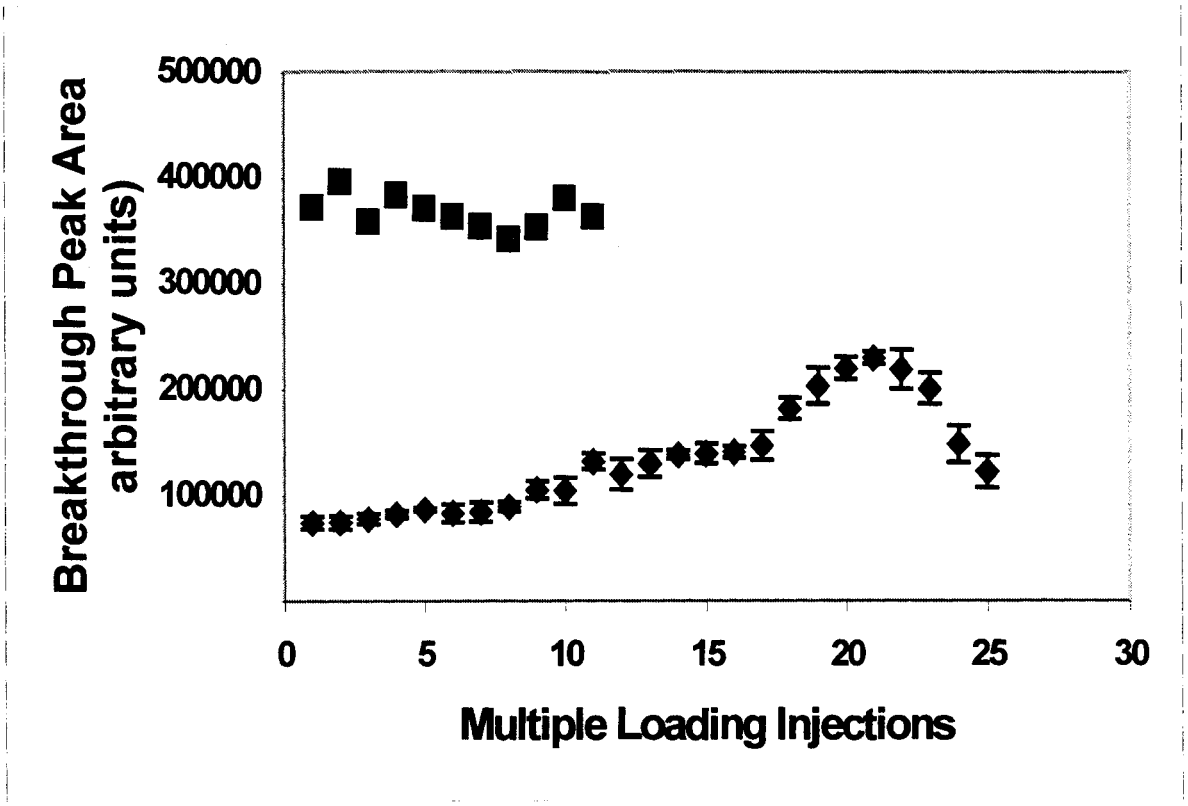


Figure 8. CFD binding saturation study (~ 40 mg of CFL MIP particles)
(■ CFD FIA peak area for comparison)

AMP

It can be concluded that AMP has quite limited binding on CFL MIP. The % binding obtained was as low as 14-20 %, compared with CFR and cephadroxil, which had 68-76 % and 78-80 %, respectively. Based on this experiment, it was found that AMP was very difficult to achieve binding saturation on this CFL MIP micro-column (Figure 9). The possible explanation is due to the limited recognition sites inside CFL MIP particles, which can molecularly match AMP molecules. Another explanation goes to the weak recognition action between AMP and recognition sites in CFL MIP, the binding action and dissociation occur simultaneously, whereas the dissociation dominates the procedure. The similar phenomenon could also be found in binding saturation study on CFD. Thus, a hypothetical deduction is provided: the more the difference in structure from CFL, the harder a binding saturation will be achieved.

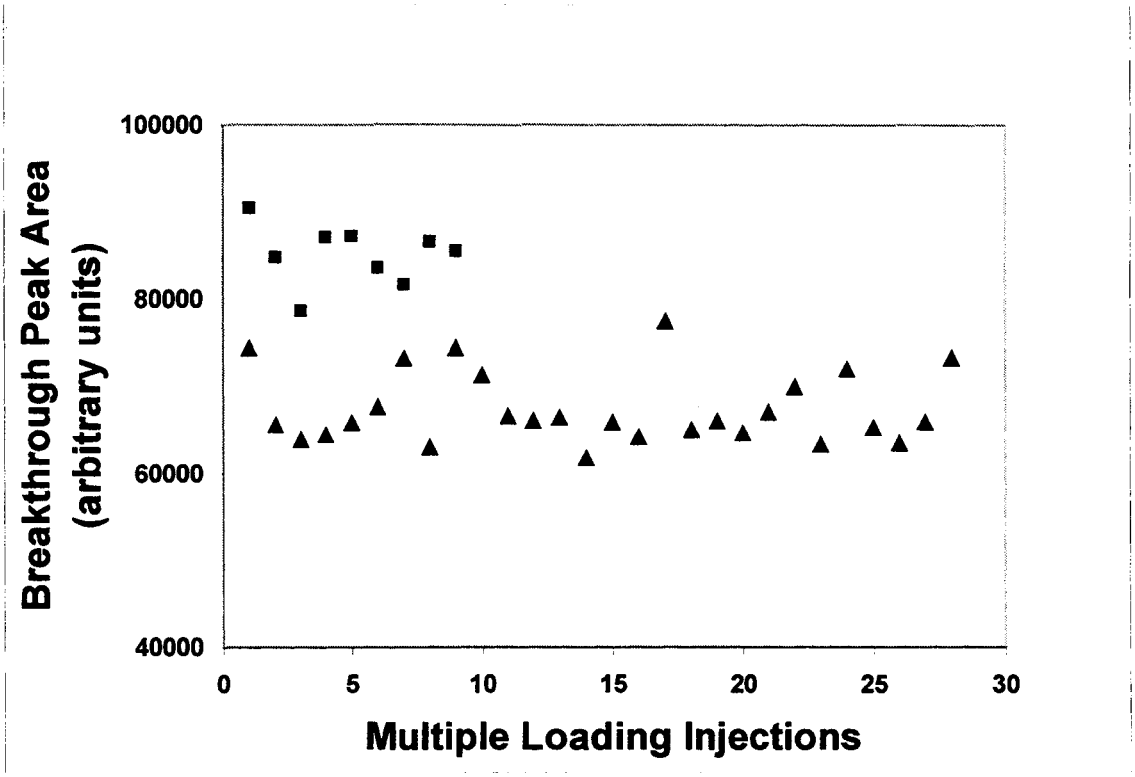


Figure 9. AMP binding saturation study (~ 40 mg of CFL MIP particles)
(■ AMP FIA peak area for comparison)

3.1.2.6 Binding selectivity of CFL MIPs

Guo and He evaluated the selectivity of this CFL MIP in a recent study, for CFL and other structurally similar compounds in aqueous media. Their results showed that the MIP exhibited a high affinity for CFL among the tested compounds. [48] An independent study was conducted in the present work, where several α-aminocephalosporins (CFR, CFD) and β-lactam antibiotics (AMP) were run through the CFL MIP micro-column individually. This micro-column configuration with on-line UV detection was a time-efficient way to test various compounds for potential interference. As summarized in Table 3, however, other α-aminocephalosporins and β-lactam antibiotics appear to have interfered (in the UV detection of CFL at 240 nm) the selectivity of CFL MIPs when added at 10-25 μg/ml. Obviously, their similarity to CFL in molecular structures allowed for a certain degree of binding under the specified dynamic conditions. It was interesting to note how an extra –OH substituent group in CFD, and one C=C bond less (changing from benzene to cyclohexadiene) in CFR, reduced the binding to 78-80 % and 68-76 % respectively. Equally surprising was the other case of AMP, which has a five-membered ring of N and S with one more $-CH_3$ substituent group and one less C=C bond to reduce the binding significantly to 14-20 %. It had previously been reported how interaction of a functional monomer with the free amino group of AMP yielded efficient binding of the MIP with AMP from aqueous solutions. [52] The present % binding results should be interpreted as dependent on the binding strength (thermodynamics) and rate (kinetics). Both parameters can potentially be utilized to develop a highly selective MISPE-DPE method for the accurate determination of CFL.

3.1.2.7 Specificity of CFL MIPs for α-aminocephalosporins

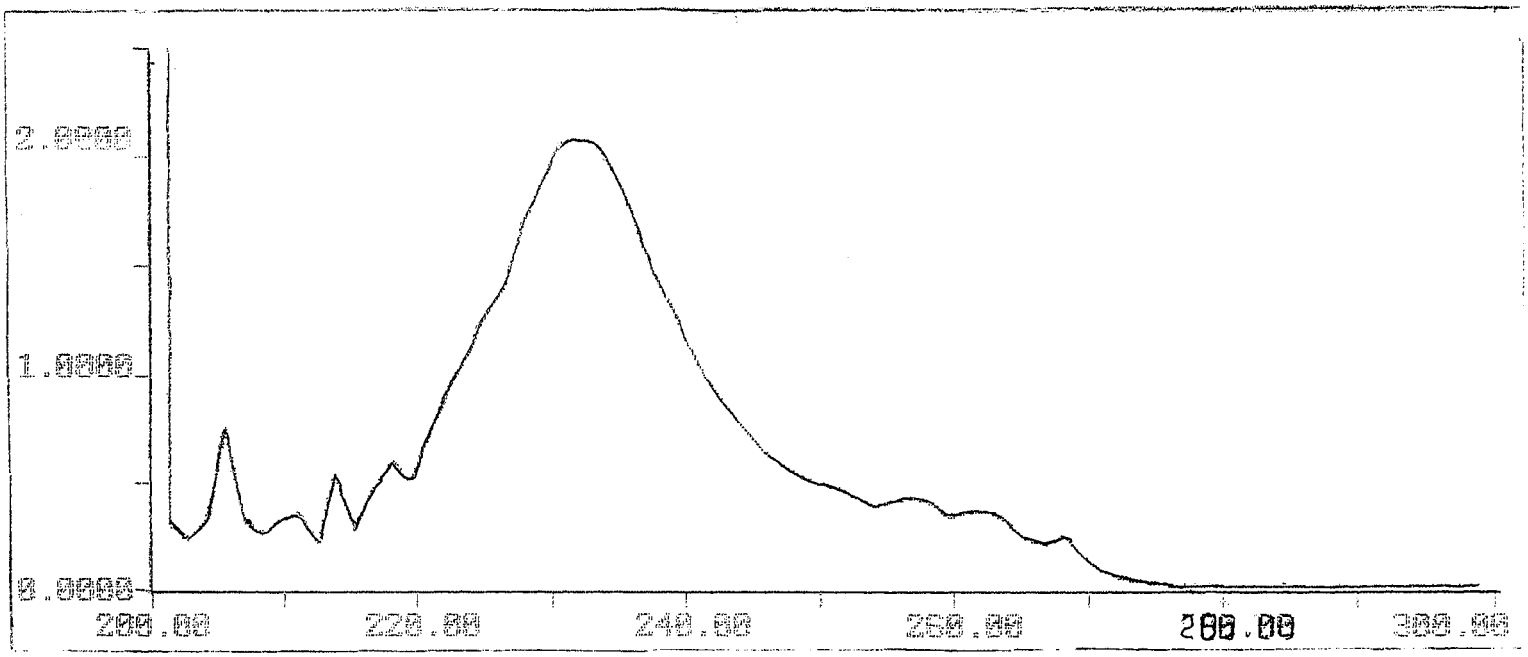
CFL, CFR, and CFD are three α-aminocephalosporins of both pharmaceutical and biomedical significance in combinatorial drug library research. Based on their measured % bindings and molar absorptivities (7552 and 8394 $M^{-1}cm^{-1}$ at 275 nm, respectively), CFR and CFD would be expected to cause significant interference in the MISPE-PE determination of CFL. Similar % binding results that were found in the earlier step of the work, suggest that the amine and groups in these molecules are responsible for interaction with the MIP. One interesting observation was the capacity of 7.3 μg for CFL binding onto the MIP micro-column, as determined by multiple loading injections of a standard CFL solution. By comparison the binding capacity for CFR was merely 1.7 μg, which is four times lower. This suggests non-specific binding of CFR molecules to only the cavities at the surface of MIP particles. No further driving forces existed to transfer the bound CFR molecules into the deeper cavities. In contrast, surface-bound CFL molecules could migrate into the deeper cavities that were actually stronger binding sites tailored for this target compound. This left the surface cavities vacant to allow for additional binding in the next sample loading. It was difficult to determine the binding capacity for either CFD or AMP, probably because of a rapid dissociation rate for the bound molecules. This speculation may explain the oscillations of breakthrough peak area observed during the binding saturation studies for CFD and AMP. The analytical implication of such labile bindings is that CFD (CFR and AMP) could possibly be eliminated from the micro-column by introducing a differential pulsed elution (DPE) step. This will be discussed in the MISPE-DPE-FPE section.

3.1.3 Elimination of the spectrometric interference from AMP by changing detection wavelength

The UV spectrum in Figure 10 shows that AMP in 1 % TFA + CH_3OH has a fairly strong UV absorbance at 240 nm, whereas at 275 nm the absorbance decreases to zero. Its molar absorptivity (at 275 nm) is significantly lower than those for CFL, CFR and CFD. This result suggested a simple way to eliminate the spectral interference from AMP by changing the detection wavelength from 240 nm to 275 nm. Verification of this design presented a 94 % binding (± 1.1 %, n = 2) for a mixture solution of 20.8 μg/ml CFL in the presence 11.2 μg/ml AMP in $CHCl_3$ (at λ = 275 nm), as compared with. 95 % binding (± 0.1 %, n = 3) for a solution of 20.8 μg/ml CFL in $CHCl_3$ (at λ = 240 nm).

Figure 10 UV spectra of AMP, CFL, CFR and CFD in 1 % TFA + CH_3OH

AMP in 1 % TFA + CH_3OH (13.3 μg/ml)



CFL in 1 % TFA + CH_3OH (20.2 μg/ml)

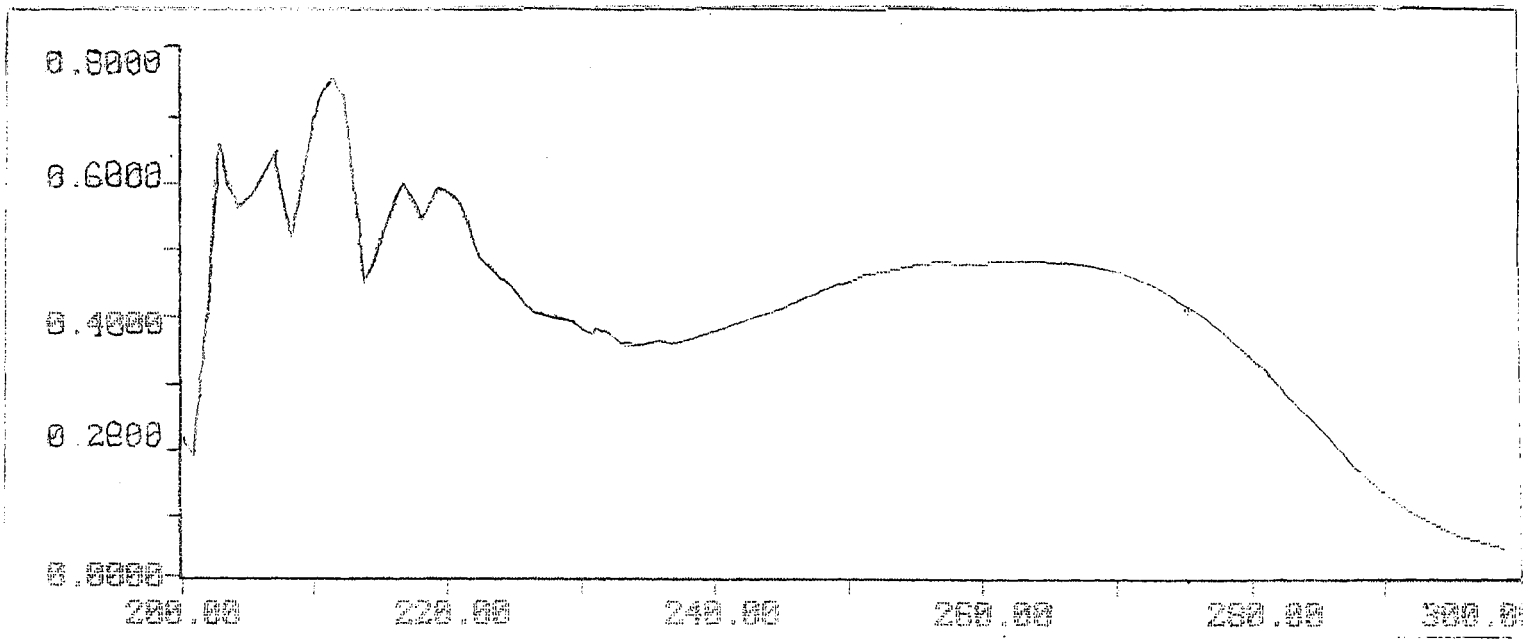
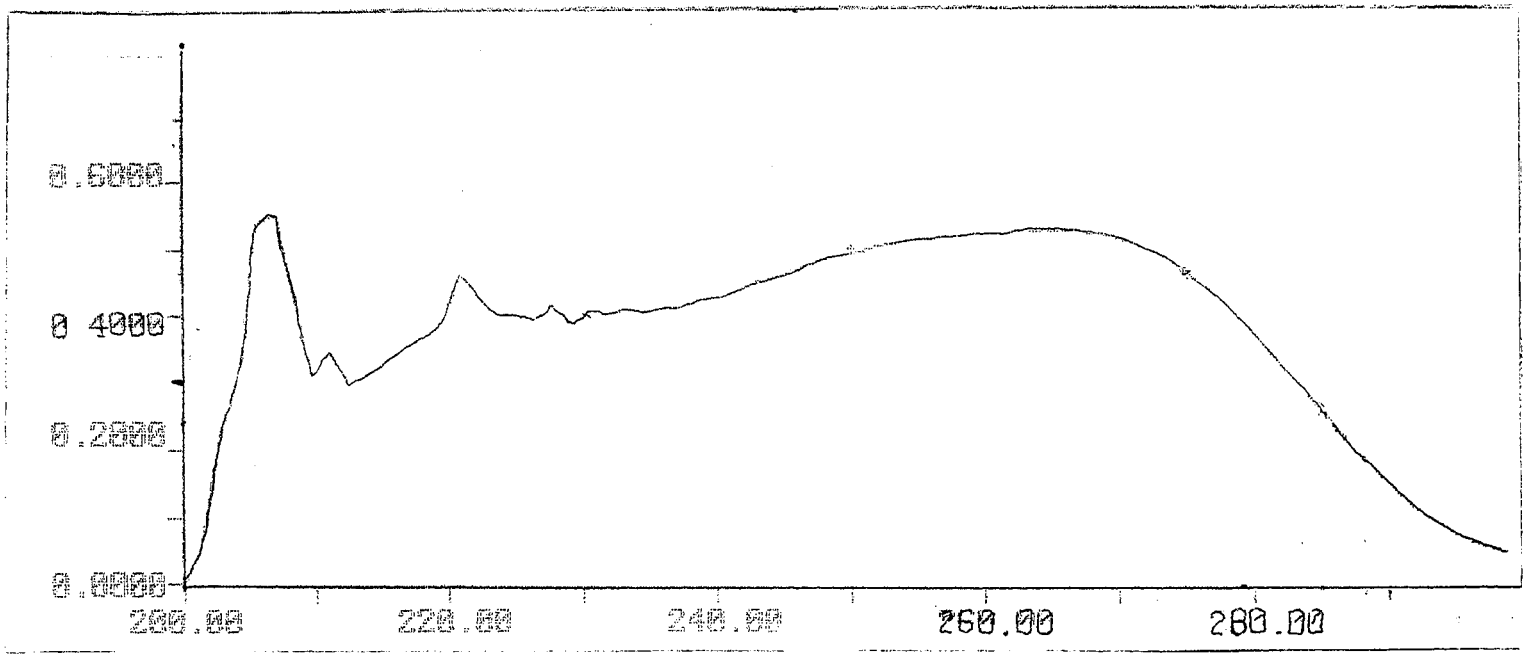
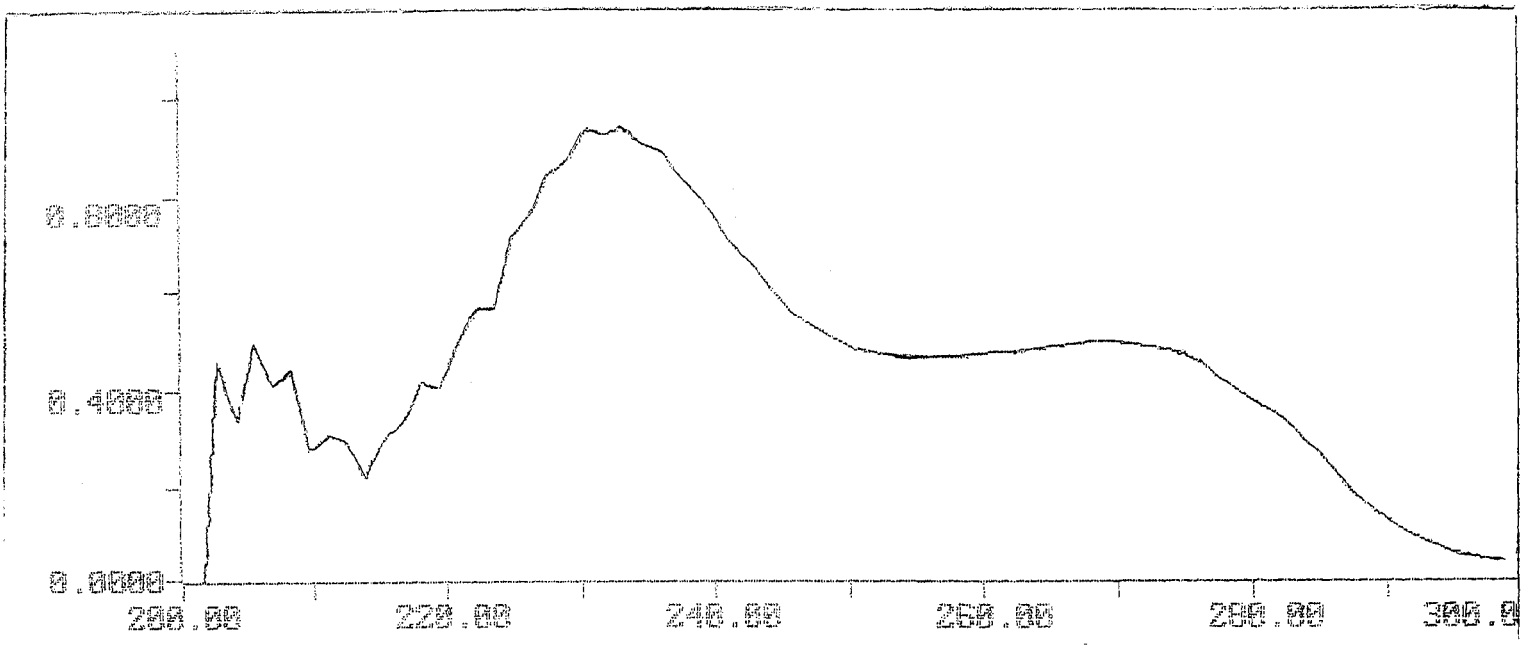


Figure 10 (continued)

CFR in 1 % TFA + CH_3OH (22 µg/ml)



CFD in 1 % TFA + CH_3OH (18.6 µg/ml)



3.1.4 Relationship of concentration and % binding

The relationship of CFL concentration and % binding was investigated by testing CFL standard solutions in the concentration range from 7-49 μg/ml (mass range 140-970 ng). As shown in Figure 11, CFL exhibited a fairly high % binding (90-97 %) over the tested concentration range, suggesting that in chloroform CFL MIPs behave well in the molecular recognition for CFL molecules.

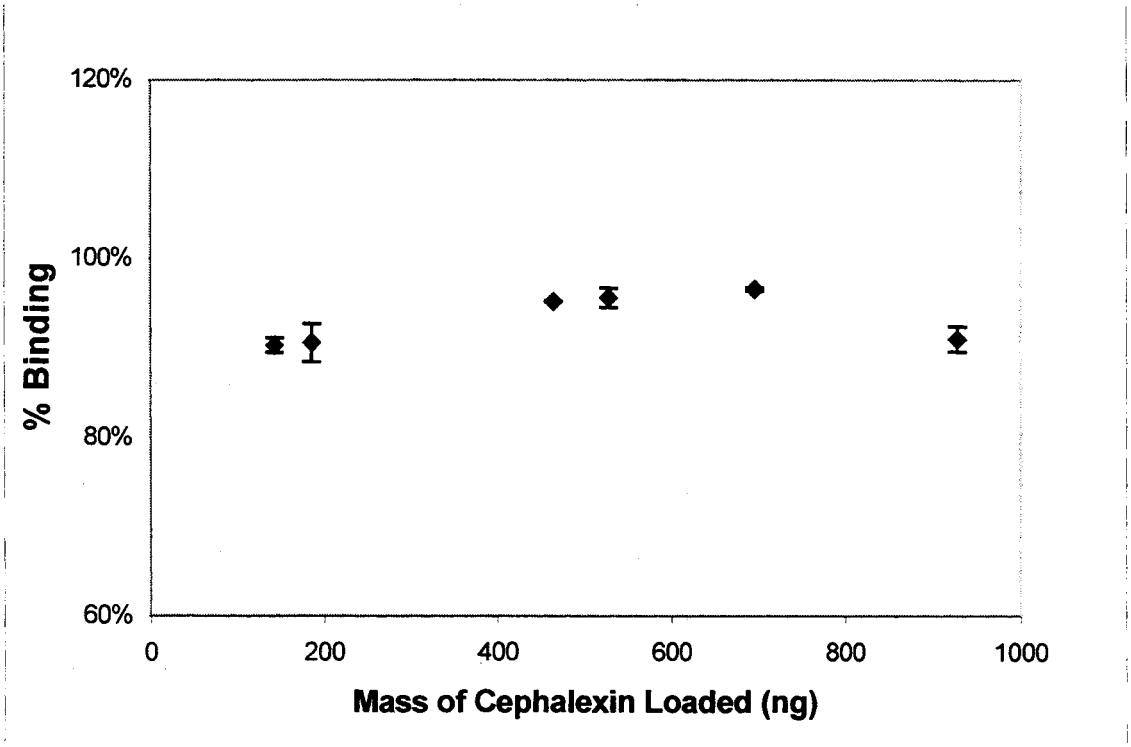


Figure 11. Relationship of % binding and mass of CFL loaded (~ 40 mg of CFL MIP particles)

3.1.5 Molecular recognition of different polymer micro-columns for CFL, CFR and CFD

--- Investigation of nonspecific binding

Control polymer micro-column

For investigation of nonspecific binding of CFL, a control polymer micro-column was used to determine the extent of nonspecific binding. The control polymer particles were synthesized by W. Mullett in the absence of imprinted molecules for comparison with his theophylline MIP micro-column. Application of control polymer micro-column proved to be very effective for the investigation of nonspecific binding. In $CHCl_3$ CFL achieved a 14-20% binding on this control polymer column, which was much higher than the 0.7%, achieved in CH_3CN.. The randomly distributed carboxylic groups inside the control polymer particles could not provide binding sites for the appropriate molecular recognition for CFL molecules, in terms of specific binding. This saturation was especially obvious when a more polar organic solvent was used as the mobile phase for MISPE, which may weaken the interaction.

Significantly different from the previous binding saturation test using the CFL MIP micro-column, binding saturation was quickly achieved on this control polymer micro-column after only three 20-μl injections of 20 μg/ml CFL standard solution ($CHCl_3$) as shown in Figure 12. When a 540 μg/ml CFL standard solution ($CHCl_3$) was used, binding saturation was quickly achieved by only one injection as shown in Figure 13. Similar phenomenon could be observed when a CFD standard solution was applied on this control polymer micro-column. As displayed in Figure 14, CFD could achieve its binding saturation after ten 20-μl injections of 20 μg/ml CFD standard solution ($CHCl_3$). Obviously, the binding sites inside the control polymer particles could not specifically distinguish CFL from its structural analogues.

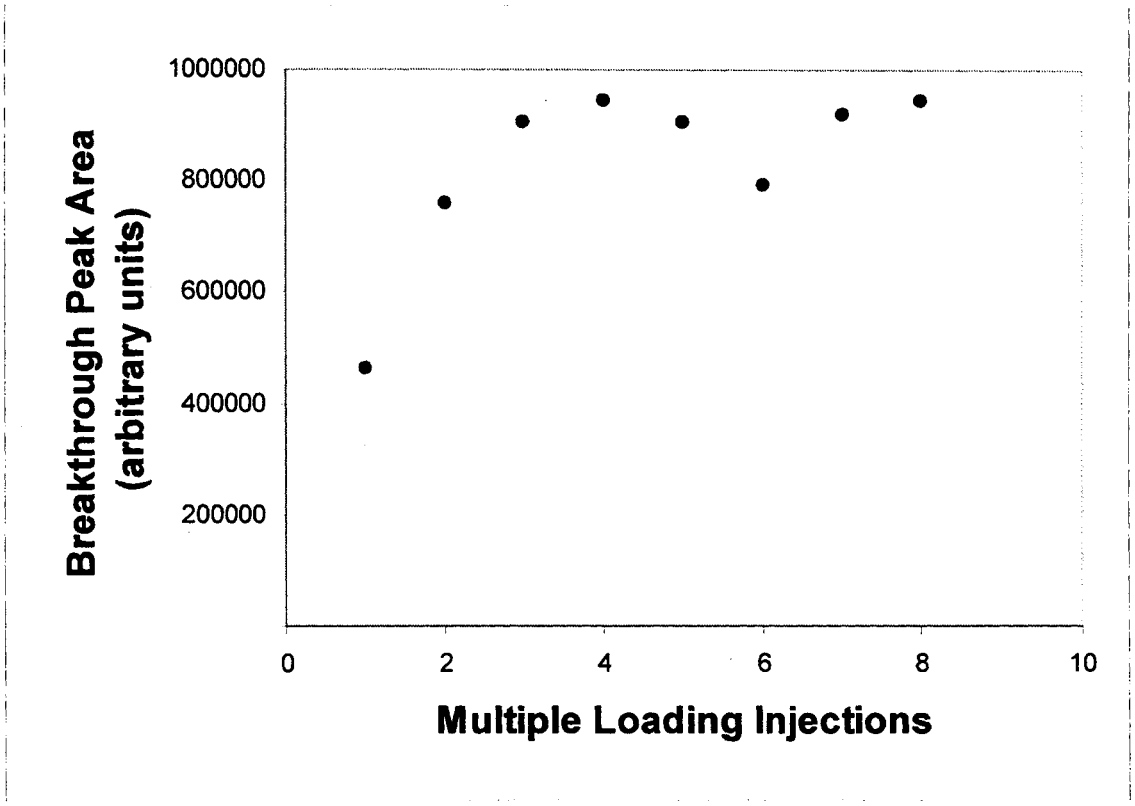


Figure 12. CFL binding saturation study on control polymer micro-column (~ 50 mg of control polymer particles) (CFL concentration: 20 μg/ml)

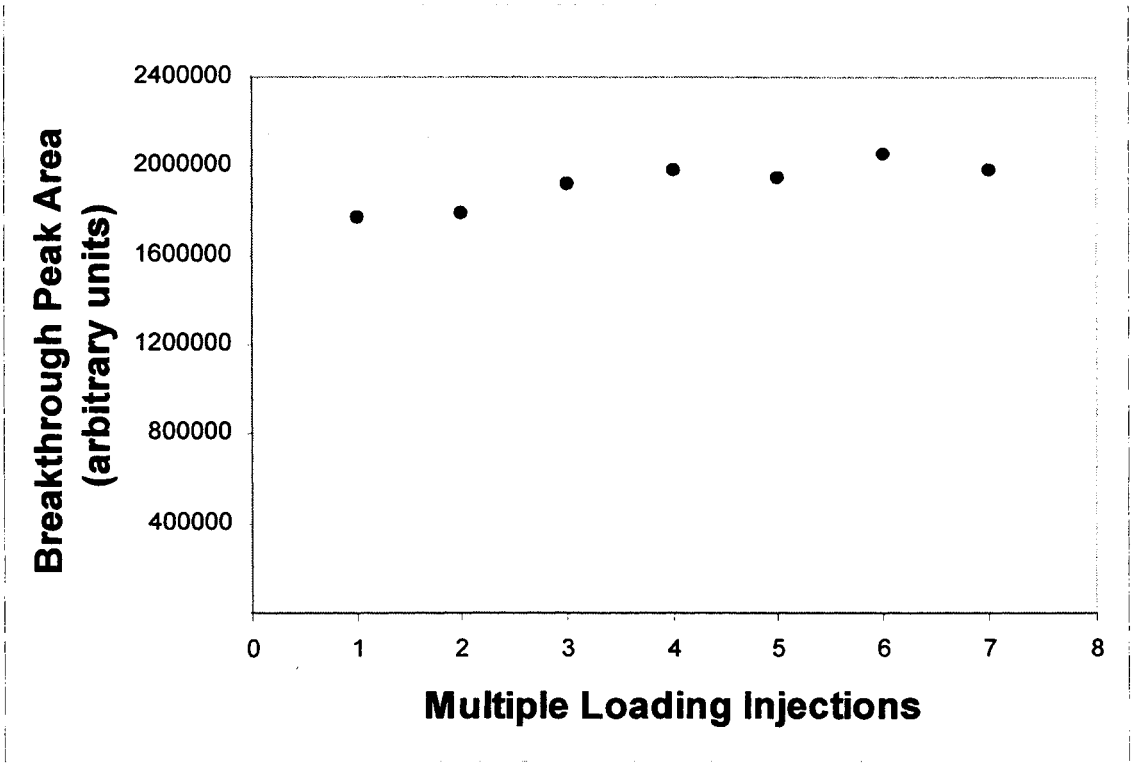


Figure 13 CFL binding saturation study on control polymer micro-column (~ 50 mg of control polymer particles) (CFL concentration: 540 μg/ml)

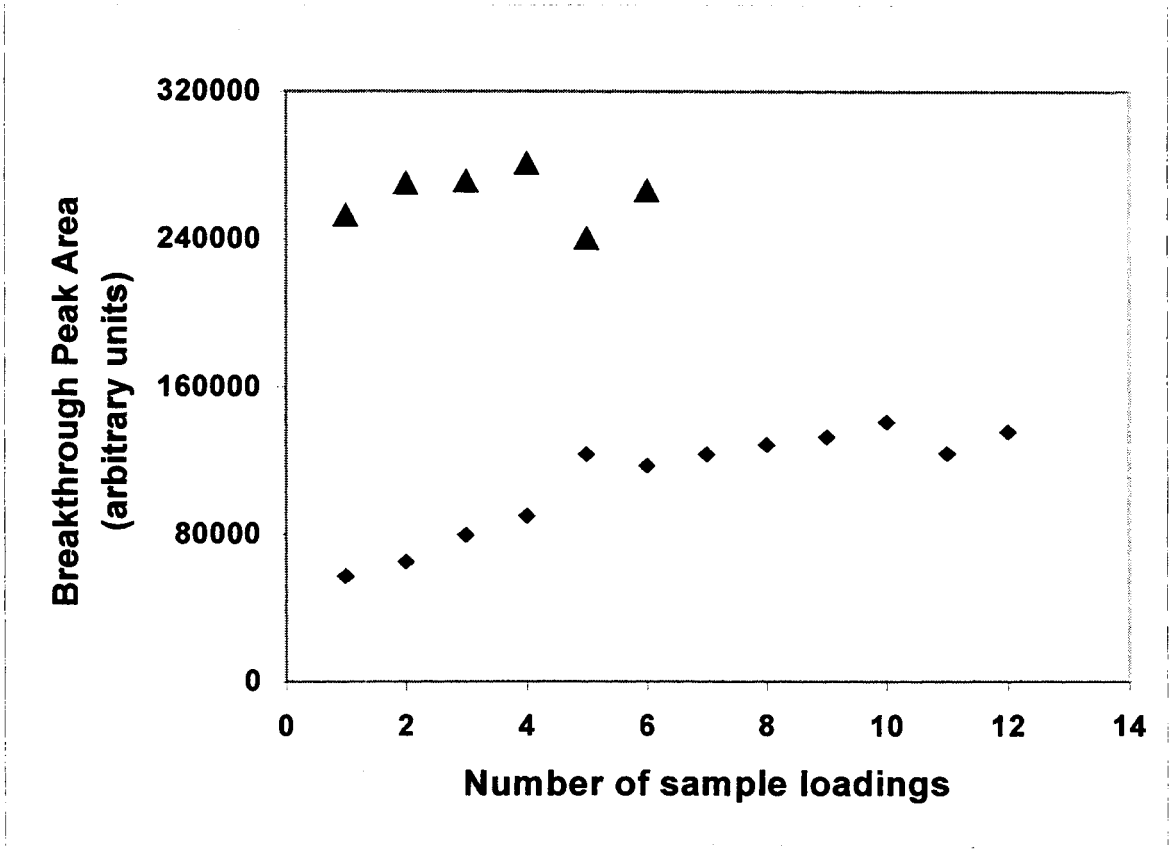


Figure 14 CFD binding saturation study on control polymer micro-column (~ 50 mg of control polymer particles) (CFD concentration: 20 μg/ml)

(▲ CFD FIA peak area for comparison)

Isoproturon MIP micro-column

Would CFL behave similarly on other MIP micro-columns as it did on the control polymer micro-column? An investigation was conducted by using an isoproturon MIP micro-column, which had the same dimensions as the CFL MIP micro-column. CFL was observed to achieve binding saturation on this isoproturon MIP micro-column after one 20-μl injection of 20 μg/ml CFL standard solution ($CHCl_3$) (Figure 15). This poor binding behavior on isoproturon MIP particles could be explained by the fact that the isoproturon MIP particles were synthesized using isoproturon as the template molecules. Therefore the distribution of carboxylic groups inside the cavities were catered for isoproturon molecules rather than CFL molecules.

The ΔPE peak area, which is proportional to the % binding, is another good parameter to evaluate binding behaviors. A comparison of ΔPE peak areas from the saturated CFL MIP micro-column, isoproturon MIP micro-column, and control polymer micro-column was displayed in Figure 16. It was found that due to different binding capacities of the three columns for CFL, significantly large ΔPE peak areas were obtained on the CFL MIP micro-column, compared with the isoproturon MIP micro-column and control polymer micro-column.

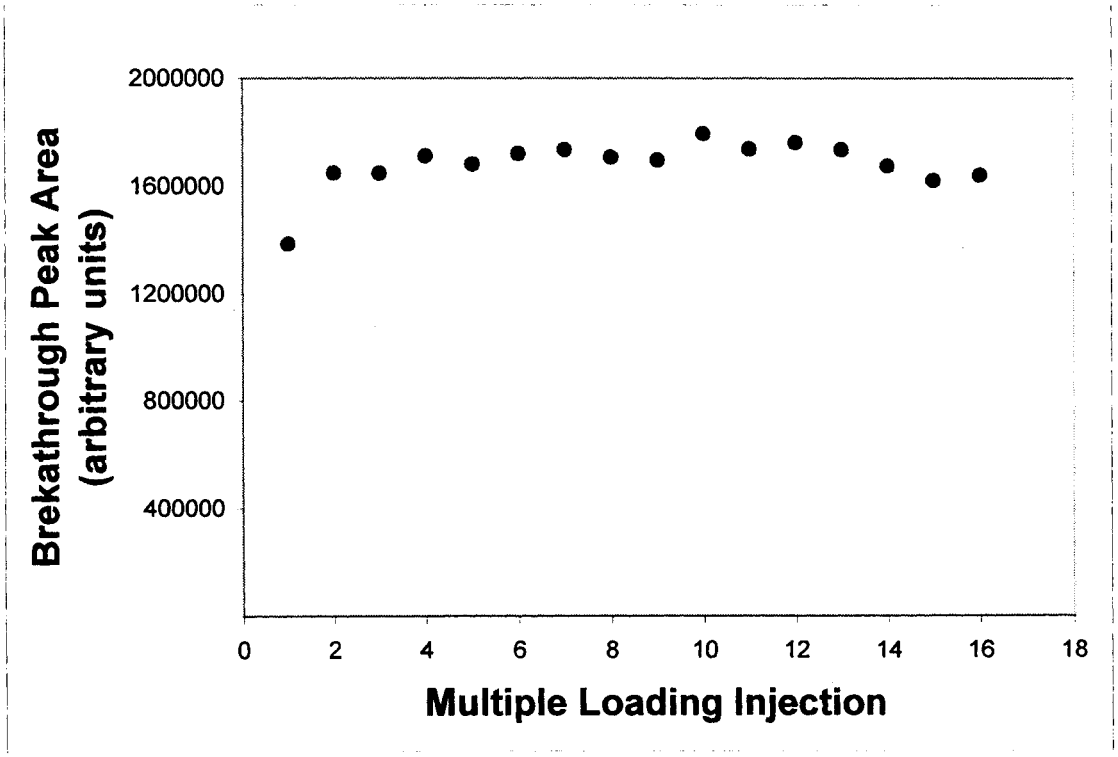


Figure 15. CFL binding saturation study on isoproturon MIP micro-column (~ 40 mg of isoproturon MIP particles) (CFL concentration: 20 μg/ml)

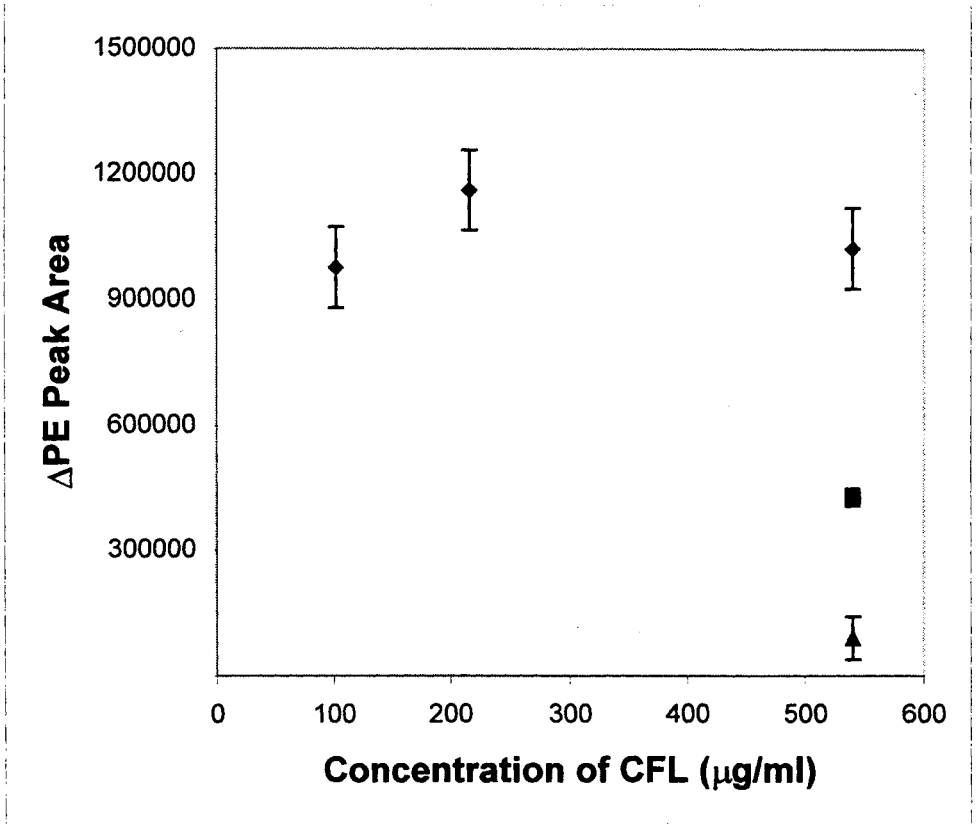


Figure 16 Comparison of ΔPE results for CFL (after reaching binding saturation): (♦) from CFL MIP micro-column; (■) from isoproturon MIP micro-column; (▲) from control polymer micro-column.

Summary

The basic difference between specific binding and nonspecific binding is their binding strengths, which depend on the number of carboxylic groups utilized by the imprinted molecule. Theoretically, in the case of CFL, specific binding was achieved by forming hydrogen bonds between all the amino groups and carboxylic groups of the CFL MIP particles. However, this cannot be realized for the control polymer or other MIP particles where the distribution of carboxylic groups was fixed not for the accommodation of CFL molecules. Since nonspecific binding can be achieved by forming only one single hydrogen bond, nonspecific binding could usually be found, but the binding strength will be weaker comparing with specific binding. This explains why once acetonitrile was used to replace $CHCl_3$ as the mobile phase, the % non-specific binding of CFL (20 μg/ml) decreased from 14 % to 0.7 %. The small and constant ΔPE peak areas obtained for the mass of CFL loaded on the control polymer micro-column can possibly be attributed to a smaller equilibrium binding constant of the control polymer particles. These results proved that the binding effects observed with the CFL MIP micro-column were based on molecular recognition and not governed significantly by any other mechanism.

3.2. Pulsed elution of CFL

Pulsed elution (PE) is normally applied with certain volume of polar organic solvent, containing organic acid. By forming stronger hydrogen or ionic binding with the analyte molecules, PE solvent may quantitatively extract the analyte molecules from the binding sites in the MIP particles. Quantitative determination is followed by measuring the extracted analyte molecules. An optimized MISPE-PE procedure for CFL was displayed in Figure 17. As illustrated in the graph, the 1st and 2nd PE peak contains almost total amount of CFL molecules extracted during MISPE, as comparing with the peak areas of the following PE peaks.

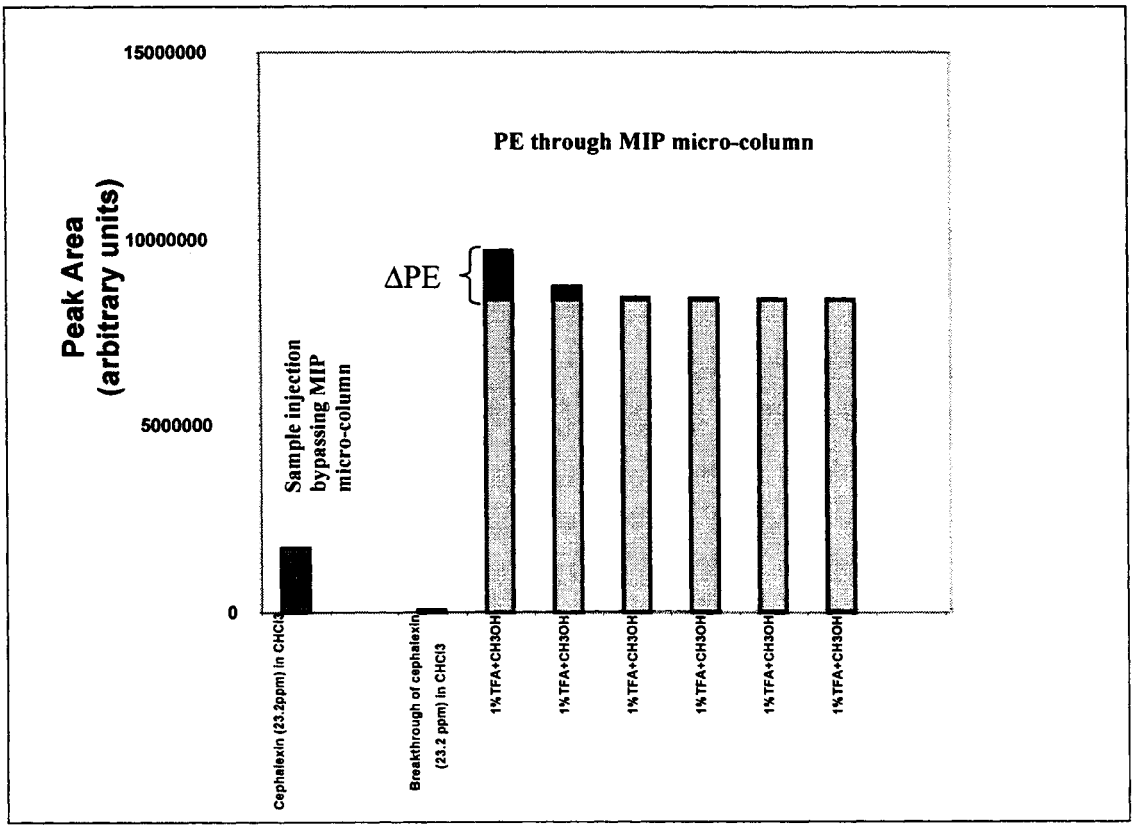


Figure 17. An optimized MISPE-PE procedure for CFL

3.2.1. How to justify an ideal pulsed elution (PE) solvent?

Application of micro-column configuration for PE, using only 20 μL of an appropriate solvent, minimized both solvent consumption and analysis time. The PE efficiency of various solvents could be determined by direct MISPE analyses of standard CFL solutions prepared in those solvents, while $CHCl_3$ was running as the mobile phase through the MIP micro-column. Comparison of the breakthrough peak area with a pre-determined flow injection analysis (FIA) peak area would yield a % binding result. A low % binding would indicate a high eluent strength (and hence good PE efficiency) for the solvent. As shown in Table 5, which summarizes the % binding results for CFL in several solvents, a trend of decreasing % binding value, which was desirable, can be seen with increasing solvent polarity. When CH_3OH was employed as solvent for the preparation of standard CFL solution, a 52 % binding only was attained. When a CH_3OH + 1 % TFA was used as solvent, a ~0 % binding was finally achieved. This indicated that CFL could not bind with the MIP sites, due to the competition of CH_3OH for the same binding sites as well as competition of TFA for the CFL molecules. The above procedure thus unambiguously identified appropriate solvents for testing in the MISPE-PE method development.

Table 5 % Binding of cephalosporin antibiotics prepared in different PE solvents onto the CFLMIP micro-column (Mobile phase: $CHCl_3$, flow rate = 0.5 ml/min)

Cephalosporin	Concentration (μg/ml)	Solvent	% Binding
CFL	23.2–56.8	$CHCl_3$	94 % (±3 %, n = 16)
	19.9	$CHCl_3$ + 0.05 % CH_3COOH	92 % (±1 %, n = 2)
	19.2	$CHCl_3$ + 2 % CH_3COOH	67 % (±1 %, n = 2)
	20.6	CH_3OH	52 % (±9 %, n = 16)
	19.5	CH_3OH + 0.5 % CH_3COOH	13 %
	19.4	CH_3OH + 2 % CH_3COOH	37 % (±2 %, n = 4)
	7.12-33.4	CH_3OH + 1 % TFA	0 % (n = 33)
CFR	20.0	$CHCl_3$	68-76 % (±2 %, n = 10)
	18.6	CH_3OH + 1 % TFA	0 % (±1 %, n = 8)

(Continued)

CFD	21.9	$CHCl_3$	78-80 % (±1 %, n = 3)
	19.5	$CHCl_3$ + 0.05 % CH_3COOH	73 % (n = 1)
	20.2	$CHCl_3$ + 2 % CH_3COOH	71 % (±1 %, n = 2)
	20.3	CH_3OH	56 % (±7 %, n = 13)
	18.9	CH_3OH + 2 % CH_3COOH	30.7 % (±0.6 %, n = 2)
	22.0	CH_3OH + 1 % TFA	0 % (±1 %, n = 9)
AMP	12.32	$CHCl_3$	14 – 20 % (±1 %, n = 3)

3.2.2 1 % TFA + CH_3OH as PE solvent

When CH_3OH was tested for the PE of CFL molecules bound onto the MIP micro-column, a PE efficiency of 84 % only was attained. When a 1 % TFA solution in CH_3OH was tested for the PE of CFL, a PE efficiency of 99 ($\pm$1.8) % was finally achieved. This PE solvent had previously been used for the elution of 4-aminopyridine from a different MIP micro-column.[53] The presence of TFA in methanol helped to overcome the hydrogen bonding between the CFL molecule and the carboxylic-acid functional groups in the MIP recognition sites, by forming stronger hydrogen bonding with CFL molecules. With a pKa of -0.25 (Table 6), TFA is a stronger hydrogen bond donor that can compete successfully with the bound CFL molecules for the binding sites. Methanol was chosen due to its powerful eluent strength (ε^0) among organic solvents (Table 4), which may also serve as hydrogen donor together with TFA. By mixing with methanol, TFA rapidly desorbed CFL from the weaker carboxylic acid group (by analogy to pKa = 4.8 for acetic acid).

Table 6. List of pKa values of carboxylic acids [54]

Carboxylic Acid	*Empirical pKa*	$H_f(RCO_2H)$ *kJ/mol*	$H_f(RCO^{2-})$ *kJ/mol*	ΔH_{ion} *kJ/mol*
Trifluoroacetic Acid	-0.25	-246.0	-344.1	-98.1
Trichloroacetic Acid	0.64	-108.9	-198.6	-89.7
Dichloroacetic Acid	1.29	-114.2	-200.6	-86.4
Nitroacetic Acid	1.68	-103.3	-191.7	-88.4
Acetylenedicarboxylic Acid	1.75	-128.8	-218.1	-89.3
Propiolic Acid	1.887	-40.3	-126.2	-85.9
o-Nitrobenzoiic Acid	2.17	-65.2	-151.9	-86.7
Glycine	2.351	-18.7	-100.5	-81.8
m-Nitrobenzoic Acid	2.45	-69.1	-152.7	-83.6
Cyanoacetic Acid	2.46	-77.8	-159.6	-81.8
Fluoroacetic Acid	2.66	-153.2	-238.5	-85.3
Malonic Acid	2.826	-201.6	-285.5	-83.9
Chloroacetic Acid	2.86	-113.3	-197.2	-83.9
Bromoacetic Acid	2.86	-99.9	-184.5	-84.6
o-Chlorobenzoic Acid	2.94	-77.2	-161.9	-84.7
Iodoacetic Acid	3.12	-89.3	-171.9	-82.6
p-Nitrobenzoic Acid	3.44	-68.0	-151.7	-83.7
Glycolic Acid	3.6	-157.9	-241.1	-83.2
Formic Acid	3.75	-103.5	-185.5	-82.0
m-Chlorobenzoic Acid	3.83	-79.7	-162.7	-83.0
p-Chlorobenzoic Acid	3.99	-79.9	-162.4	-82.5
Benzoic Acid	4.2	-73.7	-156.6	-82.9
Acrylic Acid	4.25	-82.0	-163.7	-81.7
p-Anisic Acid	4.47	-113.7	-195.4	-81.7
3-Butenoic Acid	4.68	-89.0	-170.3	-81.3
Acetic Acid	4.78	-109.1	-190.6	-81.5
Cyclobutane Carboxylic Acid	4.785	-95.9	-176.8	-80.9
Cyclopropane Carboxylic Acid	4.827	-77.8	-159.3	-81.5
Cyclohexane Carboxylic Acid	4.9	-132.3	-213.0	-80.7
Pivalic Acid	5.03	-119.4	-199.4	-80.0
Oxalic Acid	unknown	-185.5	-278.4	-92.9

3.2.3 Is there any other reagent that can be used as PE solvent?

Basic triethylamine

Opposite to the behavior of TFA, basic triethylamine had previously been used as an effective elution solvent for a propranolol-MIP.[55, 56] The carboxylic acid groups were fully regenerated without any modification by bound triethylamine molecules. However, any application of this reagent for elution of the bound CFL molecules would create a potential problem: the next injection of a CFL sample solution would require CFL to displace the triethylamine from the carboxylic acid groups. With a pKa value of 18.75,[57] triethylamine forms much stronger hydrogen bonding with the carboxylic groups inside the CFL MIP particles than CFL (pKa = 5.3-7.3) can. CFL molecules will likely be unable to displace any triethylamine from the carboxylic groups.

3 % HCl + CH_3OH vs 1 % TFA + CH_3OH

A comparison of PE efficiencies between CH_3OH + 3 % HCl (~1 mol/l, pH 1.4) and CH_3OH + 1 % TFA (~0.1 mol/l, pH 2.5) indicated that the PE was not a pH dependent behavior. As shown in Figure 18, after multiple PE with CH_3OH + 3 % HCl, as much as 85 % of the CFL remained bound on the MIP micro-column. Further PE was attained with CH_3OH + 1 % TFA, although its pH was higher than that of CH_3OH + 3% HCl. These results indicated how PE could be better achieved by using CH_3OH + 1 % TFA. Obviously, with a pKa of -0.25, the presence of TFA in methanol helped to overcome the hydrogen bonding between the CFL molecule and the carboxylic-acid functional groups in the MIP recognition site. After the above sophisticated work, the optimal solvent, CH_3OH + 1 % TFA, was hence chosen for use in all subsequent MISPE-PE analyses.

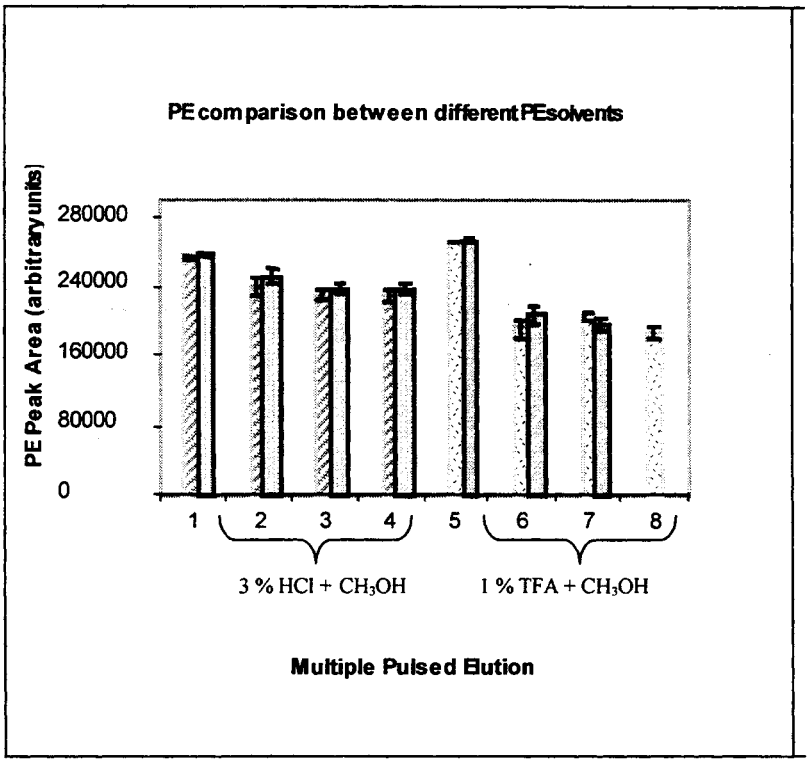


Figure 18 Comparison of PE results using different PE solvents (3 % HCl + CH_3OH and 1 % TFA + CH_3OH)

This experiment was conducted twice. The gray columns represent the 1st trial, where the dark columns represent the 2nd trial.

3.2.4 Analytical figures of merit of MISPE-PE

Beer's law was obeyed by CFL solutions over the concentration range 12-900 μg/ml in chloroform. In the present work, the molar absorptivity of CFL was measured to be 6550 $M^{-1}cm^{-1}$ at 240-242 nm, 6532 $M^{-1}cm^{-1}$ at 270 nm, and 5834 $M^{-1}cm^{-1}$ at 275 nm. FIA with UV detection at 240-242 nm yielded a sensitivity of 4.2 (±0.2) x 10^3 peak area units per ng of CFL, or 8.4 (±0.3) x 10^4 peak area units per μg/ml of CFL solution, in $CHCl_3$. Note that this FIA sensitivity decreased to 3.8 (±0.7) x 10^3 peak area units per ng of CFL in 1 % TFA + CH_3OH. From the standard calibration graph for the MISPE-PE determination of CFL (Table 8), a sensitivity of 3.1 (±0.5) x 10^3 peak area units per ng of CFL was achieved. Hence MISPE-PE has recovered 81 % of the FIA sensitivity while affording the significant merit of high analyte selectivity. On a secondary note, the sensitivity of UV detection at 275 nm for CFL in 1 % TFA + CH_3OH was found to be almost identical to that for CFL in $CHCl_3$.

The PE peak area due to 1 % TFA + CH_3OH (or blank) was quite significant. CFL has a molar absorptivity of 6550 $M^{-1}cm^{-1}$ at 240 nm, 6532 $M^{-1}cm^{-1}$ at 270 nm, and 5834 $M^{-1}cm^{-1}$ at 275 nm. Even at 275 nm, the blank produced a peak area, which was 2.2 times larger than that for a 20-μg/ml CFL solution. A RSD of 2.6 % for the blank would pose the detection limit at a modest level of 3 μg/ml (or 60 ng) CFL. Improvement of the RSD for the blank to 0.3 % by using a better solvent delivery system (Eldex cc-30s micrometer pump) afforded a lower detection limit of 0.4 μg/ml (or 8 ng) CFL.

Before being packed into the micro-column, these CFL MIP particles had been stored in the dry state at room temperature (20 - 25 °C) for more than two years. Yet they still exhibited a high molecular recognition towards CFL. This property is a unique advantage of MIP particles over the natural antibodies.

As compared with 15 ml required for HPTLC and 1 ml for HPLC, MIPSE can be finished by single injection, which only needs a 20-μl aliquot of sample solution. By changing the size of sample loop to small one, the consumption of sample solution could be even less.

3.3 MISPE-DPE-FPE

3.3.1 Differential pulsed elution (DPE) Solvent

Although CFL can achieve 90-95 % binding in these CFL MIP particles, the other α-aminocephalosporin compounds, CFR and CFD, can also achieve 68-76 % and 78-80% binding, respectively. Due to lack of sufficient specificity afforded by the CFL-MIP, DPE with an intermediate solvent to wash other α-aminocephalosporins (CFR and CFD) out of the micro-column was deemed necessary. One major requirement in the search for an appropriate DPE solvent would be that all CFR and CFD molecules were desorbed and eluted, while some CFL molecules remained in the MIP recognition sites. This was conducted methodically by changing the DPE solvent composition from 0.25 % to 15 % of acetic acid in acetonitrile. Afterwards, 20 μL of methanol + 1 % TFA was injected onto the MIP micro-column for a final pulsed elution (FPE) that desorbed any remaining molecules of bound CFL. Figure 19 shows the % FPE (or % desorption in FPE) results with various DPE solvent compositions. An increasing % of acetic acid in acetonitrile resulted in the desorption of more CFR and CFL molecules during DPE, and hence a decreasing trend of % FPE (= 1 - %DPE) was observed. As displayed in Figure 19, CFR was, however, more easily desorbed than CFL by each given % CH_3COOH in CH_3CN. Complete CFR desorption was achieved by PE with 10 % CH_3COOH in CH_3CN to yield a % FPE of zero, while some CFL remained to yield a % FPE of 41 % for quantitative analysis. As discussed before (Binding Capacity Evaluation Part), with a higher binding capacity than CFR in this CFL MIP micro-column, CFD was again proved possessing slightly stronger binding interaction in this CFL MIP particles, requiring 12 % CH_3COOH in CH_3CN to yield a % FPE of zero. The remaining CFL yielded a % FPE of 29 %. Acetic acid may be considered as analogous to the functional monomer, methacrylic acid, in the MIP.[58] Complete desorption of the remaining CFL molecules, bound in the MIP particles, was achieved by 1% TFA +CH_3OH. No detectable carryover into the next sample analysis was observed, as evidenced by a zero ΔFPE peak area.

Table 7 summarizes the DPE solvents that have been reported in the literature as required for various drug compounds bound on four different MIP micro-columns. How to quantitatively separate the structurally related drug molecules that differed either in the

position of a strong hydrogen-bonding group, or between a secondary and a tertiary amine group, was always the challenge suffered by the previous work. In those cases, different organic solvents could be tested for optimal DPE. It cannot be overemphasized how critical it was in the present work to optimize the DPE solvent by systematically increasing the % acetic acid in acetonitrile. The final increment from 10 % to 12 % made all the difference between incurring a risk of 16 % interference by CFD and enjoying an interference-free determination of CFL in the FPE step.

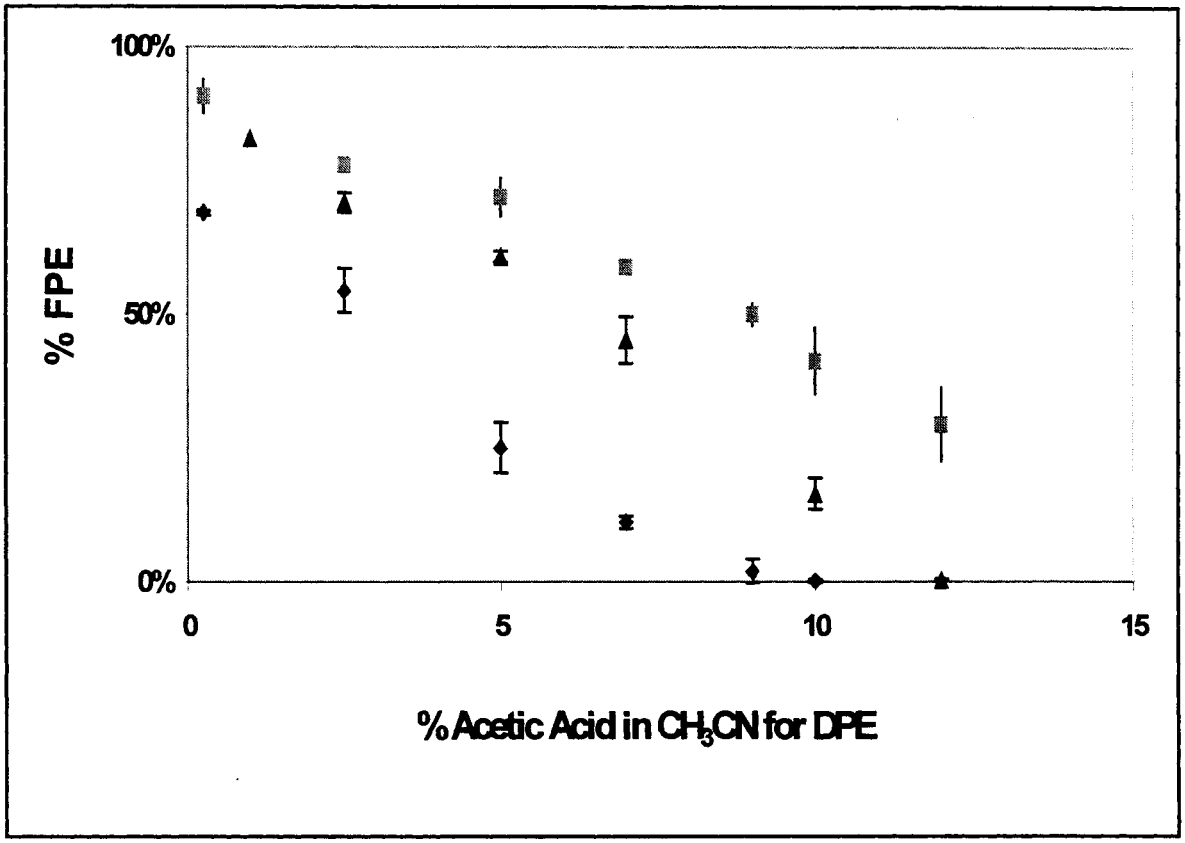


Figure 19 % FPE for various % acetic acid in CH_3CN for DPE of (■) CFL, (▲) CFD, and (◆) CFR at 20 μg/ml concentrations.

Table 7 Summary of DPE solvents for various drug molecules bound onto four different MIP micro-columns. [59-60]

Analyte	pK_a of Analyte	Interferents	pK_a of Interferents	Solvent for MISPE of Analytes and Interferents	Solvent for Elution of Interferents by DPE	Solvent for Elution of Analyte
Theophylline	8.68	Dyphylline Nicotinic acid	5.36 4.80	$CHCl_3$	CH_3CN	CH_3OH
Nicotine	8.02	Myosmine	—	CH_3CN	CH_3OH	H_2O + 1 % TFA
4-Aminopyridine	9.26	2-Aminopyridine	6.67	$CHCl_3$	DMSO	CH_3OH + 1 % TFA
CFL	5.3 and 7.3	CFR CFD AMP	2.6 and 7.3 — 2.5	$CHCl_3$	CH_3CN + 10% CH_3COOH CH_3CN + 12% CH_3COOH	CH_3OH + 1 % TFA

3.3.2 Serum analysis

In his Ph. D research, Wayne Mullet performed an extraction of human serum with chloroform first, followed by spiking the chloroform layer with theophylline. The advantage of this procedure, as he claimed, was to provide a simultaneous removal of interferences such as proteins, so as to avoid theophylline binding with albumin proteins. [61] However, in terms of in vivo serum analysis, his procedure can be arguable. Albumin proteins are present in real serum samples, and could bind with the analytes already. Therefore his design just evaded the problem presented by albumin proteins. One improvement was made in the present research regarding serum analysis for CFL. Human serum was spiked with CFL, CFR and CFD, followed by extraction with a C_{18} (octadecyl) SPE cartridge. A small volume of methanol was applied afterwards to elute the extracted analytes from the C_{18} cartridge. The eluent was collected and diluted with $CHCl_3$. This improvement was proved successful as evidenced by the observation that after MISPE of the diluted serum standard solutions ($CHCl_3$) with CFL concentrations ranging from 0.8-27 μg/ml, which is the typical therapeutic range of CFL in human serum, ΔDPE and ΔFPE peak areas showed proportional increases with CFL concentration.

3.3.2.1 Standard calibration curve of MISPE-DPE-FPE for serum analysis

Standard calibration curve of MISPE-DPE-FPE for serum analysis was constructed by recording the ΔFPE peak area versus mass of CFL loaded, after a single injection of the above diluted serum standard solutions individually, followed by DPE and FPE with 14 % CH_3COOH + CH_3CN and 1 % TFA + CH_3OH, respectively. A satisfactory linearity (R^2 = 0.9884) was found within the mass range (Table 8). Note that in this experiment, a 14 % CH_3COOH + CH_3CN solution was used as DPE solvent (mobile phase) to replace the previously used 12 % CH_3COOH + CH_3CN solution for complete elution of all the structural analogues.

3.3.2.2 Evaluation of recovery

Two serum sample solutions prepared by spiking human serum with CFL, CFR and CFD were analyzed, as described in experimental part. The recovery of this method was evaluated by performing single injection for MIPSE, with 14 % CH_3COOH + CH_3CN as the mobile phases, followed by PE with 1 % TFA + CH_3OH. The results in Table 8 indicated satisfactory recovery percentages of CFL at the specified mass levels. This confirmed the suitability of the method for the quantification of CFL.

3.3.2.3 Limits of quantification and limits of detection

The LOD and LOQ of CFL in serum were determined by analyzing serum samples spiked with CFL at relatively low concentrations of CFL (0.8–27 μg/ml) (or 20-530 ng of CFL) using the developed MISPE-DPE-FPE method. The achieved LOD for CFL (expressed as 3 x standard deviation of the serum blank) in serum was 0.3 μg/ml (or 5.1ng of CFL). The LOQ for CFL in serum was found to be 0.9 μg/ml (or 17.1 ng of CFL) (expressed as 10 x standard deviation of the serum blank). (Table 8)

3.3.3 Plasma analysis

As specified, the human serum utilized in serum analysis was minus immunoglobulin (Ig) already. Therefore, the results could not give much confident support to real sample analysis. In real serum or plasma sample, variety of biological interference, especially human protein, like IgG, might bind with analytes. As reported already that penicillin and some other β-lactam antibiotics exert their lethal effect by inhibiting the proteins that synthesize bacterial cell wall peptidoglycan. By mimicking the structure of the acyl-D-Ala-D-Ala C terminus of the peptide chain, some β-lactam antibiotics may react with PBPs to form an acyl-enzyme complex. The complex then reacts with an amino group from another peptide chain to from a cross-link. Unlike the transient nature of the penicillin-binding proteins or PBP-peptide complex, the acyl-enzyme complex formed between PBPs and β-lactam antibiotics is much more stable. In human plasma, at least 10 PBPs have been identified.

It was also reported that up to 15 % of the dose of CFL could be bound to plasma proteins.[62] Therefore, the major concern in the method development was whether the newly developed MISPE-DPE-FPE method could be applied in plasma analysis. Further investigation was conducted by using human plasma samples from anonymous patients, which were supplied by Ottawa General Hospital, to replace the human serum minus IgG.

3.3.3.1 Validation of the method

Using the same experimental procedure for plasma analysis, good linearity was obeyed within the CFL concentration range from 1.0-20 μg/ml, which is the therapeutic range of the drug in human body. The linearity expressed as a regression coefficient (R^2) is listed in Table 8. The limit of detection (LOD) and limit of quantification (LOQ) are also displayed in Table 8. Expressed as the slope of standard calibration curve, the sensitivity of the method for plasma analysis was found to be lower than for serum analysis, suggesting that some fraction of CFL molecules were bound to plasma proteins, which can not be detected by the MISPE-DPE-FPE method.

3.3.3.2 Accuracy and recovery

To confirm the accuracy of the method, further validation was performed by measuring plasma samples spiked with known quantity of CFL, CFR and CFD. As shown in Table 8, the mean recovery of 95 % (± 3 % RSD, n=3) of the added amount of CFL shows a fairly good concordance between experimental and nominal values.

3.3.4 Total analysis time

Under the present flow rate condition of 0.5 ml/min, a single MISPE-DPE-FPE analysis took 5-6 min. This was more rapid than the derivatization procedure previously reported for the determination of CFL in pharmaceutical and urine samples using 1,2-naphthoquinone-4-sulfonate (NQS) into solid-phase extraction cartridges with UV-visible detection.[63] The present MISPE-DPE-FPE analysis does not require any reaction time of 5 min, carbonate buffer of pH 10.5, or reagent of 7.1 mM NQS. It is also more rapid than

the 16-min cycle (including regeneration of the immunoreactor) required for the flow injection immunoanalysis previously reported.[64] More rapid MISPE-DPE-FPE analysis could be achieved by using a higher chloroform flow rate than 0.5 ml/min, provided that the binding efficiency stayed close to 100 %. As shown in Figure 20, for 20 μg/ml of CFL in $CHCl_3$, the flow rate could be increased to 1.25 ml/min before the % binding started to decrease. At 1.50 ml/min, it decreased to 89 (±0.4) % binding. This % binding was acceptable, considering that the linear flow velocity of $CHCl_3$ through the micro-column was as high as 5.0 cm/s and the residence time for the injected sample was as short as 1.1 s. The PE efficiency, however, dropped significantly at this high flow rate as shown in Figure 21. The PE kinetics was not fast, limiting the practical flow rate to no higher than 1.25 ml/min for the MISPE-DPE-FPE analysis. At 1.25 ml/min, a single MISPE-DPE-FPE analysis took only 2 min to complete. Further investigation of PE kinetics would be needed, to test new solvents for the efficient PE of bound CFL molecules at flow rates higher than 1.50 ml/min. Note that the % binding and PE efficiency do not have to be 100 % for the MISPE-DPE-FPE method to be useful.

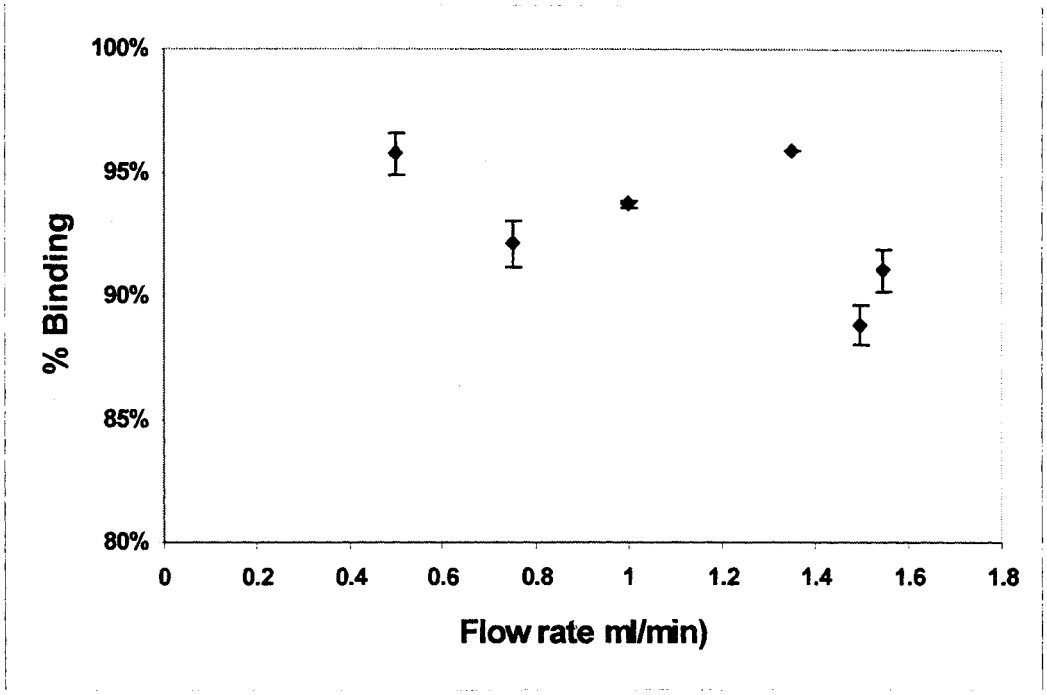


Figure 20 Dependence of % binding on flow rate

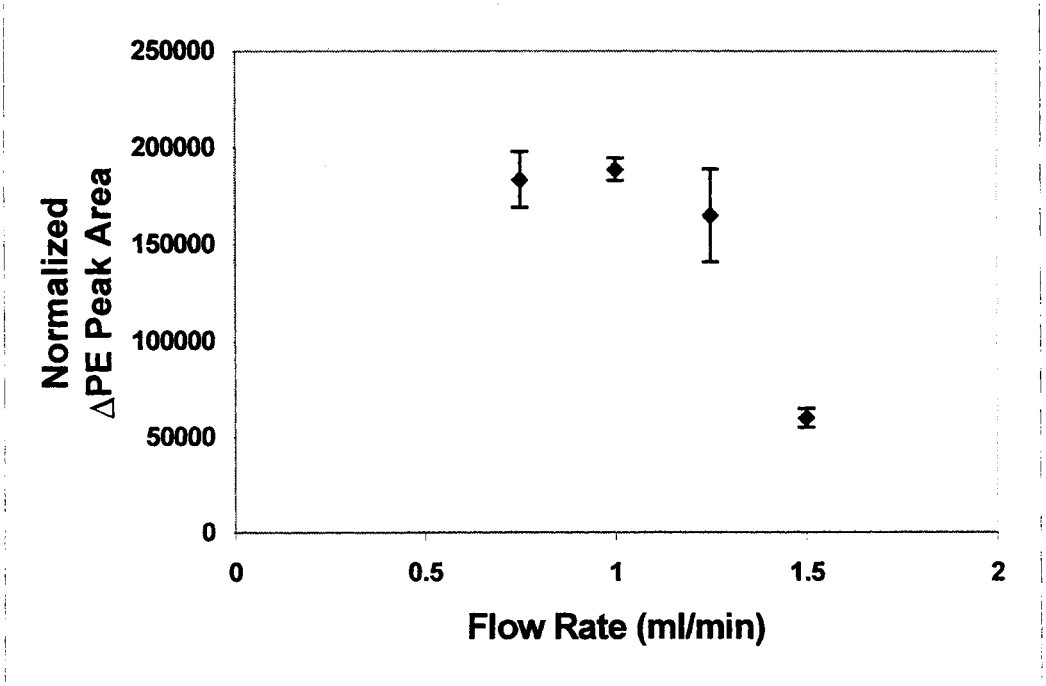


Figure 21 Relationship of ΔPE and flow rate

3.4 MISPE-PE-MS

3.4.1 Ionization of CFL, CFR and CFD

3.4.1.1 MS behavior of α-aminocephalosporin antibiotics

As shown in Figure 2, the α-aminocephalosporin antibiotics contain both amino and carboxylic groups in each molecular structure. Theoretically, they will favor both positive and negative electrospray. However, a positive electrospray mode was adopted due to two considerations. First, negative electrospray was found to be less efficient, due to $CHCl_3$ being used as the mobile phase in which dissociation of a proton from the carboxylic group becomes less possible. The second consideration was about PE solvent that contained TFA. TFA is a proton donor that actually may promote the protonation of α-amino groups, as proposed in Figure 22. By associating 1 proton from TFA, the a-aminocephalosporin molecule can be detected as a single-charged positive ion. Therefore, the choice of a positive electrospray mode is more appropriate. The initial investigation was to mimic PE procedure. CFL, CFR and CFD standard solutions (0.3 mg/ml in 1 % TFA + CH_3OH), were injected individually, through the CFL MIP micro-column, into the Quattro mass spectrometer. Their respective LC-MS spectra (Figure 23) have showed that CFL and its structural analogues could be ionized very well in the presence 1 % TFA + CH_3OH. Further investigation was conducted by performing MISPE with standard mixture containing 20 μg/ml each of CFL, CFR and CFD, followed by PE with 1 % TFA + CH_3OH. The obtained mass peaks of CFL, CFR and CFD at m/z 348, 350 and 364 (Figure 24), respectively, again proved that the ionization of these three cephalosporin compounds are good for LC-MS analysis without introducing an external ESI source.

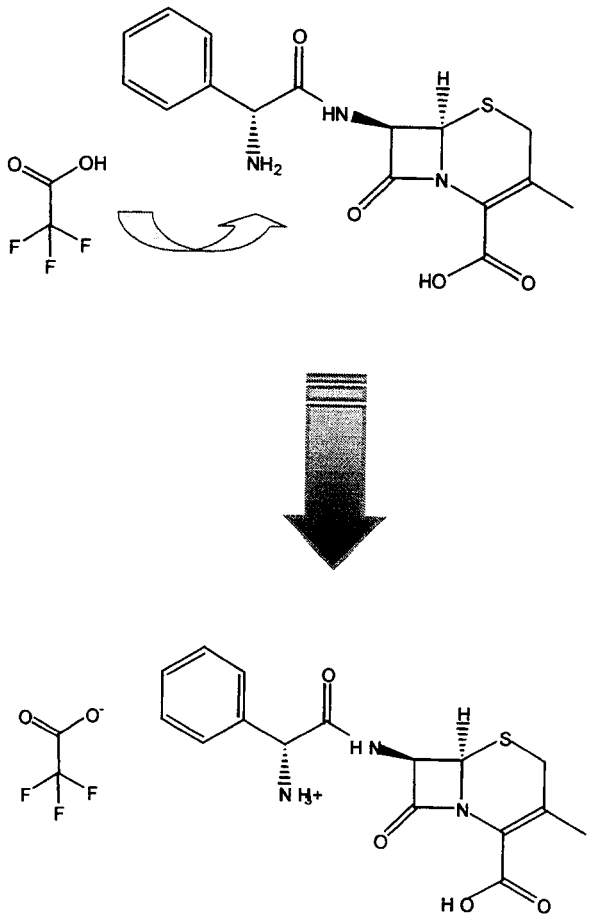
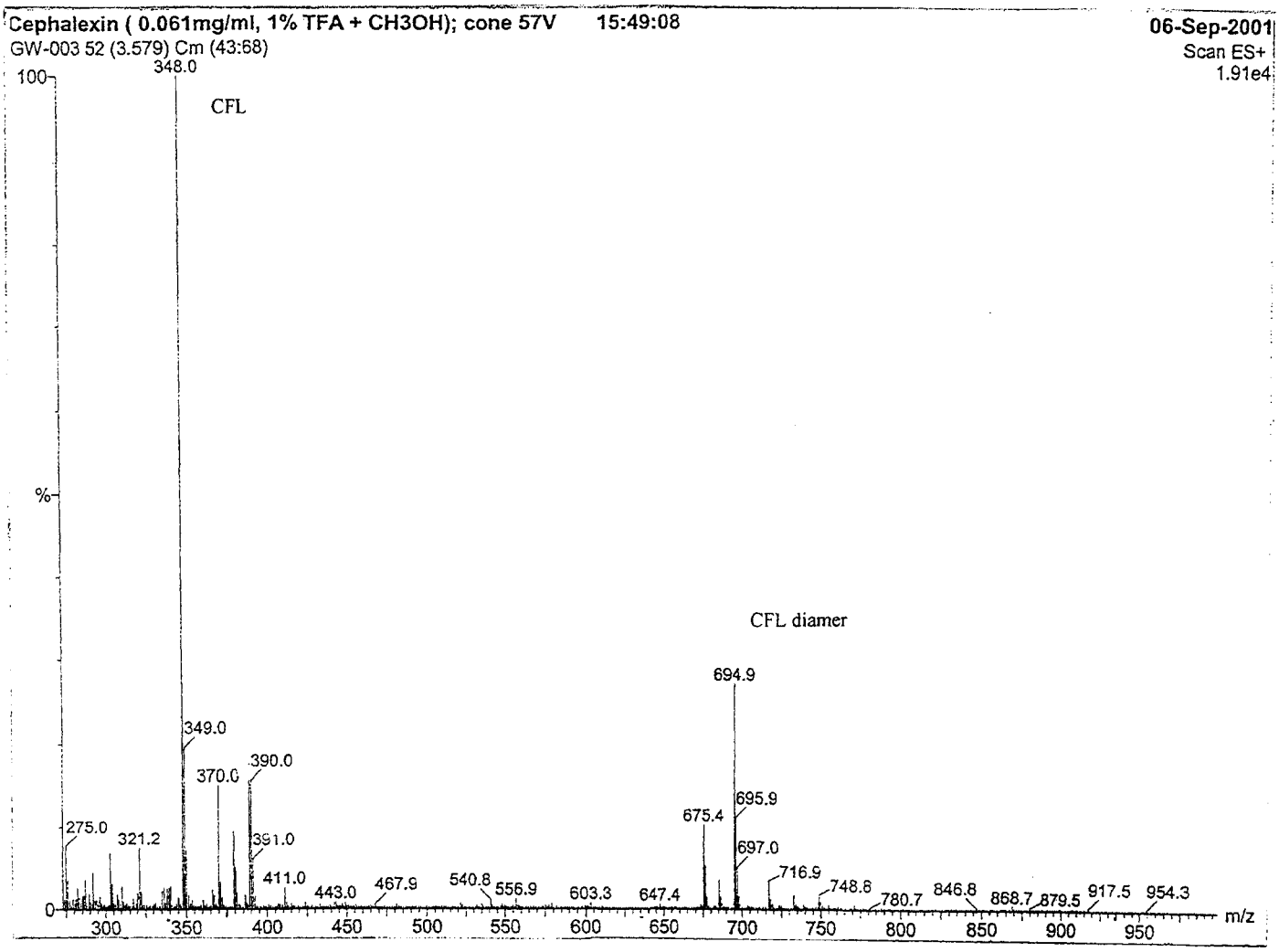
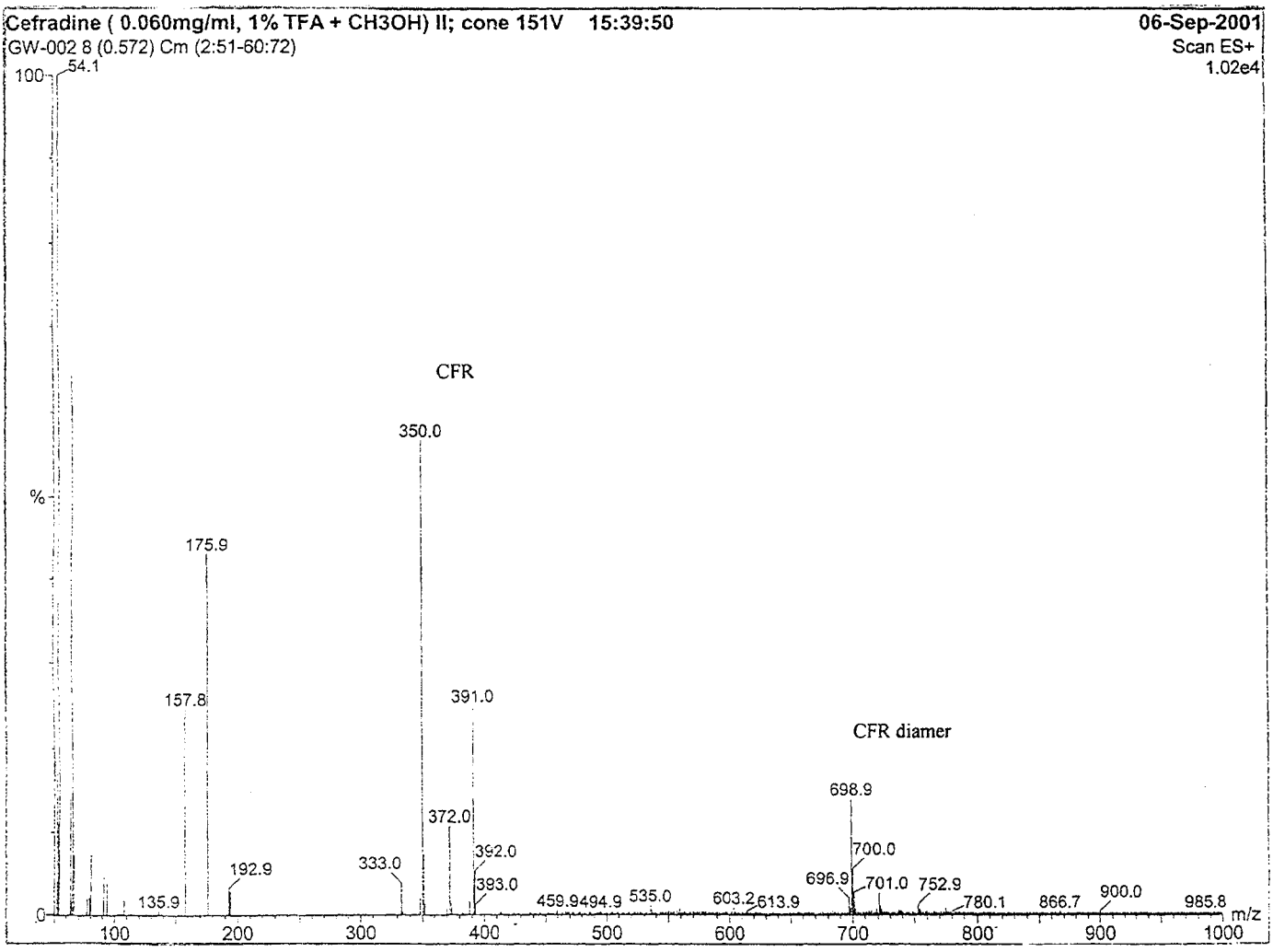


Figure 22 Ionization of CFL to a single-charged positive ion in the presence of TFA

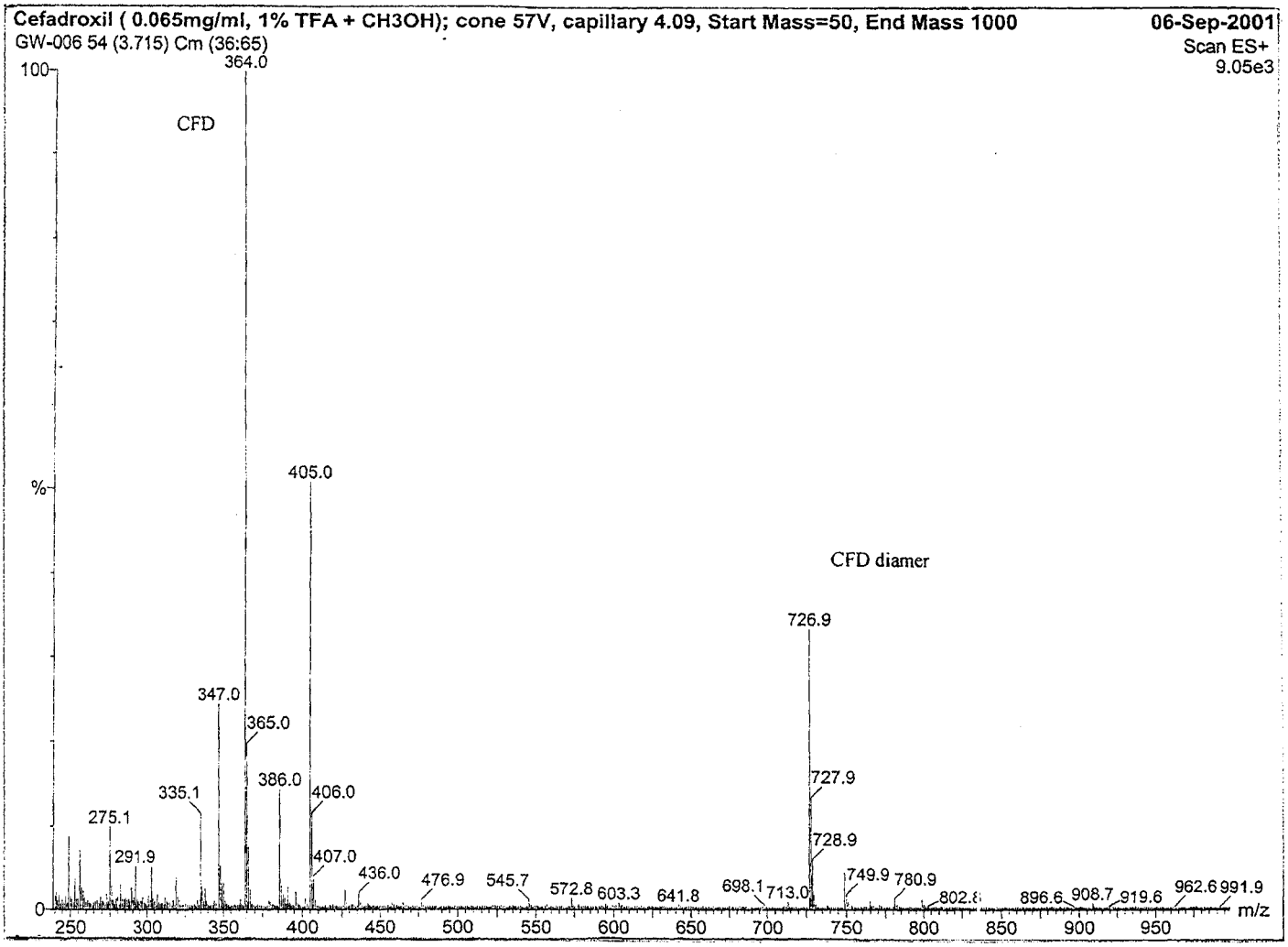
Figure 23 Mass spectra of CFL, CFR and CFD in 1 % TFA + CH_3OH (0.3 mg/ml)



A: Mass spectrum of CFL prepared in 1 % TFA + CH_3OH



B: Mass spectrum of CFR prepared in 1 % TFA + CH_3OH



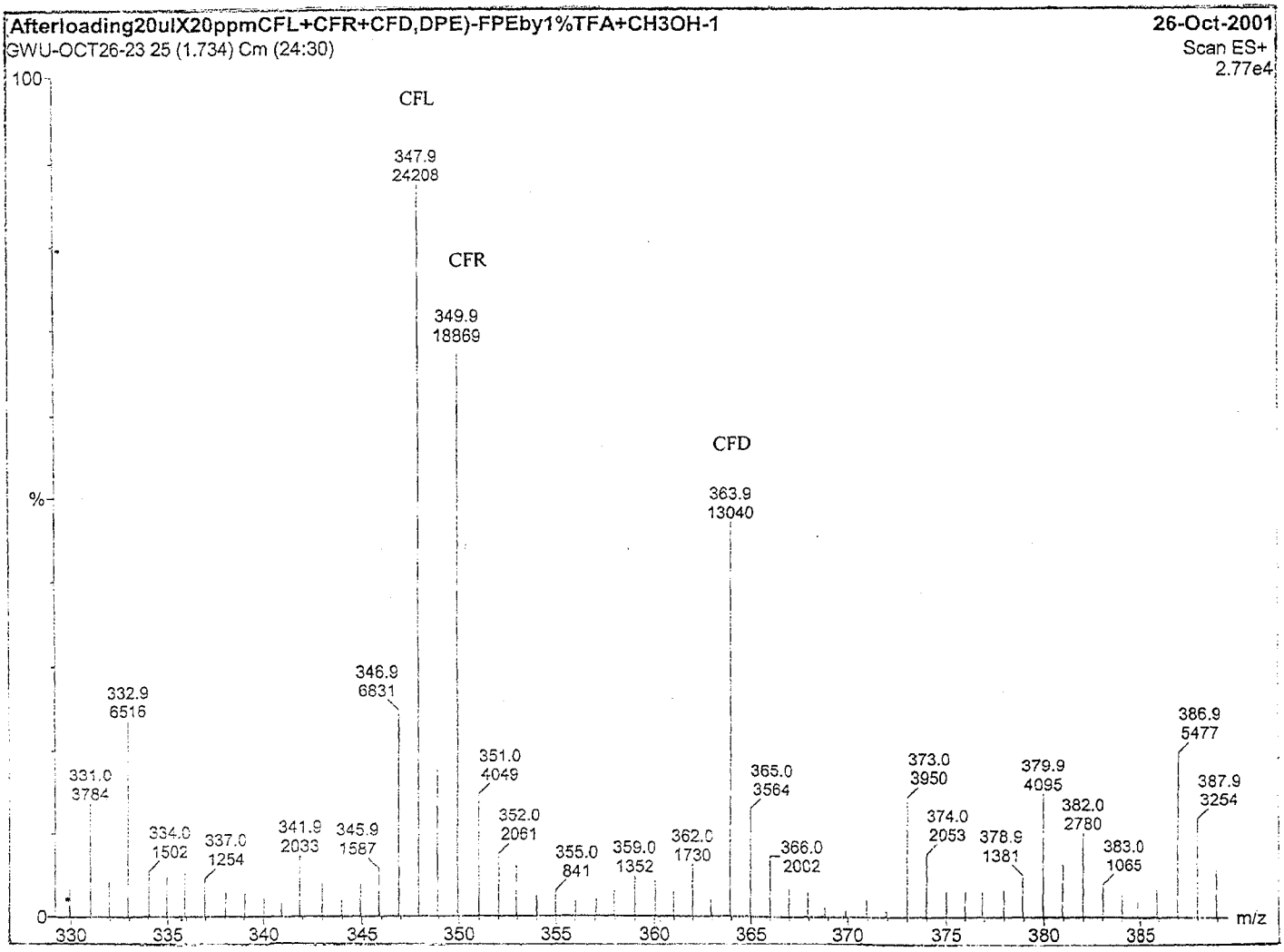
C: Mass spectrum of CFD prepared in 1 % TFA + CH_3OH

3.4.1.2 Verification of binding behavior of CFL, CFR and CFD

When a UV detector was used to monitor the MISPE procedure at λ=275nm, CFL, CFR and CFD achieved 90-95 %, 68-76 %, 78-80 **%** binding, respectively, on this CFL MIP micro-column. However, for UV detection, the % binding of each α-aminocephalosporin analyte could only be investigated separately. Therefore, it was interesting to use a mass spectrometer to verify the binding behavior of all three analytes at equal concentrations in a mixture. As illustrated in Figure 24, after PE with 1 % TFA + CH_3OH, the mass spectrum exhibited intense peaks at m/z 348, 350, and 364, respectively, for the ions of eluted CFL, CFR and CFD. Among them, CFL had the highest peak intensity of 24208 (arbitrary units), compared with the peak intensities of 18869 for CFR and 13040 for CFD. This observation confirmed the UV result that CFL may achieve the highest % binding on this CFL MIP micro-column, comparing with the other two α-aminocephalosporins.

Figure 24 Mass spectrum of CFL, CFR and CFD at m/z 348, 350 and 364

CFL (20 μg/ml) + CFR (20 μg/ml) + CFD (20 μg/ml)
MISPE with 100 % $CHCl_3$ as mobile phase, at flow rate of 0.05 ml/min, followed by PE with 1 % TFA + CH_3OH



3.4.1.3 Ionization competition between CFL and structural analogues

One major concern was about the ionization competition between CFL and its structural analogues, which may affect the accurate LC-MS quantification of CFL in the presence of CFR and CFD. Ionization competition may also come between CFL and sulindac. However this effect could be calibrated through plotting a standard calibration curve with ratio of peak intensity of CFL and sulindac vs concentration of CFL.

An investigation was launched by MISPE of 20 μg/ml CFL standard testing solution ($CHCl_3$). Afterwards, PE was performed by injecting 1 % TFA + 20 μg/ml sulindac + CH_3OH, containing CFR and CFD at concentrations ranging from 4-75 μg/ml, individually. To simplify the experiment, 1 % TFA + CH_3OH containing different concentrations of CFR and CFD, were used for PE of CFL. As can be observed in the Figure 25, a fairly constant trend of the ratio of ΔPE peak area intensity of CFL to peak intensity of sulindac could be observed, in the presence of CFR and CFD within the tested concentration range. This result declared that the ionization competition from CFR and CFD, within the above concentration range, is very weak. The ionization competition depends on the relative concentration ratio of the compound to be ionized and the interference compounds (ionization competitor). In this experiment, TFA is the ionization source for CFL, while CFR and CFD act as ionization competitors with CFL. In common sense, the higher the concentration of ionization source, the less likely the ionization competition would occur. During each time of PE, the liquid plug of 1 % (v/v) TFA (d 1.480) + CH_3OH solution actually contains ~14.8 mg/ml of TFA molecules, which is approximately 20-390 times of the amount CFR and CFD (concentration ranging from 4-75 μg/ml) tested in the experiment. Therefore, the plenty of TFA molecules available in the PE solvent offers sufficient ionization source for the eluted α-aminocephalosporin analyte molecules, and binding competition can be ignored.

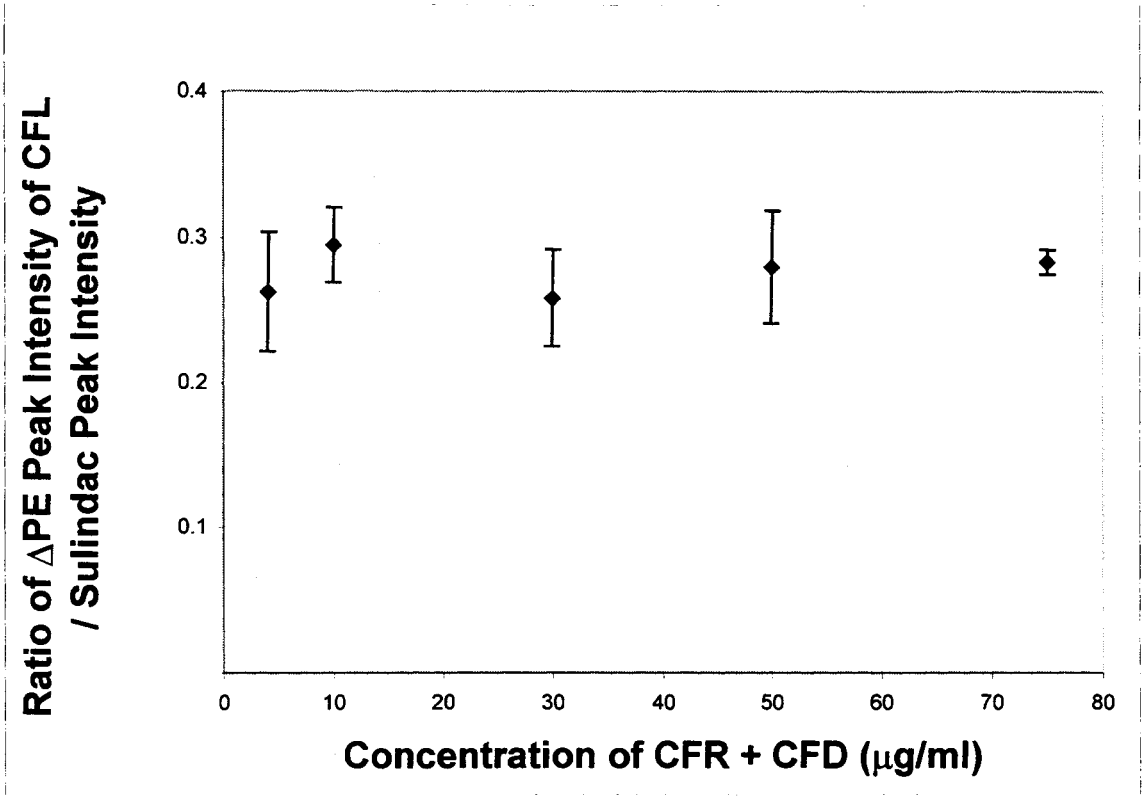


Figure 25 Investigation of ionization competition between CFL, CFR and CFD

3.4.2 Method development

CFL belongs to the first generation of α-aminocephalosporin antibiotics, the therapeutic plasma levels of which are 5-25 μg/ml. Conventional HPLC methods using either UV or photodiode array detection were found to be unsatisfactory in terms of sensitivity. LC-MS detection permits a total elimination of the background interference arising from the TFA and may allow the selective detection of CFL.

3.4.2.1 Major challenge

The development of quantitative MISPE-PE-MS analysis initially used peak intensity, which is theoretically proportional to the mass of analytes extracted by CFL MIP micro-column. However, this approach soon proved unsuccessful mainly due to fluctuations of electric signal inside the mass spectrometer. Signal fluctuations may also be induced by signal suppression or enhancement effects when samples extracted from physiological or environmental matrices are analyzed. [65] Application of the internal standard method was therefore necessary for precise quantitative analysis.

3.4.2.2 Selection of internal standard for quantification

Conventionally there are two different methods for introducing an internal standard into the analysis procedure. The surrogate introduction method involves addition of the internal standard prior to any procedures (also including extraction and purification). [66] This method compensates for signal loss attributed to sample preparation procedure. In contrast, the volumetric introduction method involves the addition of an internal standard prior to analysis particularly to address instrumental errors. Both methods can be used to compensate for quantitative errors attributed to signal suppression in LC-MS. However, for either method to be effective, the analytes and the internal standard must be eluted simultaneously, which can hardly be realized in the MISPE-PE procedure because the internal standard can not be extracted by the MIP micro-column.

The improvement to the introduction of internal standard was designed. By mixing the internal standard with the PE solvent, the extracted analytes and internal standard would be eluted simultaneously. Different compounds were tested for the best performance of

internal standard. Isoproturon initially proved to be ideal as an internal standard, due to its stable chemical properties in PE solvent and high sensitivity for ionization efficiency (Figure 26). Owing to its hazardous properties in handling, however, this compound was finally given up. Application of sulindac as an internal standard was previously reported in LC-MS spectrometric determination of celecoxib in plasma. [67] The ionization performance of this compound, in the presence of TFA, was verified by injecting sulindac standard solutions in 1 % TFA + CH_3OH, passing through the CFL MIP micro-column. The mass spectrum displayed in Figure 27 showed satisfactory peak intensity proportional to the concentration of sulindac.

Figure 26 MISPE-PE-MS spectrum for CFL when isoproturon was used as internal standard in PE solvent

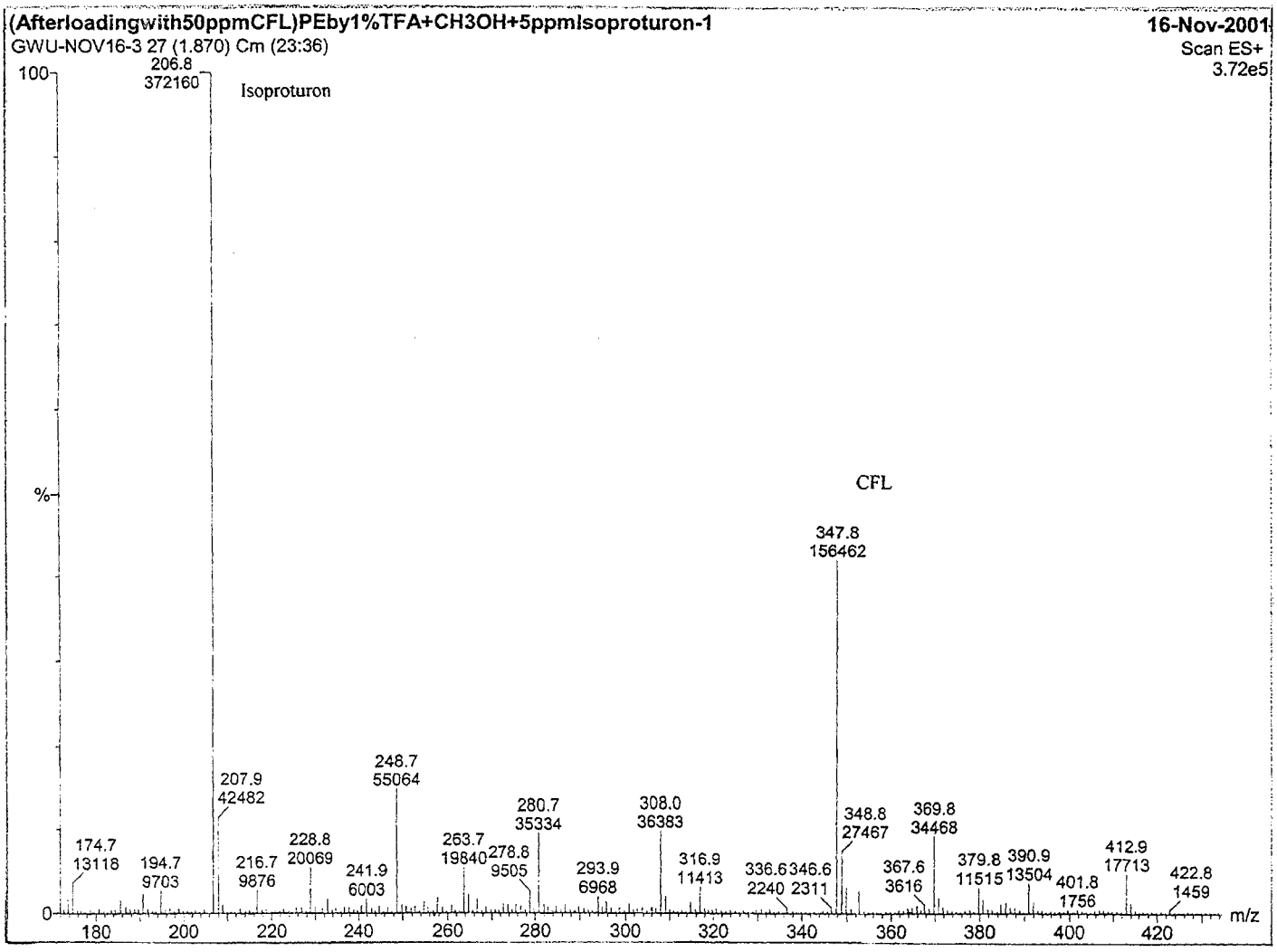
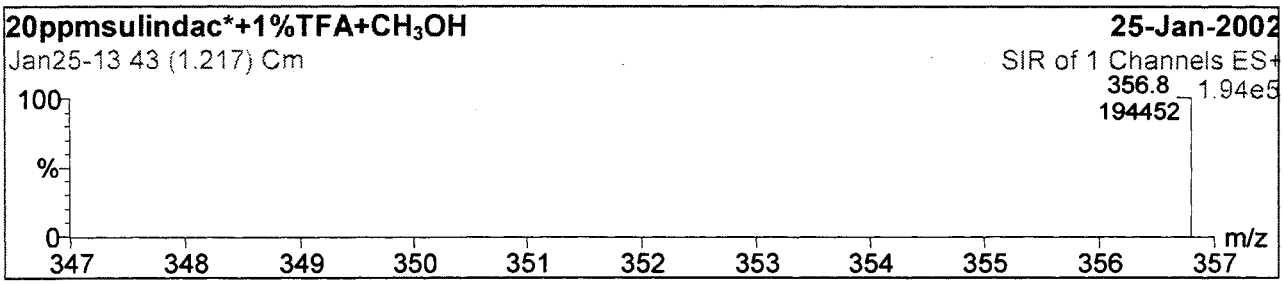
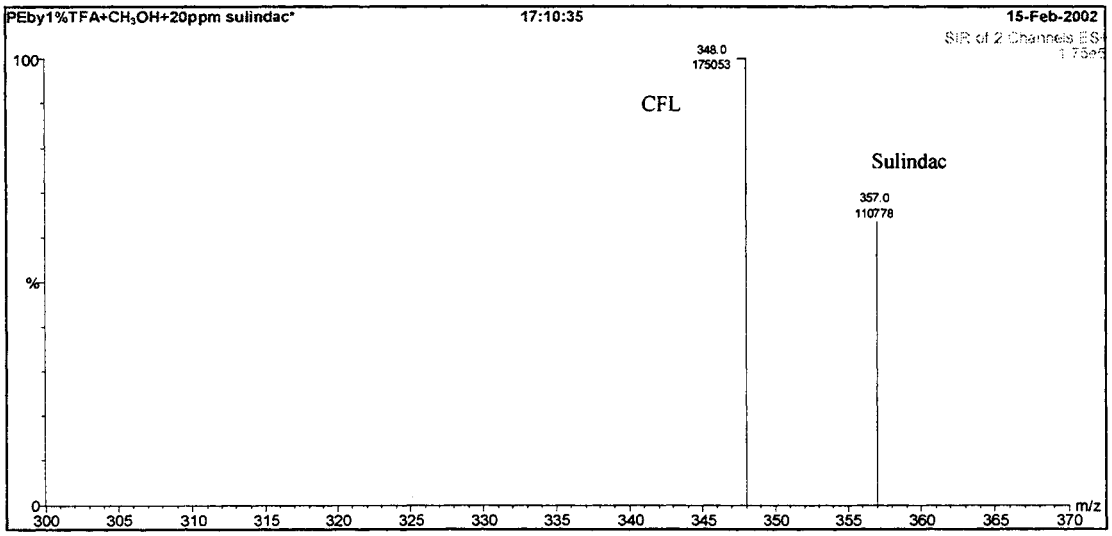


Figure 27 Investigation of using sulindac as internal standard for mass spectrometric determination of CFL



A: Mass spectrum of sulindac prepared in 1 % TFA + CH_3OH



B: Mass spectrum of MISPE-PE result
(with 1 % TFA + CH_3OH as PE solvent, containing 20 μg/ml sulindac as internal standard)

3.4.2.3 Selected ion recording (SIR)

The selected ion recording (SIR) mode is typically used in those situations where only a few specific masses are to be monitored during an acquisition. Since most of the data acquisition time is spent on these masses, the SIR mode is far more sensitive than the conventional "Full Scanning". It can be a very effective tool for improving the detection limit. In this experiment, 4 major m/z channels were set in the SIR mode: 348 (CFL), 350 (CFR), 357 (sulindac) and 364 (CFD), for monitoring the PE of these α-aminocephalosporins simultaneously. (Figure 28)

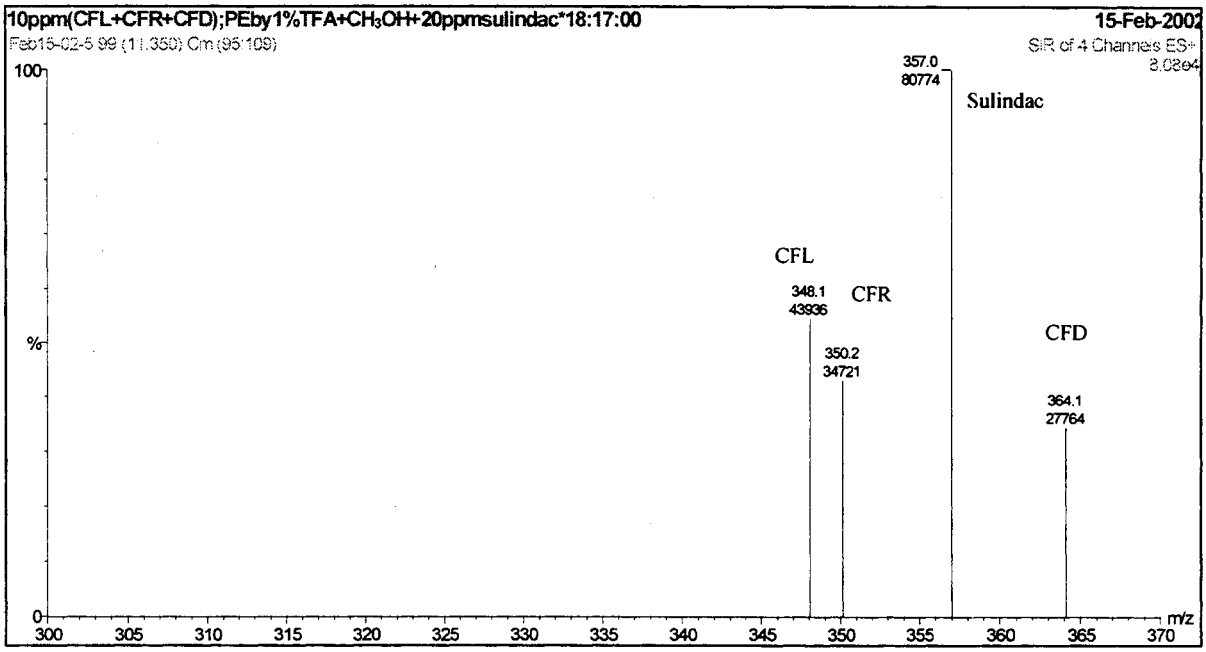


Figure 28 A selected ion recording (SIR) mass spectrum of MISPE-PE for CFL + CFR + CFD (with 1 % TFA + CH_3OH as PE solvent, containing 20 μg/ml of sulindac as internal standard)

3.4.3 Validation of MISPE-PE-MS method

3.4.3.1 Linearity

The standard calibration curve of MISPE-PE-MS for CFL serum sample analysis was constructed on the same day as for serum sample analysis. The determination of CFL in human serum sample was carried out by measuring the peak intensity ratio of CFL/sulindac vs mass of CFL loaded and regression coefficient. A good linear range (R^2 = 0.9968) was confirmed for determination from 0.25-25 μg/ml (or 5-500 ng of CFL) (Table 8).

3.4.3.2 Accuracy and recovery of the method

After extraction with an Octadecyl C_{18} SPE cartridge, human serum samples containing CFL at concentration 13.5–25 μg/ml, spiked with CFR at concentration of 60.5 μg/ml, and CFD at concentration of 31.6 μg/ml, were analyzed using LC-MS. The CFL concentration was determined from the standard calibration curve. A 93 % recovery of CFL (RD = 1.3 %) was achieved (n=2) at the above concentrations, in the presence of CFR and CFD. This confirmed the suitability of this MISPE-PE-MS for determination of CFL in serum samples (Table 8).

3.4.3.3 Limits of detection and limits of quantification

The LOD and LOQ of CFL in serum were determined by analyzing serum samples spiked with CFL at relatively low concentrations of CFL (0.25–25 μg/ml) (or 5-500 ng of CFL) using the developed LC-MS method under the described conditions (Table 2). The achieved LOD for CFL (expressed as 3 x standard deviation of the serum blank) in serum was 0.039 μg/ml (or 0.78 ng of CFL). The LOQ for CFL in serum was found to be 0.13 μg/ml (or 2.6 ng of CFL) (expressed as 10 x standard deviation of the serum blank (Table 8).

Table 8. Summary of standard calibration curves of MISPE-(DPE)-FPE in different matrices.

MISPE-DPE-FPE in Different Matrices	Slope (1/ng)	Linear Range Mass (ng) Concentration (μg/ml)	Regression Coefficient (R^2)	LOD (ng) (μg/ml)	LOQ (ng) (μg/ml)	Mean Percentage Recovery± Mean Deviation
MISPE-PE ($CHCl_3$ as mobile phase, 1 % TFA + CH_3COOH as PE solvent)	3091	12-900 0.6-45	0.9796	8.0 0.4	20 1.0	—
MISPE-DPE-FPE for serum analysis (14 % CH_3COOH +CH_3CN as mobile phase, 1 % TFA + CH_3OH as FPE solvent)	272	20-530 0.8-27	0.9884	5.1 0.3	17.1 0.9	105 ± 2 % (n=2)
MISPE-DPE-FPE for plasma analysis ($CHCl_3$ as mobile phase, 12 % CH_3COOH + CH_3CN as DPE solvent, 1 % TFA + CH_3OH as FPE solvent)	112	21-400 1.0-20	0.9987	12.8 0.6	42.6 2.1	95 ± 3 % (n=3)
MISPE-PE-MS for serum analysis ($CHCl_3$ as mobile phase, 1 % TFA + CH_3OH as PE solvent + 20 μg/ml of sulindac as internal standard)	0.0005	5-500 0.3-25	0.9968	0.8 0.04	2.6 0.1	93 ± 1 % (n=2)

3.4.3.4 Additional advantage of MISPE-PE-MS over MISPE-DPE-FPE

Coupled with the UV detector, MISPE-PE must involve a DPE step to eliminate the co-extracted structural analogues before a FPE for CFL quantification. The DPE step, although proved to be successful in eliminating structural analogues, increases the total analysis time, and it may cause variations of the ΔFPE peak area. The MS spectrometer can easily distinguish CFL, CFR and CFD simultaneously without any spectral overlap. Thus the DPE step can be eliminated. Without the involvement of a DPE step, the total analysis time of each MISPE-PE, at a mobile phase flow rate of 0.05 ml/min, is as short as 3-4 min.

3.5 Future work

This CFL MIP micro-column can be employed as an enrichment device with high selectivity for CFL. Further improvement in detection limit may involve the use of sample loop of larger-size sample loop (≥100 μl), so as to collect more CFL molecules for detectable peak intensity in serum or plasma sample solution at ultra-trace levels. It is also possible to apply this CFL MIPs as the stationary phase in HPLC analysis. The separation of cephalosporins in combinatorial library can be achieved by using a DPE solvent with a gradient elution.

CHAPTER IV

CONCLUSION

The molecular recognition properties of cephalexin-MIP particles were evaluated in the present research. The binding results confirmed a high selectivity in molecular recognition towards cephalexin. A micro-column packed with these particles was used for the fast, quantitative extraction of cephalexin from a single injection of sample solution. They proved to be stable and robust, with a unbeatable lifetime in organic solvents up to thousands of MISPE analyses for high-throughput screening. Beta-lactam antibiotics, like ampicillin, could interfere with the screening result, when present at concentrations higher than 10 μg/ml. Fortunately, the spectral interference of ampicillin could be finally eliminated by changing the detection wavelength from 240 nm to 275 nm.

Selectivity toward α-aminocephalosporins proved to be a challenge with the present MIP micro-column. The recognition sites could not 100% differentiate between molecules that are structurally dissimilar only in their non-hydrogen-bonding moieties, when their hydrogen-bonding (acceptor and donor) moieties are identical to each other. Ultimately, complete removal of cefradine and cefadroxil required an intermediate DPE step, with only a partial loss of cephalexin. Coupled with a UV detector, the quantification of CFL was finalized by applying a FPE, which eluted the remaining cephalexin from the CFL MIP micro-column. As a new analytical separation method with good selectivity, the MISPE-DPE-FPE technology makes it easy for the inexperienced chemist to select a tailor-made MIP micro-column. As a unique means of assaying nanogram levels of cephalexin, this method can be performed in a small-size laboratory, with less capital investment on instrument, as no additional HPLC analysis is necessary subsequent to MISPE. It is also superior to the current methods, in terms of selectivity, analysis time, and simplicity. By applying to human serum sample analysis, the LOD and LOQ were 0.3 μg/ml (or 5.1 ng) and 0.9 μg/ml (or 17.1 ng) of CFL, respectively, within a good linear range of 0.8-27 μg/ml (or 20-530 ng) of CFL. In plasma analysis, the achieved LOD and LOQ were 0.6 μg/ml (or 13 ng) and 2.1 μg/ml (or 42.6 ng) of CFL, respectively, within the linear range of 1.0-20.0 μg/ml (or 21-400 ng) of CFL.

A mass spectrometric detector was employed to replace the UV detector for improvement of the method. MS proved to offer higher sensitivity and freedom from spectral interference caused by the PE solvent. For the specific determination of

cephalexin and other α-aminocephalosporins found in combinatorial drug libraries, the use of mass spectrometric detection would be ideal. Each α-aminocephalosporin has a characteristic molecular mass for unequivocal peak identification in the mass spectrum during PE. By applying the internal standard method, with sulindac mixed with the PE solvent, precise quantification of eluted CFL in the serum sample was achieved. Compared with MISPE-DPE-FPE-UV, MISPE-PE-MS is more progressive. Due to the simultaneous identification and detection of CFL and all structural analogues with mass spectrometry, no DPE procedure for elimination of structural interference is necessary. The total analysis time of each MISPE-PE-MS run was as short as 3 minutes. Thanks to the high sensitivity offered by the mass spectrometer, the achieved LOD and LOQ for human serum sample analysis were as low as 0.04 μg/ml (or 0.8 ng) and 0.13 μg/ml (or 2.6 ng) of CFL, respectively, within the linear dynamic range from 0.25-25 μg/ml (or 5-500 ng) of CFL.

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