Institut für Wasserchemie und Chemische Balneologie
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Synthesis and Application of Diclofenac Molecularly Imprinted Polymers for Selective Trace Analysis

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Contents

I Introduction

1 Pharmaceutical Pollution in the Environment

What happens to medicines after they are consumed? Are they utilized in their entirety by our bodies or are they partially excreted? Where do unused and expired medicines end up? Do medicines have an effect on other organisms living in the environment? These are some of the questions people are currently trying to get the answers.

There are three main human activities that cause changes in ecosystems: habitat fragmentation, alteration of community structure, and chemical pollution [1-3]. During the past 30 years, attention to the effects of chemical pollution has focused primarily on conventional "priority" pollutants, and rightfully so. These chemicals-referred to as persistent bioaccumulative toxicants (PBT) or persistent organic pollutants (POP) include e.g. lead, mercury, and dioxin, and they continue to have highly detrimental effects over long periods of time [4, 5]. They are often bioaccumulative. Bioaccumulation refers to the tendency to increase concentration when a toxin is consumed in a successional food chain. Heavy metal, for example, mercury is found in larger concentrations in ocean dwelling fish, such as Tuna and some freshwater fish than in shellfish, and is more concentrated in bigger fish, which feed on smaller fish. Presently, people are continuing to pay close attention to their ecological consequences.

Comparatively little attention, however, has been directed to a large class of chemicals comprising pharmaceuticals and personal care products (PPCP). Pharmaceutical drugs include all the medicines used for the diagnosis, treatment, and prevention of disease; illicit or recreational drugs; veterinarian medicines including those for agricultural livestock; over-the-counter medications; and nutritional supplements and nutraceuticals. Personal care products include fragrances, lotions and creams, cosmetics, sunscreen and other consumer chemicals. The use of chemicals continues to grow worldwide. In 2000, global production of chemicals has risen over 400 million tons annually, and the chemical industry is becoming an important sector for the modern economy. But huge volume production and widespread use of chemicals may pose risks to human health and the environment. In Europe, the number of incidents of allergies, asthma, certain types of cancer and reproductive disorders has been increasing, and it is suspected that the chemicals are contributing to this trend [6-8].

In the past decade, a considerable number of reports have been published on the widespread occurrence of residues of pharmaceuticals in the environment, e.g. in aquatic and soil compartments [9-13]. Unlike agrochemicals, which are disposed or discharged into the environment on a continual basis via domestic/industrial sewage and wet-weather runoff [14], PPCP are in part subjected to the metabolism of the user; then, excreted metabolites plus some unaltered parent compounds are released into sewage water, for example, after passing through sewage treatment plants (STP). On the other hand, they are also released by drug manufacturers and through disposal of unwanted and expired drugs directly into the domestic sewage system or via leachate from landfills. Through these processes, PPCP enter the environment, where they are considered pseudo-persistent because the transformation/removal rate of PPCP from the environment is compensated by the rate of replacement, a direct result of long-term use by consumers in higher quantities and subsequent improper disposal [15-20].

As analytical techniques have become more sensitive and more widely deployed, an increasing number of drugs are being detected [21-25]. Current understanding of the long-term effects of low ppt-ppb (ng-µg L^{-1}) concentrations of such pharmaceuticals is still limited and impacts on non-target species are practically unknown [26-32]. For example, a lethal impact of diclofenac, which is one of the most widely used **n**on-**s**teroidal **a**nti-**i**nflammatory **d**rugs (NSAID) was reported on vulture populations on the Indian subcontinent [33-35].

Diclofenac is one of the most frequently detected pharmaceutically active compounds in the water-cycle. Low concentration level of diclofenac (µg L^{-1}) has been detected in influents and effluents of STP and in surface waters [36-40]. Under recharge conditions, diclofenac was also found in groundwater and in raw and treated drinking water [41]. In Germany, approximately 75 tons of NSAID are sold annually [42]. Average concentrations in the low ppb range were detected in influents and effluents of municipal STP and surface waters in Austria, Brazil, Germany, Greece, Spain, Switzerland, and the United States [41]. Diclofenac is considerably stable under normal environmental conditions. The most probable degradation pathway for in-situ elimination is photodegradation which will be influenced by additional key parameters such as eutrophic conditions, degree of suspended particulate material, or the depth of the watercourse [41].

1.1 Non-Steroidal Anti-Inflammatory Drugs

NSAID including aspirin, naproxen, ibuprofen, diclofenac etc. are a class of drugs with analgesic, antipyretic and anti-inflammatory effects; they reduce pain, fever and inflammation [43, 44]. The term "non-steroidal" is used to distinguish those drugs from steroids, which have a similar eicosanoid-depressing, anti-inflammatory action. NSAID are unusual in that they are non-narcotic. The most prominent members of this group of drugs are aspirin and ibuprofen [45]. With the isolation of salicylic acid from the folk remedy willow bark in the ninteenth century, NSAID have become an important part of the pharmaceutical treatment of pain (at low doses) and inflammation (at higher doses). Part of the popularity of NSAID is that they do not produce sedation or respiratory depression and have a very low addiction rate. Certain NSAID, including ibuprofen and aspirin, have become accepted as relatively safe and are available over-the-counter without prescription [46, 47].

1.2 Classification of NSAID

NSAID can be broadly classified based on their chemical structure. NSAID within a group will tend to have similar characteristics and tolerability. There is little difference in clinical efficacy between the NSAID when used at equivalent doses. Some common examples of NSAID are given in Table 1.

1.3 Adverse Effects of NSAID

NSAID are usually applied for the treatment of acute or chronic conditions where pain and inflammation are present. In 2001, NSAID accounted for 70 million prescriptions and 30 billion over-the-counter doses sold annually in the United States. With the aging of the Baby Boomer generation and the associated rise in the incidents of osteoarthritis and other such conditions for which NSAID are indicated, the use of NSAID may further increase**.** The widespread use of NSAID meant that the adverse effects of these relatively safe drugs have become increasingly prevalent [48-50]. These effects are dose-dependent, and in some cases severe enough to pose risks to people who consume these drugs [51,52]. Due to these reasons, powerful analytical techniques must be developed to detect these drugs in the environment.

Table 1: Structures of some representative NSAID drugs

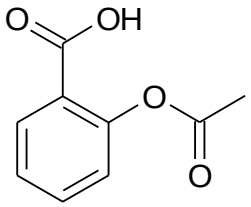
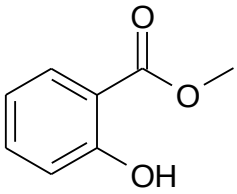
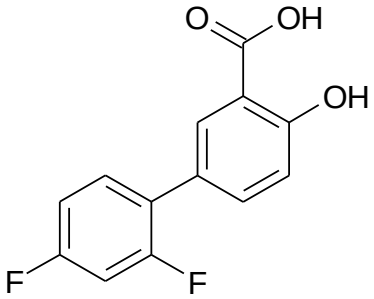
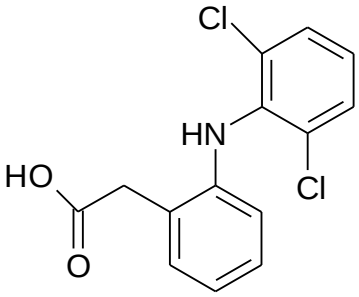
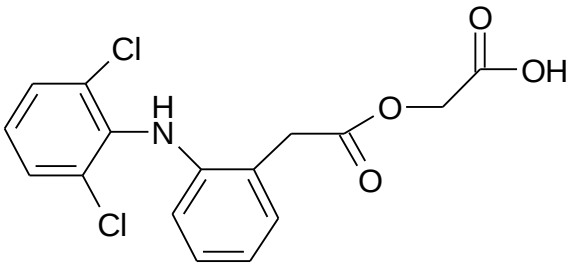
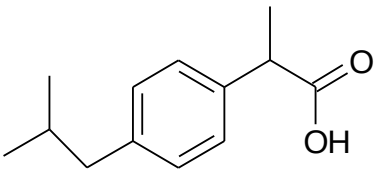
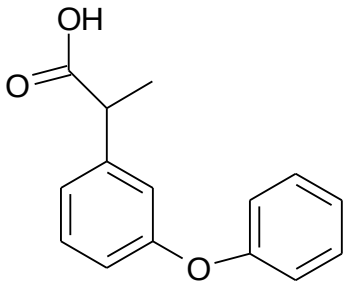
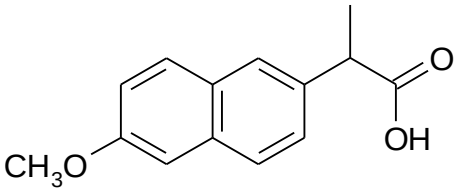
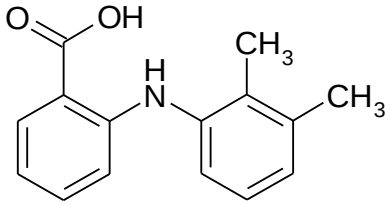
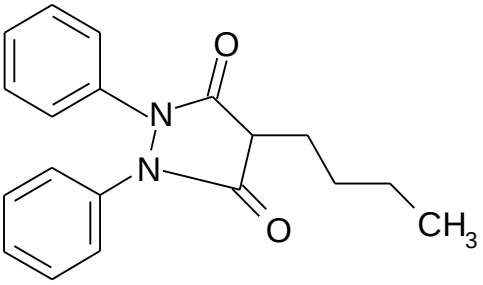
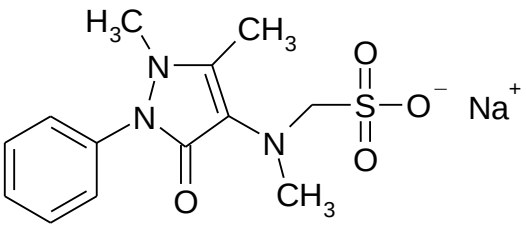
Compound class	Example	Structure
Salicylates	Aspirin	
	Methyl salicylate	
	Diflunisal	
Arylalkanoic acid	Diclofenac	
	Aceclofenac	

Table 1: **Continued**

Compound class	Example	Structure
2-Arylpropionic acids (profens)	Ibuprofen	
	Fenoprofen	
	Naproxen	
N-Arylanthranilic acids (fenamic acids)	Mefenamic acid	
Pyrazolidine derivatives	Phenylbutazone	
	Metamizole	

2 Trace Analysis of NSAID

2.1 Analytical Techniques

In nature, most NSAID are present at low concentrations (normally in the range of ng-µg L^{-1}). Thus, in order to monitor these drug residues in environment, powerful analytical techniques are needed for pharmaceutical trace analysis. Currently, gas chromatography coupled with mass spectrometry (GC/MS) and liquid chromatography coupled with mass spectrometry (LC-ESI/MS) has proven the presence of these compounds in groundwater and drinking water [53-56]. Both GC/MS and LC/MS detection have been applied to the analysis of diclofenac after preconcentration by solid-phase extraction from water samples [57-59]. Highest sensitivity was reported for GC/MS/MS with the minimum detection limit (MDL) of 0.3-4.5 ng L^{-1} and a limit of quantitation (LOQ) of 1, 5, and 50 ng L^{-1} using drinking water, surface water, and STP effluent sample, respectively. In addition, as a useful alternative capillary electrophoresis (CE/MS) was reported [60].

Immunochemical techniques can be another approach for analyzing pharmaceuticals by taking advantage of the highly selective binding by antibodies. A great number of immunoassays have been developed and used for analysis of pesticides which are found at similar levels in the aquatic ecosystem, and some tests have been applied for pharmaceutical compounds [61,62]. Available test kits for pharmaceuticals are optimized for biological specimens such as blood and urine. To our knowledge, a few groups have shown the feasibility of adapting clinical assays to the analysis of water [63-68]. In various river and drinking water samples and in wastewater, detection limits in the very low ng L^{-1} range have been achieved for hormones such as progesterone, norethisterone, ethinyl estradiol, 17 β-estradiol, testosterone, the anticancer drugs methotrexate and bleomycin. Similar results were obtained in a former study done in our laboratory on the preparation of antibodies against diclofenac. That study focused on the generation of anti-diclofenac antibodies and the development of an enzyme-linked immunosorbent assay (ELISA) for this pharmaceutical and its use for the analysis of tap water, surface water, and wastewater samples [69].

Nuclear magnetic resonance (NMR) is a spectroscopic technique [70]. In molecular imprinting, it is commonly applied to the determination of apparent dissociation constants and

the complexation-induced changes in the chemical shift of nuclei located at various points on the template and monomer molecules, thus it is a useful tool to indicate the interactions of the pre-polymerization complex [71-73]. Progressive change in the chemical shift of interacting groups is seen as the template and monomer are titrated together, rather than individual peaks for the bound and unbound forms of the template, indicating that exchange between these two extremes is fast on the NMR timescale. This echoes the point that the complexation is a dynamic process.

NMR analysis involves the absorption of electromagnetic radiation in the radiofrequency range. A sample placed in a static magnetic field is exposed to a second superimposed transverse oscillating magnetic field inducing transitions between energy levels at the respective resonance frequencies. The magnetic field experienced by a nucleus in a molecule is different from the external field with the exact resonance frequency being characteristic for the chemical environment of the nucleus (chemical shift δ). Chemically non-equivalent nuclei in a molecule are therefore differently shielded leading to separate signals in the NMR spectrum (only for nuclei with a spin quantum number $I \neq 0$). The applied magnetic fields are in the range of 1.4 to 14.1 T leading to proton resonance frequencies of 60-600 MHz. Intermolecular interactions will change the chemical environment of a nucleus, inducing changes in the chemical shift which can be monitored. NMR spectroscopy has become the main method used for structure elucidation, kinetics, and dynamics of supramolecular complexes in solution.

Complexation events such as hydrogen bond formation and π-π stacking may be observed by ^{1}H NMR titration experiments. This method of studying hydrogen bonding is a well studied field having been used in the past for applications such as studying bonding between nucleic acid bases and carboxylic acids [74] and hydrogen bonding properties of monosaccharides [75]. In NMR titration studies, measurements are performed at variable concentration of one component and at a fixed concentration of the other component. The experimental conditions are selected such that the degree of complexation is preferably high. The changes of NMR shifts of independent signals upon complex formation are used to monitor complex formation and calculate association constants.

The complex concentration was calculated as [76]:

$$[\text{Complex}] = [\text{template}]_{tot} \times (\delta_{obs} - \delta_{template})/(\delta_{complex} - \delta_{template}),$$

where $[\text{template}]_{tot}$ is the total template concentration, $\delta_{template}$ is the shift of the free template molecule, $\delta_{complex}$ is the chemical shift of the complex and δ_{obs} is the observed chemical shift. Association constants were determined differently, maintaining a constant amount of template and titrating increasing amounts of functional monomer. The association constants were then calculated using the model described by Atwood [77] to calculate association constants in weak complexes based on changes in observed NMR signal shifts, which in this case leads to:

$$\delta_{obs} = \delta_{template} + (\delta_{template} - \delta_{complex})/(2[\text{Monomer}]_0) \times \{[\text{Monomer}]_0 + [\text{Template}]_0 + 1/K - ([\text{Monomer}]_0 + [\text{Template}]_0 + 1/K)^2 - 4[\text{Monomer}]_0[\text{Template}]_0)^{1/2}\}.$$

Additional variables are: $[\text{Monomer}]_0$ is the total monomer concentration, $[\text{Template}]_0$ is the total template concentration, and K is the association constant. Computational methods allow the association constant and the complexation induced shift for the fully complexed compound from a fast ligand exchange to be obtained. For a slow exchange between substrate and the ligand, which is less frequent, the separate signals for the free and complexed species may be used to directly determine K and the stoichiometry of the complex.

2.2 Trace Enrichment by Solid Phase Extraction

It is known that most drugs exist in the environment at low concentrations. In order to detect drug residues in the environmental samples, useful separation techniques are needed for trace enrichment and purification prior to analysis. To determine diclofenac and other non-steroidal anti-inflammatory drugs in water samples, a pre-enrichment step is necessary. Presently, solid-phase extraction (SPE) is the most widely used procedure to extract traces of organic compounds from environmental samples [78-81]. In addition, SPE could be automated or even performed in-line by direct connection to the chromatographic systems.

SPE is an increasingly useful sample preparation technique. With SPE, many of the problems associated with liquid/liquid extraction can be prevented, such as incomplete phase separations, less-than-quantitative recoveries, use of expensive, breakable specialty glassware,

and disposal of large quantities of organic solvents [82-86]. SPE is more efficient than liquid/liquid extraction, yields quantitative extractions that are easy to perform, is rapid and can be automated. Solvent use and lab time are reduced. SPE is used most often to prepare the liquid samples and extract semi-volatile or non-volatile analytes, but can also be used with solids that are pre-extracted into solvents.

2.2.1 Types of SPE Packing Materials

SPE materials are good for sample extraction, concentration, and cleanup. They are available in a wide variety of chemistries, adsorbents, and sizes. Different types of solid phase extraction packing materials are shown in Table 2.

2.2.2 SPE Separation Process

(1) Selective extraction:
A selected SPE packing material will bind selected components of the sample, either the compounds of interest or the sample impurities. The selected components are retained when the sample passes through the SPE tube or disk (the effluent will contain the sample minus the adsorbed components). Then, either collect the adsorbed compounds of interest through elution, or discard the tube containing the extracted impurities (shown in Figure 1).

(2) Condition SPE tube packing:
To condition the SPE tube packing, it should be rinsed with up to one tube full of solvent before extracting the sample. Normally, a volume of 5-10 mL is used**.**

(3) Selective washing:
The compounds of interest and the impurities are retained on the SPE packing when the sample passes through; the impurities are rinsed through with wash solutions that are strong enough to remove them, but weak enough to leave the compounds of interest behind.

(4) Selective elution:
The adsorbed compounds of interest are eluted by a solvent that leaves the strongly retained impurities behind.

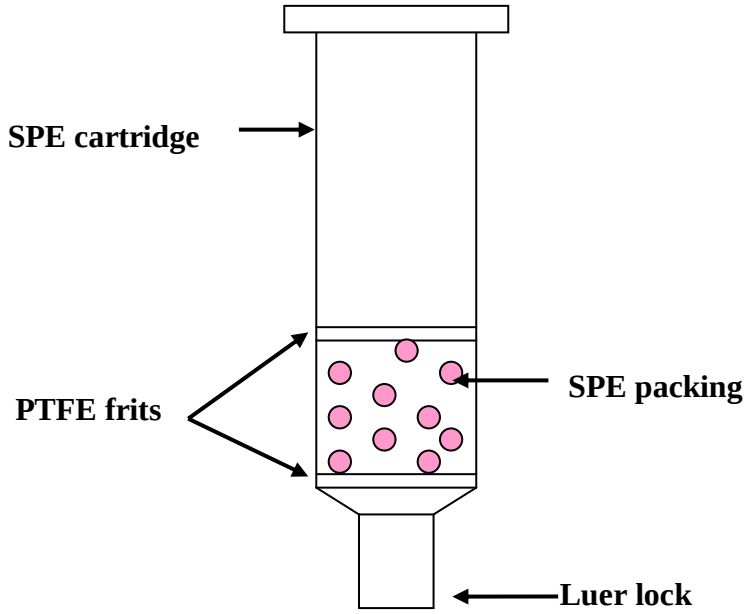


Figure 1: Diagram of a typical SPE tube and packing

2.2.3 Disadvantages of SPE Techniques

The SPE technique has been proved to be a very useful analytical method for trace sample enrichment and preconcentration in the environmental field. However, many types of organic compounds, mainly polar ones, are not completely extracted from predominantly aqueous solutions with typical SPE packing materials; moreover, silica-based materials have considerable pH liability. These drawbacks may be overcome by using chemically modified packing materials which have enhanced surface hydrophilicity and improved extraction efficiencies, due to the possibility of polar interaction between the polar analytes and the functional groups of these compounds. These chemically modified materials have greatly improved the sample extraction efficiency; however they are not selective for a specific analyte. Due to this limitation sorbent material with higher selectivity, such as molecularly imprinted polymers (MIP), are increasingly developed and applied for the selective extraction of target analytes from different environmental samples.

Table 2: Most commonly used SPE packing materials

Silica-based packing materials		
LC-18	octadecyl bound endcapped silica	For reverse phase extraction of non polar to moderately polar compounds, such as drugs antibiotics, dyes, caffeine, fat soluble vitamins
LC-8	octyl bound endcapped silica	For reverse phase extraction of non polar to moderately polar compounds, such as drugs antibiotics, dyes, caffeine, fat soluble vitamins
LC-4	butyldimethyl bound endcapped silica	Less hydrophobic than LC-8 or LC-18. For extraction of peptides and proteins
LC-Ph	phenyl bound silica	Slightly less retention than LC-18 or LC-8 materials, For reverse phase extraction of non polar to moderately polar compounds, especially aromatic compounds
LC-CN	cyanopropyl bound endcapped silica	For reverse phase extraction of moderately polar compounds, normal phase extraction of polar compounds, weak cation exchange for carbohydrates and cationic compounds
LC-Diol	diol bound silica	For normal phase extraction of polar compounds
LC-NH2	aminopropyl bound silica	For normal phase extraction of polar compounds, weak anion exchange for carbohydrates, weak anions and organic acids
LC-Si	silica gel with no bound phase	For extraction of polar compounds, such as alcohols, amines, drugs, dyes, herbicides, pesticides, ketones, organic acids, steroids
Alumina-based packing materials		
LC-alumina A	acidic pH ~5	For anion exchange and adsorption extraction of polar compounds such as vitamins
LC-alumina B	basic pH ~8.5	For adsorption extraction of polar compounds, cation exchange
LC-alumina N	neutral pH ~6.5	For adsorption extraction of polar compounds, with pH adjustment, cation or anion exchange. For extraction of vitamins, antibiotics, essential oils enzymes, glycosides and hormones
Magnesium silicate based packing materials		
LC-Florisil		For adsorption extraction of polar compounds, such as alcohols, amines, drugs, dyes, herbicides, pesticides, ketones, organic acids, steroids
ENVI-Florisil		For adsorption extraction of polar compounds, such as alcohols, amines, drugs, dyes, herbicides, pesticides, ketones, organic acids, steroids
Graphitized carbon-based packing materials-nonbound carbon phase		
ENVI-carb	nonporous	For adsorption extraction of polar and nonpolar compounds
ENVI-carb C	nonporous	For adsorption extraction of polar and nonpolar compounds

2.3 Molecularly Imprinted Polymers

A molecular imprinted polymer is a highly cross-linked polymer that was formed in the presence of a template molecule that is extracted afterwards, thus leaving complementary configuration to the template molecule in terms of size, shape, and functional groups [87-94]. These customized synthetic polymers are provided with highly specific recognition ability for target template molecules and they can be used to fabricate sensors, catalysis or for separation methods [95-99]. The functional mechanism of these molecularly imprinted polymers is similar to antibodies or enzymes [100-104].

2.3.1 Background

It is known from biochemistry that a living system controls its activity through enzymes. Most enzymes act specifically with only one substrate to produce products. The basic process where enzymes catalyze chemical reactions begins with the binding of the substrate to the active site on the enzyme. The active site is the specific region of the enzyme which combines with the substrate. The binding of the substrate to the enzyme causes changes in the distribution of electrons in the chemical bonds of the substrate and ultimately leads to the formation of products. The active site has an unique geometric shape that is complementary to the geometric shape of a substrate molecule, which means that enzymes specifically react with only one or a few similar compounds. The specific action of an enzyme with a single substrate can be explained by "lock and key" analogy. In this analogy, the lock is the enzyme and the key is the substrate. Only the correctly sized key (substrate) can fit into the key holes (active site) of the lock (enzyme), while incorrectly shaped or sized substrate molecules do not fit into the enzyme.

In 1930s, a theory for the diversity of antibody formation was developed when it encounters with antigen. The remarkable specificity of antibody-antigen complexes found in the chiral recognition of D- and L-tartranilic acid was attributed to the interactions of the chemical groupings of the nascent antibody with the antigen during antibody biosynthesis. Each structural unit of the antibody would be selected and oriented to fit the local configuration and features of the antigen surface.

These theories were further elaborated by Linus Pauling [105], who postulated that the great diversity in antibody formation was due to the formation of different three-dimensional configurations of the antibody polypeptide chain induced by the interaction with the antigens. Following these "instructive" theories on antibody diversity, the antibodies would be able to change their 3D structure in order to form as many interaction points as possible with the epitopes of the antigens. Thus, the antibody combining sites were "moulded" with the antigen as a template in a casting procedure, i.e. they were molecularly imprinted with the antigens.

Those models from Pauling's theory laid the foundation for the area of molecular imprinting. After two decades, F. H. Dickey who was inspired by the theory of Linus Pauling as to how antibodies were formed began to create affinity for dye molecules in silica gel [106, 107]. Dickey's silica gels can be considered to be the first imprinted materials.

2.3.2 The Molecular Imprinting Process

Molecular imprinting in organic polymers first appeared in the 1970s when covalent imprinting in vinyl polymers was reported by G. Wulff and his co-workers [108, 109]. Another type, non-covalent molecular imprinting was introduced a decade later by K. Mosbach and his co-workers [110, 111].

The common principles of molecular imprinting are summed-up in the scheme shown in Figure 2. One or more types of polymer-forming components (functional monomers) are arranged in a complementary configuration to the template molecules (Figure 3), either by the formation of covalent bonds, or by self-association. The matrix-forming material (cross-linker) and a porogenic solvent are also added and the whole mixture is cured to give a porous material containing nascent imprint sites of a complementary shape, size and functionality to the template molecules.

The chemical strategies employed in imprinting can be divided into covalent, non-covalent, metal-ion mediated imprinting, etc., in terms of the interactions used in the imprinting step.

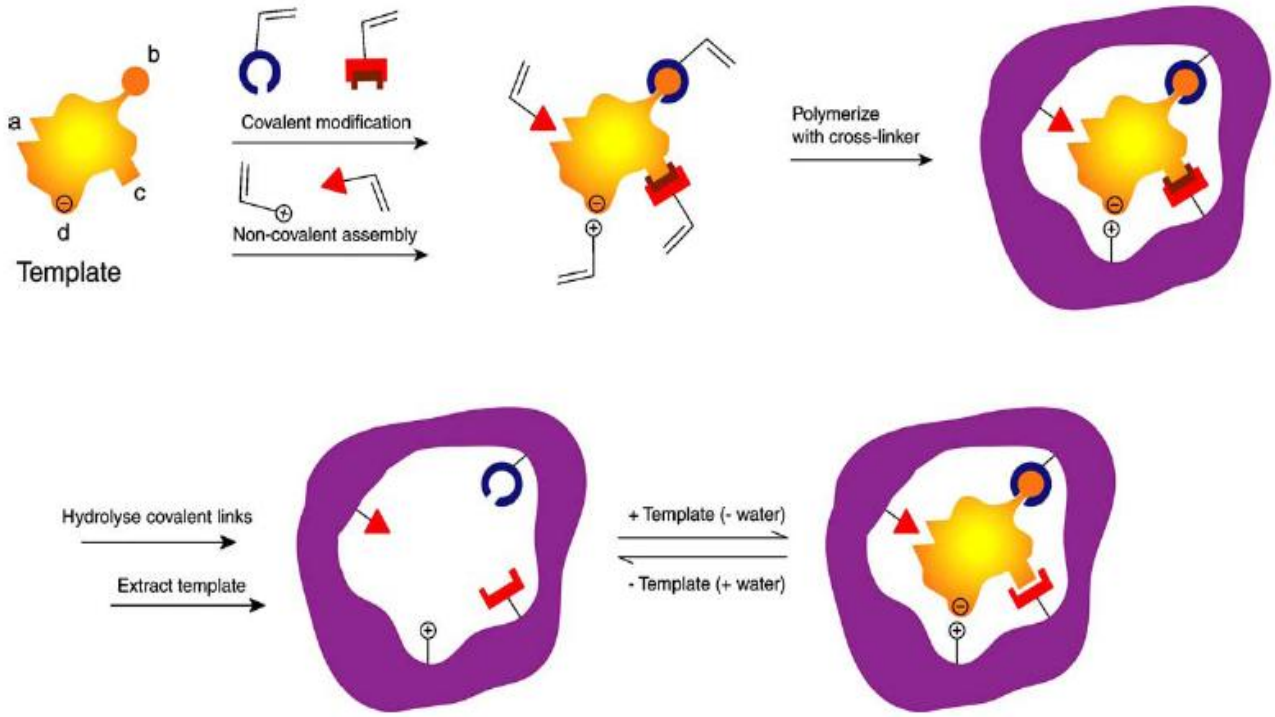


Figure 2: Schematic representation of the imprinting process showing some of the interactions used in creating affinity in the binding site for template. The interactions with functional motif groups (a-d) of the template represent the following imprinting strategies: (a) non-covalent hydrogen-bonding with methacrylic acid, acrylamide, etc.; (b) reversible covalent interaction such as a boronate ester; (c) semi-covalent (sacrificial spacer) method; (d) electrostatic interaction with an oppositely charged monomer. Rebinding/release of the template from the final polymer site involves a facile condensation/hydrolysis reaction at site (b). Template removal involves a chemical step such as hydrolysis, if covalent bonds are to be broken. If the rebinding step involves a condensation reaction (covalent imprinting), one or more molecules of water will be released on binding the template

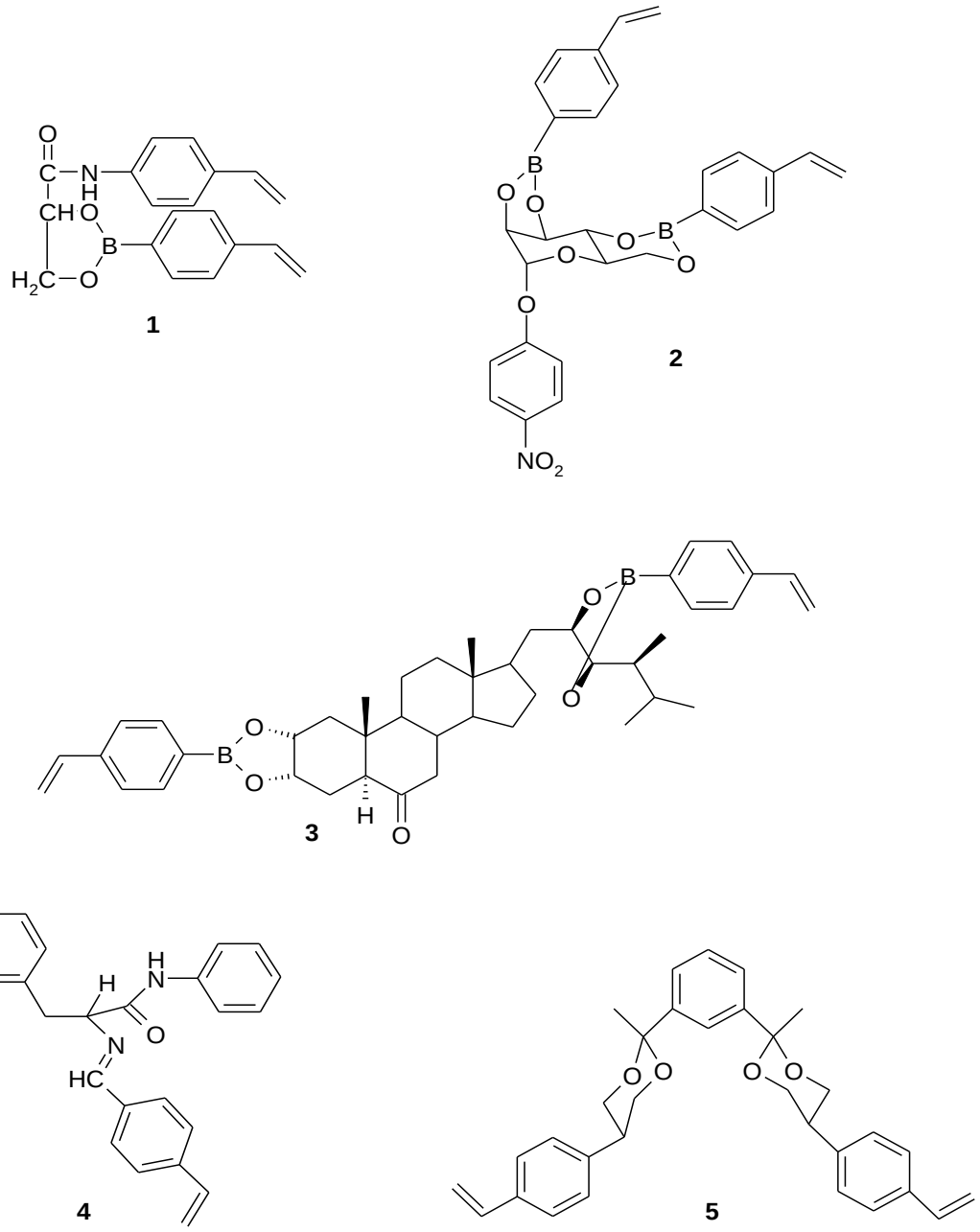


Figure 3: Template monomer structures used in reversible covalent imprinting template monomer derived from (1) glyceric acid [108]; **(2) 4-nitrophenyl-α-D mannopyranoside-2,3:4,6-di-*o* (4-vinylphenylboronate)** [109]; **(3) Bis-boronate ester of castasterone** [112]; **(4) Schiff's base of phenylalanine anilide with 4-vinylbenzaldehyde** [113]; **(5) Bis-ketal of 1,3 diacetylbenzene with a polymerizable diol** [114]

2.3.3 Covalent Molecularly Imprinting

Covalent imprinting refers to molecular imprinting strategies whereby the template and polymerizable units are attached by covalent bonds to form a template-monomer by a chemical reaction independent of polymer formation. Copolymerization of this template-monomer with a high proportion of cross-linker, in a porogenic solvent, results in a polymer which has template covalently bound within the polymer body. Removal of this template and subsequent rebinding step will both involve chemical reactions and the rebound template will be indistinguishable from template immediately following polymerization.

2.3.3.1 Imprinting with reversible covalent bonds

The classical methods of covalent imprinting involve readily reversible condensation reactions to prepare template-monomers. All of these assemblies require mild aqueous conditions to hydrolyze template from the polymer. The relatively specific structural requirements of covalent methods places rather narrow limitations on which templates can be imprinted in this way, namely, 1,2- and 1,3-diols, aldehyde, ketone and amine. That the rebound state is identical to the base prepared polymer is seen as a distinct advantage of these methods, in which all binding sites should more or less resemble one another. In addition, as there is no excess of functional monomer, nonspecific binding may be greatly reduced. The requirement for synthesis of the template-monomer is the biggest drawback of any covalent imprinting technique. A template-monomer of this type may be very sensitive to the presence of water, thus preventing the use of emulsion and suspension polymerization methods.

2.3.3.2 Imprinting with strong covalent bonds

Early work carried out by Damen and Neckers [115-117] and Shea and Thompson [118, 119] employed template strategies in which strong covalent bonds, notably ester bonds, were used to assemble the polymerizable template monomer species, followed by incorporation into divinylbenzene based matrices. The templates were removed by treatment with a suitable reagent or by hydrolysis. Rebinding to the polymers involved reaction of an acyl chloride with the alcohol component or displacement of bromide by a salt of the carboxylic acid. While

these were valuable experiments, rebinding through carboxylic ester formation is not practical for the vast majority of applications for which MIP are currently used.

2.3.4 Non-covalent Molecular Imprinting

Non-covalent molecular imprinting is the simplest method of producing selective polymers by using non-covalent interactions. All the synthesized imprinted polymers in this Ph.D project were based on this strategy for molecularly imprinted polymerization.

Non-covalent imprinting uses the typical forces of attraction between molecules such as hydrogen bonds, ion-pairs, dipole-dipole interactions and van der Waals forces to generate adducts of template and functional monomers in solution. Unlike those used in covalent imprinting, these adducts are unstable and dynamically rearrange on a time scale relevant to the imprinting process. One of the key developments in this area of imprinting is the emergence of methods aimed at generating much more stable adducts that will lead to a greater yield of more uniform receptor sites. The use of non-covalent interactions can be traced back to the earliest reports of imprinting in silica matrices, but it has been popularized by the work of Mosbach's group [110], which showed that this approach was a viable method for producing receptors in imprinted polymers. Today, it is the predominant method used because it offers much more flexibility in terms of the functionalities on a template that can be targeted. It also requires much less chemistry synthesis than the pre-synthesis of covalent adducts.

2.3.4.1 Imprinting with a single functional monomer

This is the simplest approach to non-covalent imprinting, and it is by far the most widespread in this field. Even in this situation, however, the nature of the pre-polymerisation complexes is far from simple and a number of interactions need to be taken into account. In addition to the desirable template-functional monomer interactions, there may also be interactions of either the template or the functional monomer with the cross-linker. The former may be important in defining or refining functional receptor sites, whereas the latter is probably undesirable, since it shifts the equilibrium away from the desired template monomer interactions (cross-linker is usually present at higher concentration than both the template and functional monomer).

Additional complications can arise from self-association of functional monomer. This is a particular problem with carboxylic acids, which have a strong tendency to dimerize.

During the past 20 years, many different functional monomers (shown in Figure 4) have been tested in non-covalent imprinting. Some have found wide utility, while others have been used only in one or a few specialized situations. Despite the apparent drawback of strong dimerization under the conditions in non-covalent imprinting, carboxylic acid-based monomers, principally methacrylic acid have been by far the most successful. This probably stems from the fact that they have relatively few bonds with rotational degrees of freedom and their ability to interact in various ways with the template: as hydrogen bond donors, hydrogen bond acceptors and ion-pair formation, as well as weaker dipole-dipole interactions. Methacrylic acid also benefits from the bulk of the methyl group, which probably restricts rotation and conformational flexibility and also provides additional van der Waals interactions to help defining the general shape-selective elements of the receptor sites.

In a few cases trifluoromethyl acrylic acid has proved superior, probably due to its enhanced acidity [120-122]. Vinylbenzoic acid might be considered an attractive option due to the bulk and π-electron system of the aromatic ring. However, in practice, it has generally provided disappointing results. This may be largely due to its relatively poor reactivity ratios with typical methacrylate cross-linkers. This emphasizes that many factors relating to dynamics during polymerization itself, and not only the equilibrium in a pre-polymerization mixture, need to be taken into consideration. There is by now a considerable use of more acidic monomers, such as sulphonates, phosphonates and phosphates. These are particularly used to form ion pairs when carrying out imprinting under aqueous conditions. In principle, they should be highly effective, but their performance is probably compromised by the large number of bonds with relatively unrestricted rotational degrees of freedom in the structures of the commercially available members of this group. Phosphonates and phosphates have also found utility in imprinting based on metal ion complexation.

From the basic monomers, the vinyl pyridines have been the most widely applied. In their basic form, these represent electron rich π-electron ring systems, which allow them to interact strongly with electron deficient aromatic rings, as well as through acid-base interactions and hydrogen bond acceptance or donation if in the conjugate acid form. These monomers often interact strongly with templates, which suggest that they should be highly successful in

imprinting. They have proved useful in many cases, but their use can also have significant drawbacks. A particular problem is the very strong π-π interactions that occur during template rebinding in aqueous conditions. At first sight, this might be expected to be beneficial, but in practice it generally leads to extremely high levels of nonspecific binding of analytes to the polymer, so much that imprinted and reference polymers often show identical abilities in that they both effectively bind near 100% of the template. This situation arises when using vinylpyridine imprinted polymer. The polymers are highly effective adsorbents for SPE, but imprinting has no beneficial effect in terms of affinity or selectivity over the reference polymer. Normally, vinyl imidazole should be an interesting monomer, but it has been little used, due to its lack of commercial availability and the relative difficulty of preparing it in the laboratory.

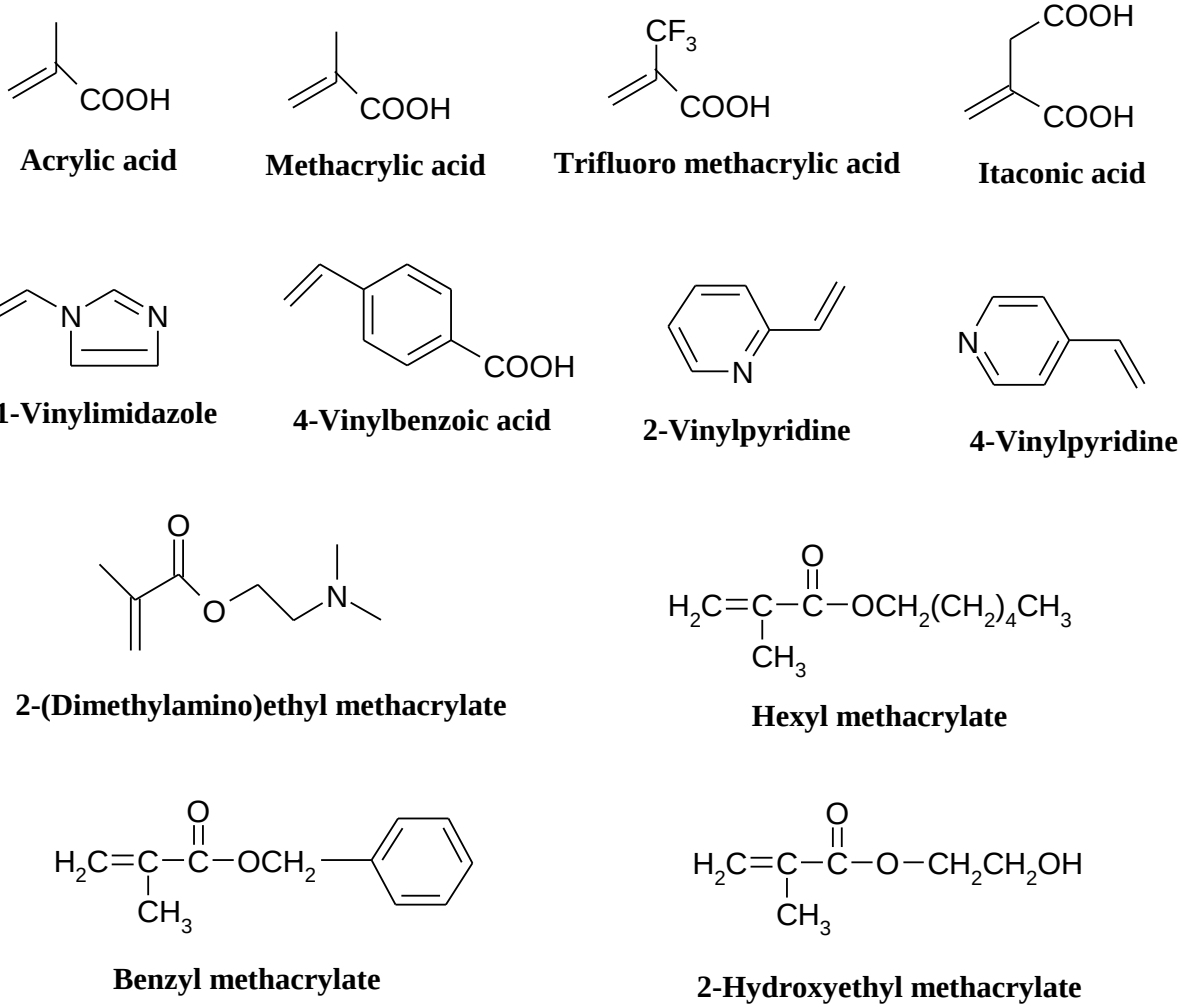


Figure 4: Selection of functional monomers for non-covalent imprinting

2.3.4.2 Imprinting with combination of monomers

It seems highly attractive to combine the specific interaction potential of a variety of different monomers; however, in reality it is far from simple. In practice, almost all imprinting methods use a mixture of monomers, since the cross-linker is an essential part of the recipe. Although the cross-linker often contains no strongly interacting functional groups, it will surely also interact with both the template and monomers, since it is generally present in significant molar excess. Interesting work by Spivak has shown that if the cross-linker is appropriately functionalised, no further functional monomers are required to achieve effective imprinting [123, 124]. In order to be successful, the formed adducts between the template and the functional monomers need to be stronger than any interactions between the functional monomers.

Computational imprinting has predicted combination imprinting recipes that have worked well in practice, though the analysis may not have been extensive enough to show that the monomer mixture performed better than any optimized single monomer formulation. There has been some work done to demonstrate molecular imprinting with mixtures of functional monomers. Early work by Ramström et al. used mixtures of methacrylic acid (MAA) and 2-vinylpyridine (2-VP) in the imprinting of amino acid derivatives [125]. They showed that when the carboxyl terminus was free, better imprinting occurred with the mixture of MAA and 2-VP than with either monomer alone. This may be due to a strong interaction between the 2-VP and the carboxyl group enhancing the affinity of the receptor since MAA and 2-VP have similar pKa. However, the two monomers may also interact strongly with one another.

Mixtures of acrylamide and 2-VP have been used in a series of studies by Meng et al. [126]. This cocktail has been shown to give good results with a variety of templates, and has the benefit that the monomers are likely to interact less strongly than in the above case. This gives them more opportunity to interact with the template, leading to functional receptor sites.

2.3.4.3 The nature of pre-polymerization complex

It is generally assumed that in non-covalent imprinting a pre-polymerization complex is formed between the template and functional monomers, which is then incorporated into the

growing polymer network. While this provides a good static model from which predictions and optimization strategies can be developed, it is by no means certain that this is how it really works dynamically as the process of polymerization proceeds. That adducts exist in the prepolymerization mixture is indisputable, but this is an equilibrium average state of a highly dynamic system. It seems likely that during polymerization, small sections of polymer structure will develop carrying multiple functional groups that will have their local environment and configuration influenced by a template. This will produce a cooperative effect since the multiple interactions will display a much higher avidity than single monomer-template interactions. As polymerization proceeds, these structures will further develop by changing shape and by the addition of more functional monomers, leading to a higher avidity that traps the template in the receptor site until it matures to completion. When considering factors pertinent to the design of non-covalently imprinted polymers, any factor that strengthens the interaction between a single functional monomer and a template will also enhance the avidity effect in the dynamic maturation model.

The potential for multiple sites of interaction between functional monomers and template in the pre-polymerisation complexes demonstrates the complexity of the system (Table 3). Factors such as the polarity and hydrogen bonding strength of the solvent and the polymerization temperature will affect interactions more strongly than others. In general, hydrogen bonding interactions are favoured by low polarity solvents and lower temperatures, whereas ion pair and other strong dipolar interactions are favoured by polar solvents. In practice, a compromise between solvent polarity and solubility of the template usually needs to be made. Since the solvent also determines the structure and porosity of the imprinted resin, this also places some constraints on the system. Thus the choice of solvent is limited to a short list comprising of chloroform, dichloromethane, acetonitrile and toluene for predominantly hydrogen-bound complexes. Some information about the strength and stoichiometry of monomer-template interactions can be obtained by spectroscopic means including UV-Vis spectrometry and NMR [127-129].

Table 3: Non-covalent interactions used in the imprinting process

Polymerizable binding groups	Binding sites to the matrix	References
Electrostatic interaction		
methacrylic acid	amine	[130, 131]
acrylic acid	amidine	[132]
itaconic acid	amine	[133]
sulfonic acid	amine	[134]
2-vinylpyridine, 4-vinylpyridine	acid	[135]
	in combination with covalent interactions	[136, 137]
	in combination with carboxylic acid binding groups	[138]
amine	phosphate (ATP)	[139]
Hydrogen bond formation		
methacrylic acid	amide	[140]
	amide in combination with electrostatic interaction	[141-143]
acrylic acid	purine base	[144]
ethylene dimethacrylate	nucleoside	[145]
Other interactions		
Charge transfer interactions	often in combination with covalent interactions	[146]
Hydrophobic interactions	often in combination with covalent interactions	[147]

The ratio of functional monomer to template is clearly an important parameter in the polymerization mixture and the optimum value rarely corresponds to the complex stoichiometry predicted by spectroscopic means. Too much functional monomer may contribute to high non-specific binding while too little will result in a poor yield of imprinted sites due to inadequate complexation of the template. Ratios are determined in practice by a combination of experience and trial or by combinatorial methods. Mayes and Lowe [148] have shown, however, that high affinity sites are still created in a morphine imprinted polymer prepared with methacrylic acid to template ratios of 50:1, 150:1 and even 500:1 as well as the normal ratio of 4:1. The lower yield of imprinted site was not a limitation for the highly sensitive radio-ligand binding assay conditions used and the high concentration of

functional monomer relative to template ensured that the entire template was fully complexed in the pre-polymerisation mixture. Control of the polymerisation temperature is seen as important in helping to stabilise the interactions between template and monomer. Consequently, initiators with low decomposition temperatures and photochemical initiation at low temperature have been reported. It should be noted, however, that free radical polymerisation is an exothermic process and a typical monolithic imprinted polymer forms a gel-like material very early in the polymerisation process.

2.3.4.4 Advantages and disadvantages

The most obvious strength of the approach is the relative simplicity of the process itself. Little or no synthetic chemistry is needed. It is generally accepted that stronger interactions between functional monomer and the template at this stage lead to better receptor sites in the final polymer. The other great strength of the approach is the range of chemical functionalities that can be targeted. Using covalent imprinting, only a few different types of functional groups can be targeted. With non-covalent approaches, almost any functionality can be addressed. Some, such as ion-pairs or hydrogen-bonding groups, are rather easy to target. Others, such as aromatic rings, may need more subtle approaches. Since such a wide range of monomers is available, suitable interactions can be arranged with any functional group. Other complicating factors, such as unwanted interactions with cross-linkers or self-association between functional monomers need to be considered and special care is needed as systems increase in complexity.

Three significant drawbacks are also associated with the non-covalent approach. The first is the heterogeneity of the receptor sites produced. Since a variety of solution adducts of functional monomer and template are present in the pre-polymerization mixture, often a heterogeneous population of receptor sites results. These ranges from the very high affinity (dissociation constant K_d in the nM range) to very low affinity (K_d is mM range) and this system is often compared with that of polyclonal antibodies. For certain applications, this range of receptor affinity might be beneficial since it can increase the dynamic range of some assay systems, but in general it is a disadvantage, which makes the materials more difficult to work with.

The second problem is the fact that a lot of functional monomer is spread around the polymer network, outside receptor cavities. This leads to the very non-specific single point interaction between analyte molecules and the polymer, which is undesirable for most practical applications e.g. it increases background signals in sensors and reduces the purity of extracts in SPE. However, it is also possible that this non-specifically anchored functional monomer increases the hydrophilicity of the polymer surface. As a result, it may increase non-specific interactions through e.g. hydrogen bonding, and at the same time, it may reduce non-specific binding due to van der Waals interactions. The balance of these effects will depend entirely on the specific nature of the polymer and the matrix in which it is being applied.

The third problem in non-covalent imprinting is the very low yield of functional high-affinity receptor sites relative to the amount of template present in the pre-polymerization mixture. It could be that template-monomer adducts present prior to polymerization are destroyed during the violent and dynamic process of radical based network polymerization, due to conformational or thermal effects. It could be attributed to non-real chain cross-linking, giving inadequate rigidity to maintain receptor conformational integrity. It also could be that most receptor sites are located in inaccessible regions of the polymer, while this seems less likely since the vast majority of template can be extracted, suggesting that these sites should be accessible for rebinding. Thus, the large amount of wasted template in non-covalent imprinting remains a problem, and contributes to issues such a template leaching.

All these fundamental problems have driven research towards specifically designed monomers and stoichiometric imprinting, in an attempt to generate a higher yield of more homogeneous binding sites, more similar to that produced by covalent imprinting while also maintaining the fundamental advantages of the easy reversibility of the binding interactions and the range of templates that can be used.

2.3.5 Template Molecules in Molecular Imprinting

A wide variety of template molecules have been imprinted in various imprinting protocols [149-154]. A selection of different compounds is displayed in Table 4. Template analytes such as drugs, herbicide, toxins, hormones, amino acids, carbohydrates, co-enzymes, nucleotide bases and proteins have been successfully used for the preparation of selective recognition materials. Of the imprinting strategies used, it has become evident that non-

covalent interactions between the template molecule and the functional monomers for imprinting are more versatile. The apparent weakness of these interaction types, when considered individually, may be overcome by allowing a multitude of interaction points simultaneously. Together with the fast association and dissociation kinetics of these bonds, many possible combinations can be tested before the correct partners associate to each other; furthermore, the use of non-covalent interactions in the imprinting step closely resembles the recognition pattern observed in nature.

Table 4: List of a few of compounds used as template molecules in imprinting

Compound Class	Example	Compound Class	Example
Drugs	Theophylline	**Amino acids**	Phenylalanine
	Diazepam		Tryptophan
	Morphine		Tyrosine
	Ibuprofen		Aspartic acid
	Chloramphenicol		
	Naproxen	**Carbohydrates**	Galactose
			Glucose
Herbicides	Atrazine		Fucose
	Metsulfuron-methyl		
	2,4-dichlorophenoxyacetic acid	**Co-enzymes**	Pyridoxal
Mycotoxins	Ochratoxin A	**Nucleotide bases**	Adenine
Hormones	Cortisol	**Proteins**	RNase A
	Enkephalin		Urease

2.3.6 Cross-linkers in Polymerization

Since a very high degree of cross-linking (70-90%) is required for achieving specificity, only a limited number of cross-linkers have been utilized in the molecular imprinting (Figure 5). The solubility of the cross-linker itself in the pre-polymerization solution and the solubility of the monomerized template species reduce the number of possible alternatives. Nevertheless,

several different cross-linkers have been tried with different degrees of success [155, 156]. Originally, isomers of divinylbenzene were used for cross-linking of styrene and other functional monomers into polystyrenes. Later, it was found that acrylic or methacrylic acid based systems could be prepared with much higher specificity. Ethylene glycol dimethacrylate (EGDMA) and trimethylolpropane trimethacrylate (TRIM) are presently commonly employed in several systems [157]. In recent years, several other cross-linkers have been studied. Thus, tri- and tetrafunctional acrylate cross-linkers, such as pentaerythritol triacrylate (PETRA) and pentaerythritol tetraacrylate (PETEA) have been used for the preparation of peptide-selective molecularly imprinted polymers [158, 159].

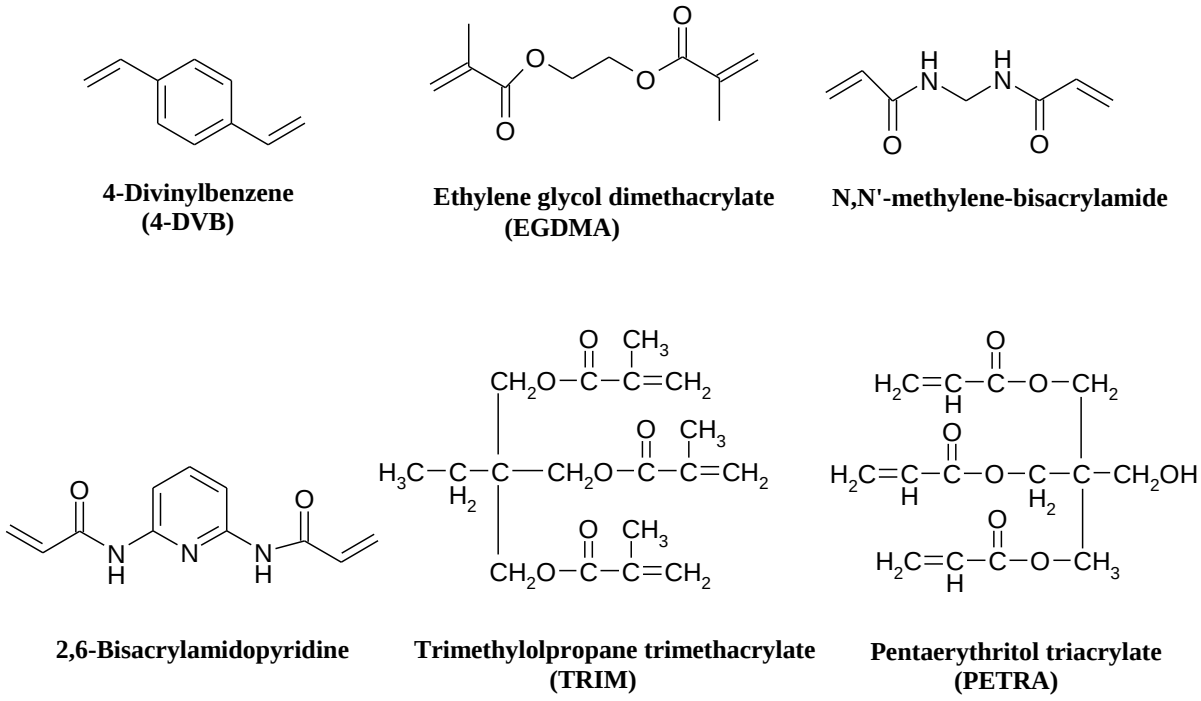


Figure 5: Selection of functional cross-linkers for non-covalent imprinting

2.3.7 Choice of Porogens

The solvent (porogen) plays an important role in the outcome of a molecular imprinting process, a role which is particularly pronounced in self-assembly systems [160, 161]. To be a porogen in the imprinted polymerization, the solvent governs the strength of non-covalent interactions in addition to its influence on the polymer morphology. Generally, the more polar the porogen, the weaker the resulting recognition effect becomes, as a consequence of the

influence of the solvent polarity on non-covalent interactions [162-164]. The best imprinting porogens, for accentuating the binding strengths, are solvents of very low dielectric constant, such as toluene and dichloromethane. The use of more polar solvents will inevitably weaken the interaction forces formed between the print species and the functional monomers, resulting in poorer recognition. On the other hand, the influence of porogen on the structure of the prepared polymers may compensate for this apparent drawback on the specific surface area and the mean pore diameter of the polymer structure is dramatically dependent on the type of porogen used. Thus, acetonitrile (ACN) as a fairly polar solvent leads to more macroporous polymers than chloroform (Table 5). A lower surface area and a lower macroporosity may lead to diminished recognition, because of lower accessibility to the sites.

Table 5: Polar and non-polar solvents as porogens in molecular imprinting

	Name	Bp (°C)	Dielectric constant
High polar solvent	Water	100	80
	N,N-dimethylformamide	153	38.3
	Methanol	68	33
	Acetonitrile	81	36.6
Low polar solvent	Dichloromethane	39.8-40	9.1
	Tetrahydrofuran	66	7.52
	Chloroform	60.5-61.5	4.8
	Hexane	69	2.02
	Toluene	111	2.4

In the recognition step, similar questions about the choice of solvent arise. Since all non-covalent forces are influenced by the properties of the solvent, non-polar solvents normally lead to the best recognition. When applying the polymers to gradually more polar solvents, the recognition is diminished. Also, the morphology is affected since the swelling of the polymers is dependent on the surrounding medium. Thus, swelling is most pronounced in chlorinated solvents, such as chloroform and dichloromethane (DCM), as compared to, e.g. ACN and tetrahydrofuran (THF). This swelling behavior may lead to changes in the three-

dimensional configuration of the functional groups taking part in the recognition in the polymer binding sites resulting in poorer binding capability. As a rule of thumb, the best choice of recognition solvent should be more or less identical to the imprinting porogen in order to avoid any swelling problems, although this is not necessarily a prerequisite. The polymer swelling taking place when polymers are prepared in organic porogens and subsequently used in aqueous phase, is not necessarily a gross obstacle, however. The swelling in water is approximately the same as for many other solvents, such as acetonitrile

In this work, in order to compare the effect of porogen solvent on the binding performance of the imprinted polymers, different solvents such as methanol, ACN, DCM, chloroform and toluene were tried as porogens in the imprinted polymerization process.

2.4 Using NSAID as the Template Molecule

Molecularly imprinted polymers synthesized using non-steroidal anti-inflammatory drugs as the template molecule were normally applied to the following two aspects: (1) used as the stationary phase in LC for enantiomeric separations; (2) used as the selective sorbent in the SPE procedure [165-167].

According to our knowledge, MIP has been applied to extract different NSAID, mainly naproxen and ibuprofen, by several research groups. Haginaka et al. synthesized uniform-sized MIP for ibuprofen, naproxen and ketoprofen, with multistep swelling and thermal polymerization method [168, 169]. Later, corresponding polymers were selectively modified with a hydrophilic external layer to obtain restricted access media (RAM)-MIP and used in an on-line SPE system coupled to the HPLC [170]. Using similar technique, Suedee et al. [171] prepared MIP using S-ibuprofen and S-ketoprofen as template molecules to examine enantioselective release for racemic drugs. Caro et al. [172, 173] extracted naproxen from urine samples with molecularly imprinted solid phase extraction (MISPE) materials and later synthesized imprinted polymers using ibuprofen as the template molecule. The ibuprofen was extracted from a mixture of NSAID including naproxen, fenoprofen, and diclofenac from river water and wastewater samples. The combination of an experimental design, multivariate analysis method and a high-throughput technique was developed in the screening and evaluation of MIP for piroxicam [174]. Recently, Farrington and Regan reported the rational design, generation and testing of a MIP for ibuprofen [175]. These MIP were capable of

recognizing ibuprofen in aqueous samples with acceptable selectivity over the structurally related analogous naproxen and ketoprofen. To investigate the chemical and physical nature of the recognition, NMR studies were carried out into the nature of the pre-polymerization complex formation. Similarly, O'Mahoney et al. [176] performed theoretical, NMR spectroscopic and X-ray crystallographic studies of MIP using naproxen as a model template. Unlike the papers mentioned above, in which 4-vinylpyridine or 2-vinylpyridine was used as functional monomers, the imprinting of ibuprofen using methacrylic acid as the functional monomer was described by Hung et al. [177]. The MIP was succesfully applied as HPLC stationary phase for the separation and analysis of ibuprofen and ketoprofen.

To date, Tunón-Blanco and coworkers have reported the use of diclofenac as template molecule as well as methacrylates as functional monomer and cross-linker for the development of voltammetric sensors [178-180]. For example, the pre-polymerization mixture was deposited onto the surface of a glassy carbon electrode and a polymer film was obtained after spin coating and in situ thermal polymerization. Voltammetric sensing relied on the selective extraction of the template into the MIP-film followed by release of this compound from the recognition sites and detection at the electrode surface. As a main limitation, the fouling of the electrode surface by oxidation products of diclofenac was reported. Recently, this group published the preparation of diclofenac MIP grown inside the pores of preformed chromatographic-grade silica beads through thermal polymerization [180]. After removal of the composite material, the particles were used for MISPE in human urine and the extracts were analyzed by differential pulse voltammetry. However, this detection method did not show a very low detection limit, i.e., diclofenac could only be quantified at mg L^{-1} concentration level.

The aim of this study was to synthesize non-covalently imprinted polymers using diclofenac ($C_{14}H_{10}C_{l2}NO_2H$) as the template molecule. This polymer was applied the first time in the form of MISPE to selectively extract diclofenac from complex surface water and wastewater samples.

II Results and Discussion

3 Molecularly Imprinted Polymerization using Diclofenac as the Template Molecule

In nature diclofenac (2-[(2, 6-dichlorophenyl) amino] benzene acetic acid) exists in an anion form, generally. Commercially, diclofenac is offered as sodium salt. Thus, prior to the molecularly imprinted polymerization using diclofenac acid as the template molecule, diclofenac can be obtained by conversion and extraction from the acidic solution of diclofenac sodium salt ($C_{14}H_{10}C_{l2}NO_2Na$) (shown in Figure 6). The diclofenac was kept in a brown-colored glass bottle and stored at low temperature before being used as the template molecule in molecular imprinting.

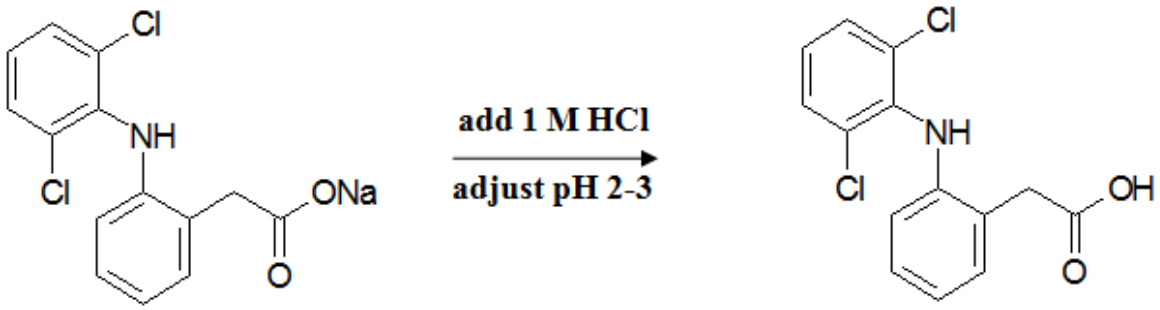


Figure 6: Procedure for the converting of diclofenac sodium salt into the diclofenac acid.

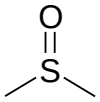
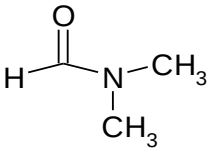
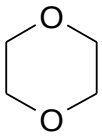
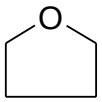
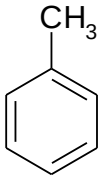
3.1 Physical and Chemical Properties of Diclofenac

3.1.1 Solubility of Diclofenac in Organic Solvents

In MIP synthesis usually a homogeneous liquid system is applied. All the components should be fully soluble and miscible to each other. This is a prerequisite for molecular imprinting. Because it is used as the template molecule, the solubility of diclofenac acid in commonly

used solvents had to be checked out before carrying out any molecular imprinting experiments. Data are summarized in Table 6.

Table 6: Solubility of diclofenac in various organic solvents

Solvent	Structure	Solubility
Dimethyl sulfoxide (DMSO)		Excellent
Dimethylformamide (DMF)		Excellent
Dioxane		Excellent
Acetonitrile (ACN)	$CH_3C{\equiv}N$	Good
Methanol	CH_3OH	Good
Tetrahydrofuran		Good
Dichloromethane (DCM)	CH_2Cl_2	Partial
Chloroform	$CHCl_3$	Partial
Hexane	$CH_3(CH_2)_4CH_3$	Insoluble
Toluene		Insoluble

3.1.2 UV-Vis Spectroscopic Properties of Diclofenac

UV-Vis spectroscopy was performed on diclofenac standard solutions, which were prepared in methanol, acetonitrile, octanol and ethyl acetate, respectively. From the UV-Vis spectra in Figure 7, it can be seen that the maximum UV absorption wavelengths are in the range of 275-285 nm. In the weak hydrogen bond acceptor-ethyl acetate, the maximum UV absorption wavelength of diclofenac is ~275 nm; in acetonitrile, the maximum UV absorption wavelength is ~278 nm; in the weak hydrogen bond donor-octanol, the maximum UV absorption wavelength is ~270 nm and in the strong hydrogen bond donor-methanol, the wavelength shifts to ~285 nm. These UV spectra helped to determine the absorption wavelength of diclofenac prepared in different kinds of organic solvents.

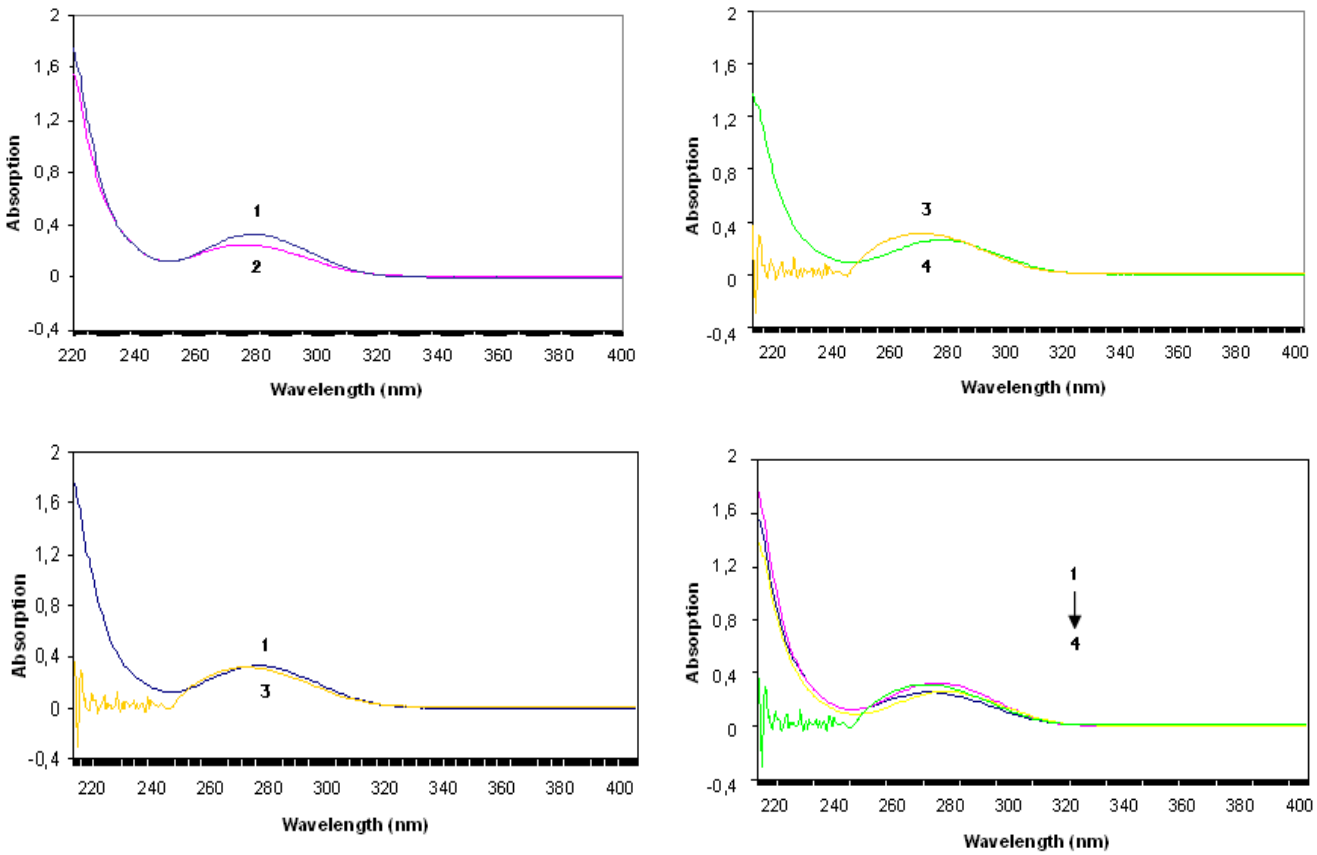


Figure 7: UV-Vis spectra of diclofenac. Diclofenac was prepared in different organic solvents at a concentration of 45 mg L^{-1}: (1) ethyl acetate, (2) acetonitrile, (3) octanol, and (4) methanol.

3.1.3 Chemical Functions of Diclofenac

In molecular imprinting, the non-covalent imprinting normally uses the typical forces of attraction between molecules such as hydrogen bonds, ion-pairs, dipole–dipole interactions and van der Waals forces to generate adducts of template molecule and functional monomers in monomer solution. Selected as the template molecule in molecular imprinting, diclofenac has several functional groups which could form different intermolecular interactions between the functional monomer and the template, i.e. the carboxyl group -COOH and the amine group -NH in diclofenac may interact with basic monomers such as 2-vinylpyridine (2-VP) /4-vinylpyridine (4-VP) or interact with acidic monomers i.e. methacrylic acid (MAA) to form different non-covalent bonds.

Four possible interactions could be formed between diclofenac and the functional monomer as follows:

1) Ion-pair forming between the carboxyl group -COOH of diclofenac and nitrogen atom in the pyridine ring of 2-VP/4-VP.

2) Hydrogen bonding between amine -NH group of diclofenac and nitrogen atom in the pyridine ring of 2-VP/4-VP.

3) Hydrogen bond could also be formed between the amine group -NH of diclofenac template and the carboxyl group -COOH of the functional monomer MAA.

4) In addition, π-π stacking between the aromatic rings of diclofenac and that of the vinylpyridine could also be formed [76, 181, 182]. This π-π stacking can further enhance the interaction between the template molecule and the functional monomers.

These four interactions between diclofenac and the functional monomer could be seen in Figure 8-10:

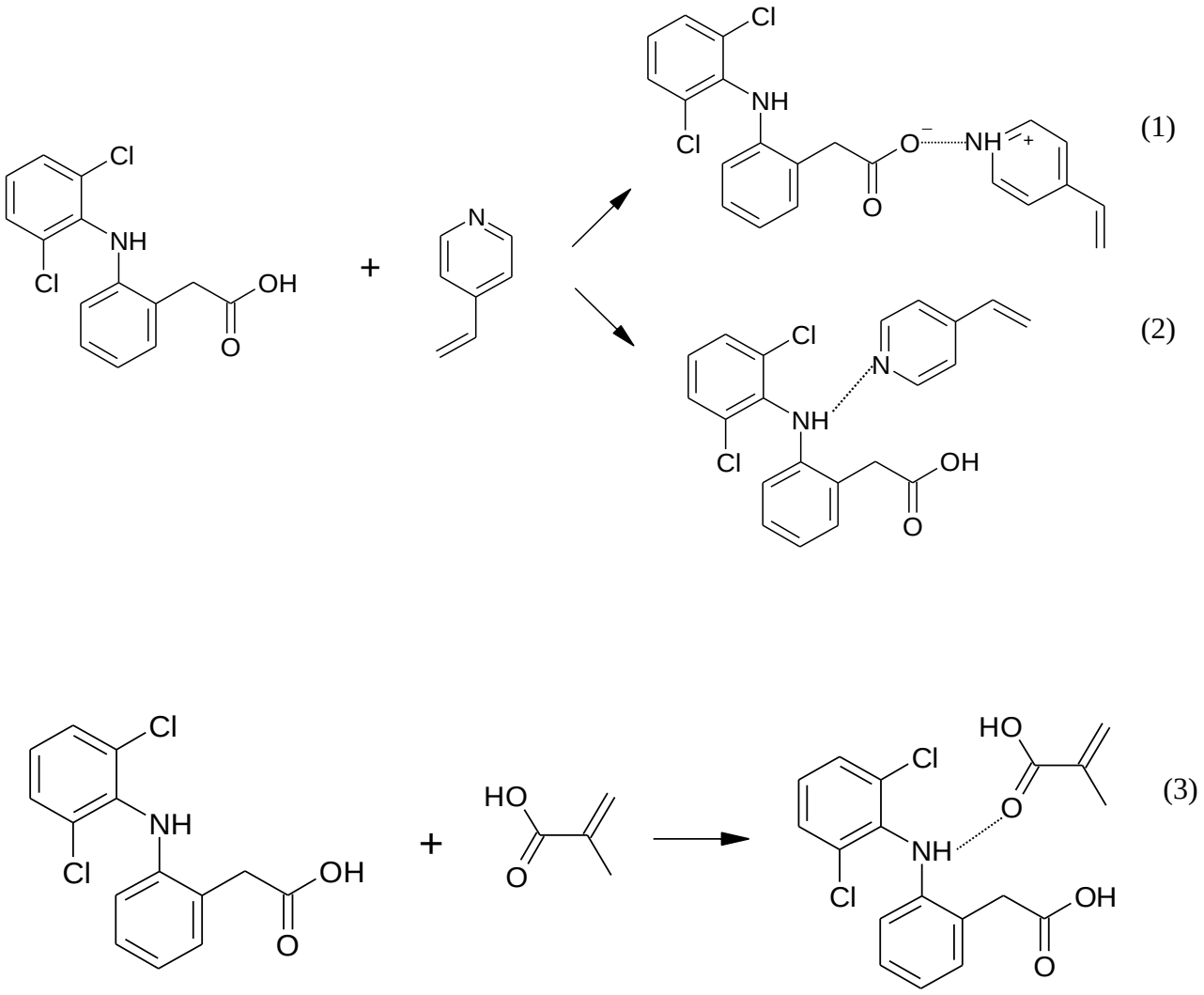


Figure 8: Possible interactions between the template molecule and the functional monomers in the pre-polymerization complex: (1) Shows the ion-pair formation between the -COOH of diclofenac and the N atom on pyridine ring of 4-VP; (2) shows the hydrogen bond formation between -NH of diclofenac and the N atom on pyridine ring of 4-VP; (3) shows the hydrogen bond formation between -NH of diclofenac and the -COOH of methacrylic acid.

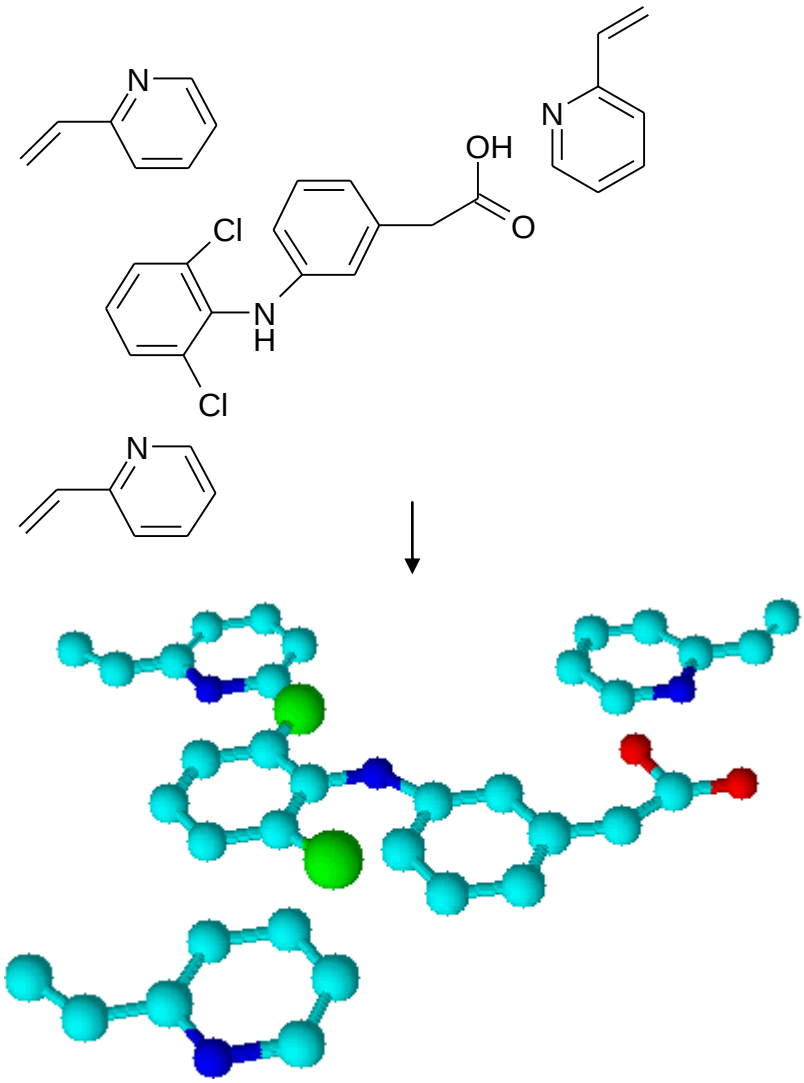


Figure 9: Proposed conformation of the π-π stacking interaction between diclofenac and the vinylpyridine (2-VP).

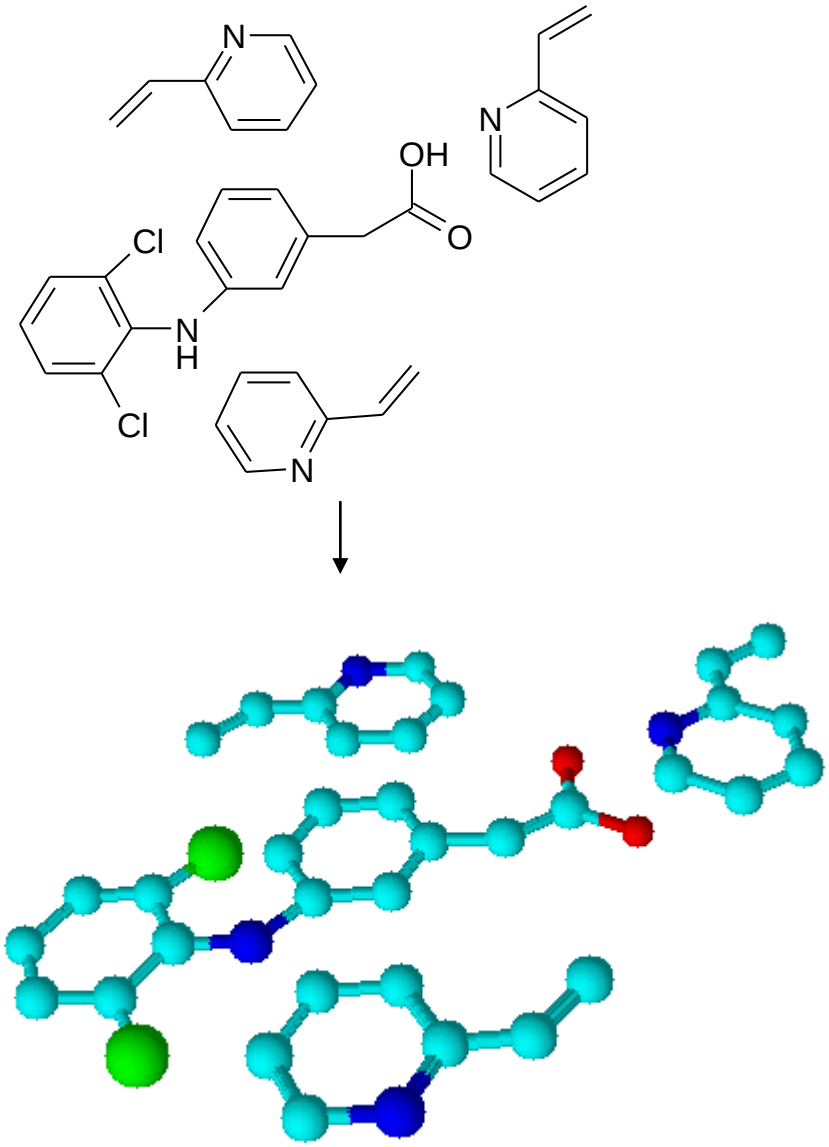


Figure 10: Proposed conformation of the π-π stacking interaction between diclofenac and the vinylpyridine (2-VP).

4 Synthesis of Diclofenac Imprinted Polymers using 4-VP as the Functional Monomer

Although a few research groups have been successful using the photo-polymerization approach to synthesize MIP [183-191], most researchers in the field of molecular imprinting still prefer to use traditional thermal free radical polymerization approach to synthesize different MIP.

In this project, the thermal free radical polymerization technique was utilized for the preparation of molecularly imprinted polymers using diclofenac (free acid) as the template molecule. Azobisisobutyronitrile/2,2′-azobis(2-methylpropionitrile (AIBN) was employed as the initiator. The temperature of polymerization was set at 60 ºC (shown in Figure 11).

At the beginning, a pre-polymerization complexation step was performed by mixing the components in the monomer system at room temperature to facilitate interaction between the functional monomer and the template molecule. At this stage, many research groups usually cool down the template-monomer complex temperature, i.e., incubate the mixture in ice to stabilize the formed complex. In this work, the pre-polymerization mixture was incubated at room temperature for 20 min instead of incubation at low temperature.

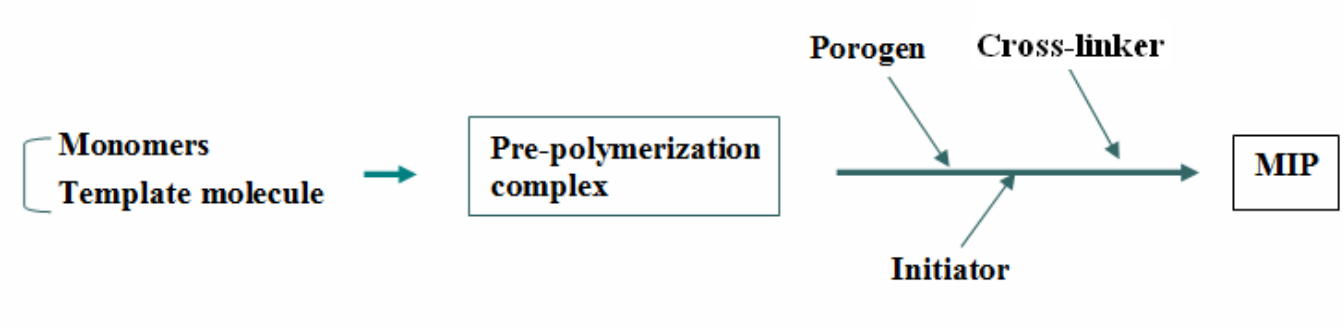


Figure 11: The flow chart of the general procedure of molecularly imprinted polymerization.

4.1 Functional Monomers

Diclofenac (pKa 3.9) is a weak organic acid which could form non-covalent interactions with basic functional monomers such as 2-VP or 4-VP. Vinyl-pyridine is fundamentally heterocyclic aromatic compound with similar chemical properties. There is a pair of electrons on the nitrogen atom, which is not delocalized into the aromatic π-system, thus these

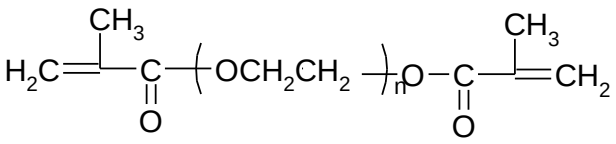
chemicals are basic with chemical properties similar to tertiary amines [192-194]. They can be protonated by reaction with organic acids and forms positively charged aromatic polyatomic ion pairs [195-198].

4-VP (pKa 5.51) is a colorless liquid with a distinctively putrid odor, and it would be fast oxidized to red colored liquid when exposed in air. Normally, certain amounts of inhibitors (such as 100 ppm hydroquinone) are present in commercial products, so a purification step is necessary to remove all inhibitors in the monomers prior to any polymerization. In this project, 4-VP was firstly chosen as the functional monomer. It was purified for the use in the following molecularly imprinted polymerization. During the purification, a vacuum distillation of the monomer was done by heating 4-VP at 85 °C in an oil bath. 4-VP (with boiling point of 62-65°C at 15mm Hg) was evaporated at 40-42 °C (at vacuum ~15 mbar). The distilled 4-VP solvent was collected for the later polymerization.

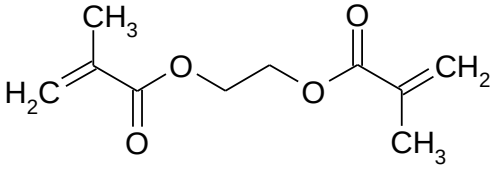
4.2 Purification of Cross-linkers

Several cross-linking compounds are commonly used in the thermal molecular imprinting, such as polyethylene glycol dimethacrylate (PEGDA), ethyleneglycol dimethacrylate (EGDMA) and divinylbenzene (DVB), as shown in Figure 12.

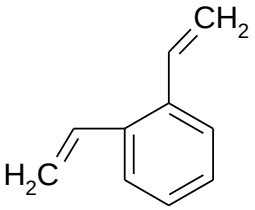
PEGDA is a condensation polymer of ethylene oxide and water, with the general formula $H(OCH_2CH_2)_nOH$, where n is the average number of repeating oxyethylene groups typically from 4 to about 180. The low molecular weight members from n=2 to n=4 are diethylene glycol, triethylene glycol and tetraethylene glycol respectively, which are produced as pure compounds. The low relative molecular weight compounds (up to 700) are colorless, odorless viscous liquids with a freezing point of -10 °C (diethylene gycol).



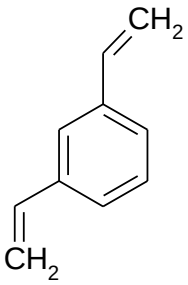
Polyethylene glycol dimethacrylate (PEGDA)



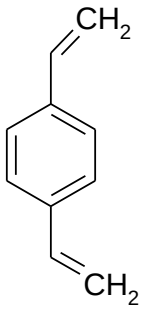
Ethyleneglycol dimethacrylate (EGDMA)



***o*-Divinylbenzene (*o*-DVB)**



***m*-Divinylbenzene (*m*-DVB)**



***p*-Divinylbenzene (*p*-DVB)**

Figure 12: Structures of the commonly used cross-linkers in the process of molecular imprinted polymerization.

PEGDA is termed in combination with a numeric suffix which indicates the average molecular weights. One common feature of PEGDA is its water solubility. It is also soluble in many organic solvents including aromatic hydrocarbons (not aliphatics). PEGDA is often used as a functional comonomer for flexible plastics and as a crosslinking agent between the molecular chains of polymers.

EGDMA is a diester formed by condensation of two equivalents of methacrylic acid and one equivalent of ethylene glycol. It has the structural formula: $CH_2=C(CH_3)$-CO-O-CH_2-CH_2-O-CO-$C(CH_3)=CH_2$. It is one of the most commonly used cross-linkers in radical copolymerization reactions.

DVB often acts as a cross-linking agent in ion-exchange polymerization. It can exist in the form of three structural isomers. The isomers differ in the positioning of the vinyl groups. They may be in the ortho position (variously known as 1,2-diethenylbenzene, 1,2-

divinylbenzene, *o*-vinylstyrene, *o*-divinylbenzene); the meta position (known as 1,3-diethenylbenzene, 1,3-divinylbenzene, *m*-vinylstyrene, *m*-divinylbenzene) or the para position (known as 1,4-diethenylbenzene, 1,4-divinylbenzene, *p*-vinylstyrene, *p*-divinylbenzene). The commercial form of divinylbenzene contains the three isomeric forms, but the meta isomer demonstrates.

All these cross-linkers must be purified prior to the polymerization. The purification procedure of these cross-linkers is as follows:

A clean plastic cartridge was blocked with filter papers and glassy wool at bottom. Then aluminium oxide (basic) was packed in the cartridge. After the cartridge was ready, the cross-linker was loaded into the plastic cartridge. Inhibitors in the cross-linkers would interact with aluminium oxide and were retained in the cartridge during the elution process, thus these inhibitors were separated from the cross-linker.

The purified cross-linker eluent was collected and was further filtrated by a 0.45 µm size filter unit to remove small aluminium oxide particles mixed in the solvent. The cross-linker solution was stored in dark at low temperature until polymerization.

4.3 Synthesis of MIP using Different Cross-linkers

For non-covalent molecular imprinting, the complementary intermolecular interaction between a template and the functional monomer is a critical factor for precise molecular recognition. Another important factor in molecular imprinting is the cross-linker whose type and quantity is also important for attaining high affinity of MIP (Figure 13). In this experiment, to find out the best performed cross-linker, imprinted polymers with different cross-linkers (PEGDA, EGDMA and DVB) were prepared in ACN using 4-VP as the functional monomer (Tables 7-9). The non-imprinted polymer (NIP) was prepared in the same way but without the addition of the template diclofenac.

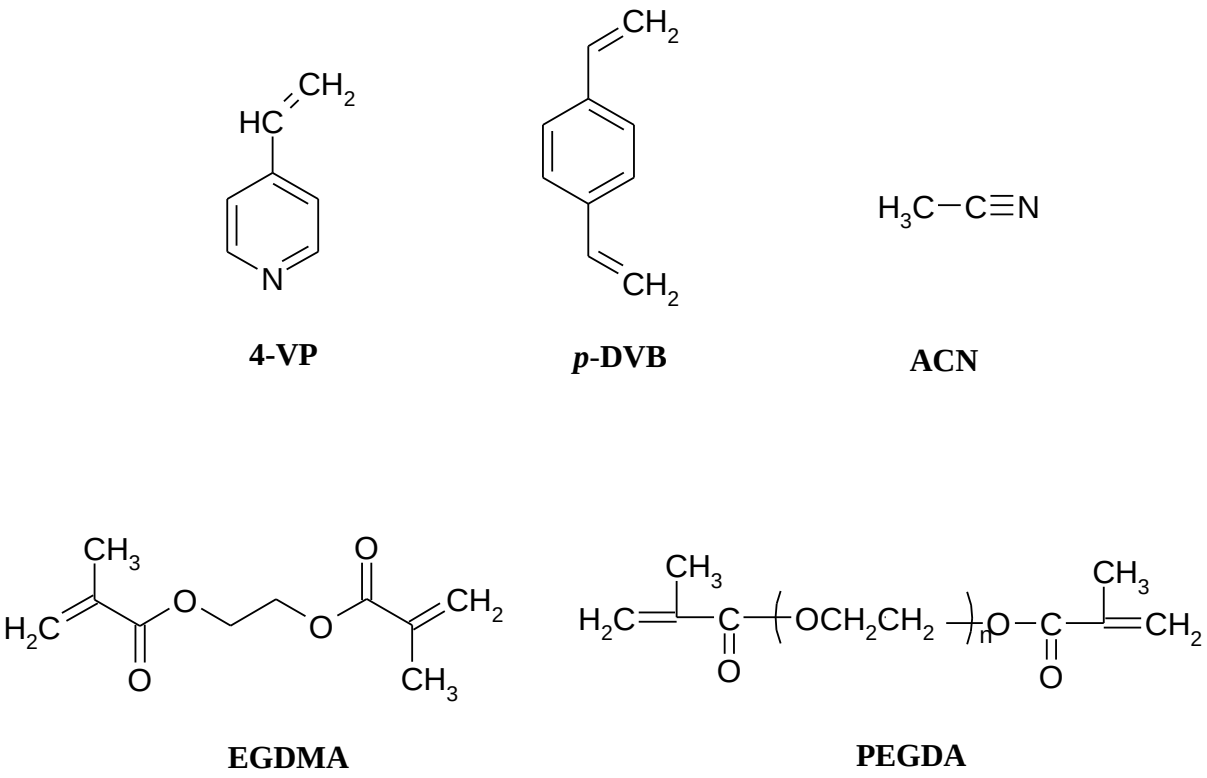


Figure 13: Synthesis of different molecularly imprinted polymers using 4-VP as the functional monomer, ACN as the porogen solvent and the cross-linkers of DVB, EGDMA and PEGDA were compared in the polymerization.

Table 7: 4-VP monomer system with PEGDA as cross-linker and ACN as porogen

	MIP 1	**NIP 1**
Diclofenac (mg)	87.0	/
4-VP (µL)	140	140
ACN (mL)	1.2	1.5
PEGDA (mL)	2.48	2.48
AIBN (mg)	19.0	19.0

Table 8: 4-VP monomer system with DVB as cross-linker and ACN as porogen

	MIP 2	NIP 2
Diclofenac (mg)	87.0	/
4-VP (µL)	140	140
ACN (mL)	1.5	1.5
DVB (mL)	1.28	1.28
AIBN (mg)	19.0	19.0

Table 9: 4-VP monomer system with EGDMA as cross-linker and ACN as porogen

	MIP 3	NIP 3
Diclofenac (mg)	87.0	/
4-VP (µL)	140	140
ACN (mL)	1.2	1.5
EGDMA (mL)	1.38	1.38
AIBN (mg)	19.0	19.0

4.4 Post-treatment of the Imprinted Polymers

After molecularly imprinted polymerization was completed, hard polymers of NIP and MIP were obtained, and these polymers need further treatment for evaluation and characterization.

4.4.1 Grinding, Sieving and Sedimentation

The obtained MIP was crushed, ground in a mechanical mortar, and then wet sieved by acetone using two sieves (Fritsch GmbH, Idar-Oberstein, Germany). The pore size of the top sieve is 63 µm (mesh no. 230), and the pore size of bottom one is 32 µm (mesh no. 450). Thus the collected particle size fraction is between 32-63 µm. The polymer was then sedimented to

eliminate fine particles. The sedimentation was performed in ~20 mL acetone; after 1 h, the supernatant was discarded and fresh acetone was added to the precipitated MIP particles. The fine particles were separated after 5 to 6 cycles of sedimentation and decantation.

4.4.2 Removal of Template Molecules from MIP

Before the imprinted polymer materials can be used for any real application, the template molecules have to be removed from the highly cross-linked polymers by suitable extraction solvents [199]. The extent of template removal depends on the requirements of the subsequent application. Therefore, in preparative applications incomplete removal may be a marginal problem whereas in analytical applications, bleeding of non-extracted template is likely to cause quantification inaccuracies. An additional problem is the legal implications of template bleeding when attempting to prosecute for illegal drug use. So selecting a good approach for template removal is critical to achieve good results on the extraction of analytes from environmental samples.

4.4.2.1 Soxhlet extraction

Soxhlet extraction is a common approach to wash away the template molecules in the imprinted polymers. Typically a Soxhlet extraction could result in the removal of up to 99% of the template. In this work, Soxhlet apparatus added with polar organic solvent (methanol) was used overnight to remove the template. However, the binding results have shown that a small portion of the template remains unextracted even after long time and extensive washing using the polar organic solvent with addition of acetic acid. The remaining template can constitute a problem if it would bleed from the polymer during the washing step of the template-MIP rebinding procedure, giving erroneous results and an increased LOQ. This problem would hamper the use of MIP for later application of trace level analysis. Thus, a better template extraction approach should be developed for template removal.

4.4.2.2 Ultrasonication

Microwave assisted extraction has been proven to be perhaps the most effective tool in achieving efficient extraction of the template [200, 201]. Since microwave assisted extraction

was not available in the laboratory, ultra-sonication extraction was used as the main extraction method for the template removal from the synthesized MIP.

The polymer particle fraction of size 32-63 µm was collected. After sieving, these particles were then ultra-sonicated in MeOH/HOAc (9:1, v/v) for 15 min, followed by centrifugation to remove the supernatant solvent. This procedure was repeated a few times until the template molecule could not be detected in the supernatant solution (at 278 nm). Then, the particles were sonicated again in methanol three times for 15 min per cycle to remove residual acetic acid. Finally, the extraction solvent was removed by centrifugation and the particles were dried under vacuum. As a blank, NIP was similarly treated, although it is in the absence of the diclofenac molecule. This procedure is shown in Figure 14.

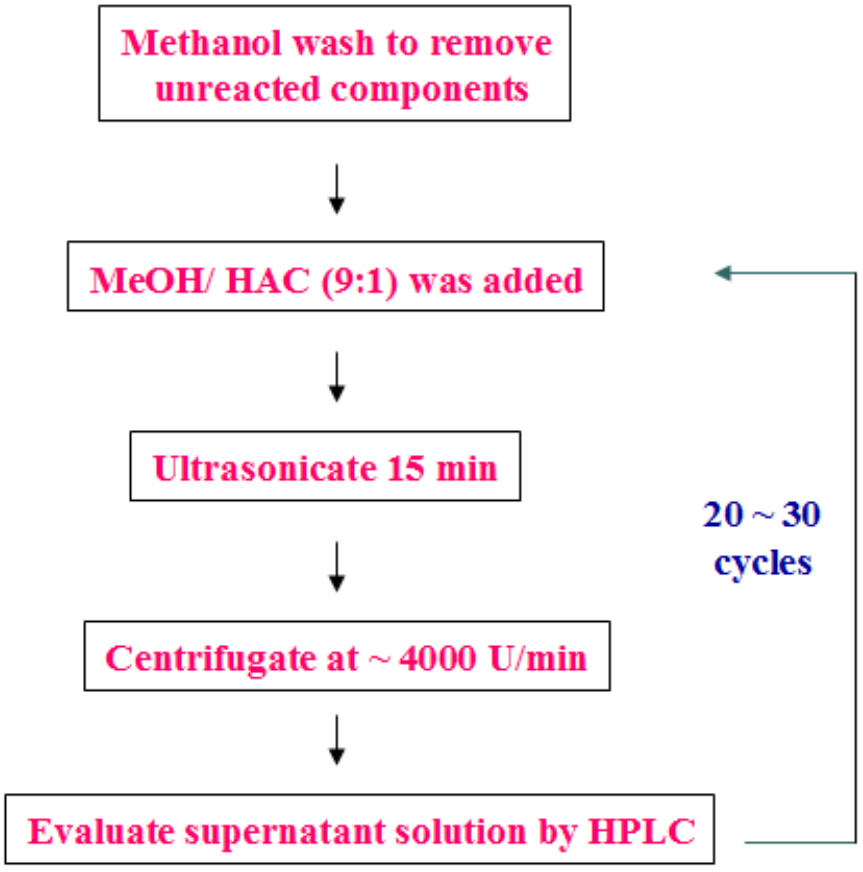


Figure 14: Flow chart of the template removal from MIP by ultra-sonication in MeOH/HOAc (9:1, v/v) mixture.

4.5 Evaluation and Characterization of MIP

After ultra-sonication in MeOH/HOAc (9:1, v/v) solution, the template molecule and other non-reacted components were removed from the MIP. However, prior to any binding tests, the MIP should be further evaluated to see whether the residual template has been removed thoroughly from the highly cross-linked polymer. Only when template residue can not be detected the MIP can be characterized by HPLC in terms of MIP binding performance [202-207]. In this work, HPLC combined with a photo diode array (PDA) detector (HPLC/DAD) was employed for evaluation and characterization of the diclofenac imprinted polymers [208-211].

4.5.1 Chromatographic Conditions

Analysis was performed on a Dionex BioLC system equipped with a DG-2410 degasser, ternary HPLC pump, PDA-100 photodiode array UV-visible detector, and an AS-50 thermal compartment column oven (Dionex, Germering, Germany). Chromatographic separations were carried out with a Phenomenex Synergi Max-RP column 250x4.6 mm i.d. (4.0 µm particle size). Injection was performed with an AS-50 autosampler connected with a 25 µl sample loop.

The polymer chromatographic evaluation was carried out using an isocratic mode. ACN/H_2O (60:40, v/v) with addition of 0.1 M acetic acid was used as the mobile phase at a flow rate of 0.8 mL min^{-1}. The wavelength of the PDA detector was set at 278 nm.

A binary mobile phase with a gradient elution was used for the MISPE experiments in the HPLC analysis. Solvent A was Milli-Q water (1 L) with the addition of 0.1 M acetic acid and solvent B was ACN (1 L) with the addition of 0.1 M acetic acid. The gradient profile was 40-66.5% of B from 0 to 16 min which was raised to 85% in 10 min, followed by 10 min of isocratic elution, 85-40% of B to return to the initial composition in 15 min. The column oven temperature was set at 35 °C, the temperature of auto-sampler tray was set at 24 °C and the flow rate was 0.6 mL min^{-1}.

For the analyte extraction by C-18 SPE cartridge, the C-18 SPE was preconditioned with 5 mL of MeOH followed by 5 mL of pure water. Additionally, the water sample was acidified to pH 3 and was then sucked through the cartridge by negative pressure. After the passage of

the water sample, the C-18 SPE cartridge was washed with 10 mL pure water and dried continually by the vacuum. The analytes were then eluted with 3 mL of MeOH. Solvent removal and residue reconstitution were the same as in the previous MISPE procedure

4.5.2 Chromatographic Evaluation

The solvent extraction efficiency of the prepared MIP particles can be evaluated by chromatographic analysis (Figure 15). Under isocratic elution, the peak retention of diclofenac in the chromatograms during the solvent extraction is at 6.95 min. After a few cycles washing with MeOH/HOAc (9:1, v/v), the concentration of diclofenac in the extracted supernatant solution will continue to decrease until it cannot be detected (at 278 nm). The extraction of the template molecules retained in the imprinted polymer was evaluated and analyzed by HPLC/DAD.

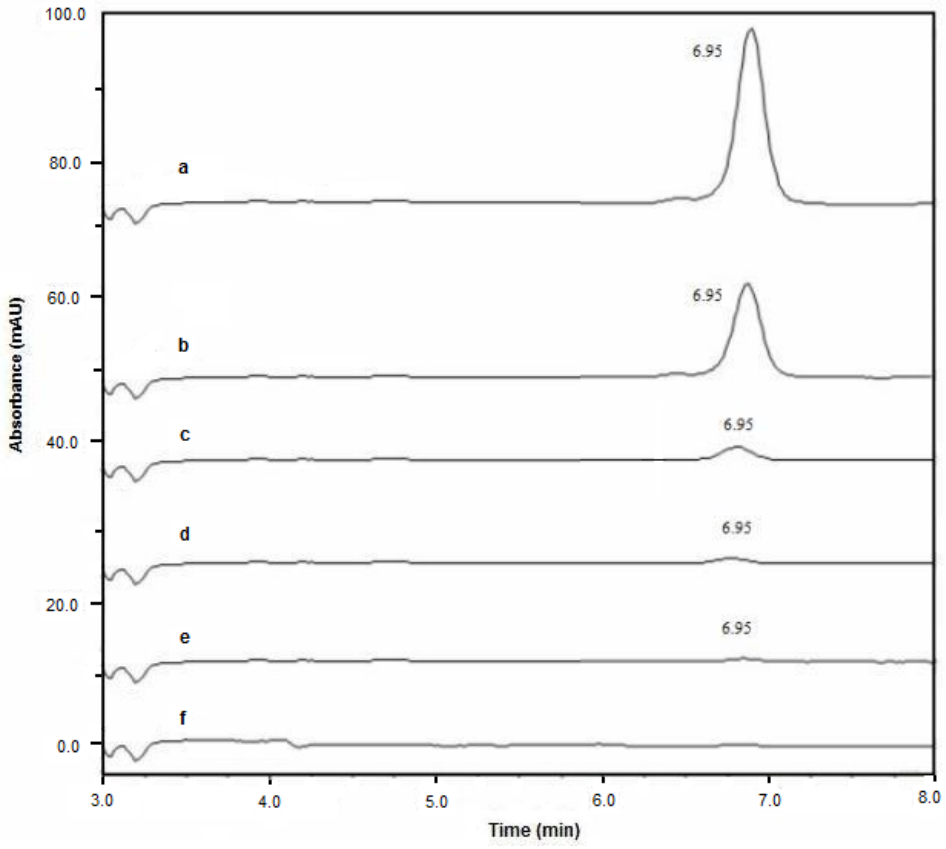


Figure 15: Chromatographic evaluation of the MIP template extraction efficiency in MeOH/HOAc (9:1, v/v) solvent mixture assisted with ultrasonication. (a) supernatant solution of MIP after 1st extraction, (b) 2nd extraction, (c) 3rd extraction, (d) 4th extraction, (e) 5th extraction and (f) 6th extraction.

4.5.3 UV-Vis Spectroscopic Study of the Interaction between Diclofenac and the Monomer 4-VP in Pre-polymerization Complex

Recognition of imprinted polymers for the target analyte is based on the complementary interaction between the specific functional groups of the template molecule and the polymer. Therefore, the study of the intermolecular interaction between template-functional monomer prior to polymerization would be important to probe the recognition mechanism of the imprinted polymers and their binding performance. In this study, the interaction between diclofenac and 4-VP in the pre-polymerization stage in porogen acetonitrile was estimated with UV-Vis spectroscopic analysis. Since the cross-linker and the initiator have less influence on the interaction between template and functional monomers, the studies of the UV-Vis spectroscopic analysis were performed in the absence of the cross-linkers and the initiator AIBN.

The effect of 4-VP on the UV-spectra of diclofenac in acetonitrile solution was tested. The results indicated that the maximum UV absorption wavelength of 4-VP remained unchanged in the presence of diclofenac, even though a large excess of diclofenac was added. However, the small difference of UV-spectra of 4-VP with and without diclofenac could not certify the interaction between them. On the other hand, it is noted that there are several different functional groups in the template diclofenac. So, the absorption spectrum of diclofenac would obviously change if it interacts with 4-VP by hydrogen bond or other interactions i.e. ionic interaction. The UV-spectra of diclofenac (2.6 µmol L^{-1}) in the presence of various concentrations of 4-VP (from 0.0 µmol L^{-1} to 342 µmol L^{-1}) are shown in Figure 16. As expected, the maximum UV absorption wavelength of diclofenac shifted remarkably to shorter wavelengths along with an increase in the concentration of functional monomer 4-VP. The shift of the UV maximum absorption band could be due to the hydrogen bonding effect on the π-π absorption band of a molecule whose chromophore acts as a proton donor [212]. Also, from the molecular modeling study (Figure 17, analyzed with the molecular modeling software Weblab ViewerPro 4.0 from MSI Molecular Simulations Inc., San Diego, CA, USA), it is more clear to observe the possible interaction between functional monomer 4-VP and the carboxyl group in the diclofenac molecule. It may be concluded from the UV spectra that the functional monomer 4-VP bound effectively to the template analyte diclofenac in the pre-polymerization solution, and a complex of diclofenac and 4-VP was formed.

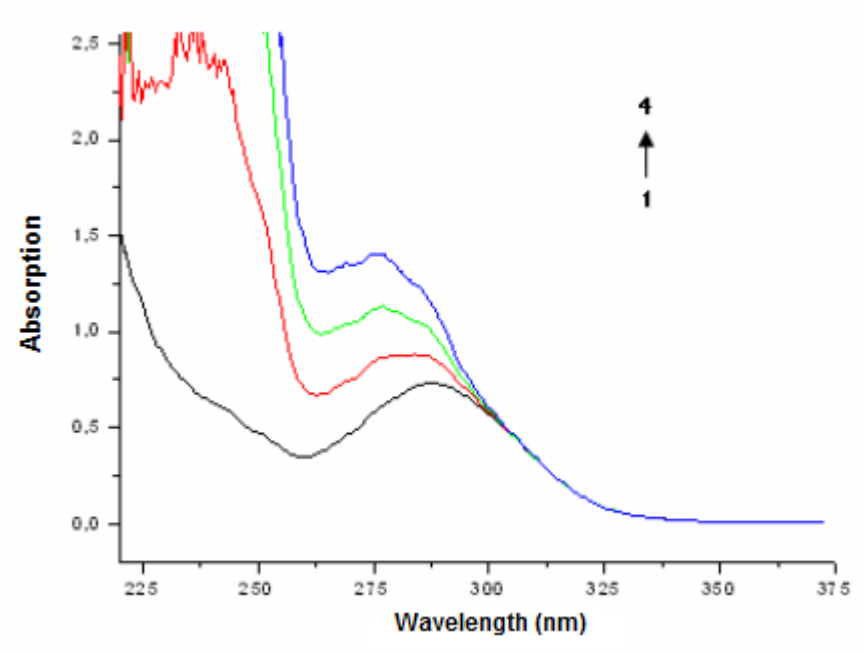


Figure 16: The UV spectra of diclofenac with addition of various concentrations of 4-VP in ACN. Concentration of diclofenac: 2.6 µmol L^{-1}. Concentration of 4-VP: (1) 0, (2) 114, (3) 228, (4) 342 µmoL^{-1}.

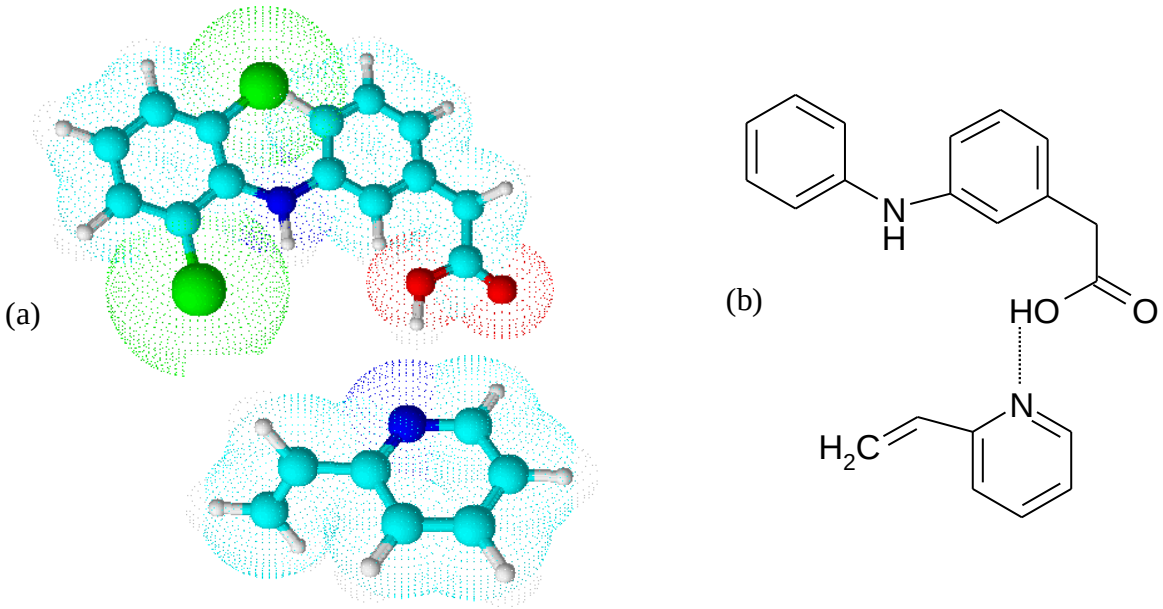


Figure 17: The possible interaction format between template diclofenac and 4-VP molecules based on the molecular modelling studies. (a) molecular model; (b) plane structure.

4.5.4 Comparison of MIP prepared with Different Cross-linkers

To evaluate the binding performance of MIP prepared with different cross-linkers, standard solutions of a known concentration (0.4 mg L^{-1}) of diclofenac were prepared. Each standard solution (1 mL) was pipetted and mixed with 10 mg of the prepared imprinted polymer particles in a 1.5 mL glass vial. The mixtures were incubated on a microplate shaker (EAS 2/4 from SLT Labinstruments, Crailsheim, Germany) for 30 min, and then rapidly filtrated. The concentration of the free diclofenac in the filtrate solution was measured using HPLC. The amount of diclofenac bound to the MIP was calculated by subtracting the concentration of free diclofenac from the initial diclofenac concentration.

Table 10: Binding of diclofenac (0.4 mg L^{-1}) on polymers prepared with different cross-linkers

		Functional monomer 4-VP	
MIP	**Cross-linker**	**MIP binding (%)**	**NIP binding (%)**
1	PEGDA	4.1	2
2	DVB	24	15
3	EGDMA	45	12

The binding results of each imprinted polymers were summarized in Table 10. From the binding test results, it is seen that among the MIP prepared with the three cross-linkers (MIP **1**, **2** and **3**), only MIP **3**, which was prepared with EGDMA as the cross-linker, shows high binding affinity for the template. MIP **1**, which was prepared with PEGDA demonstrated very low binding of the template. This is mostly due to this cross-linker's incompatibility (less soluble in other organic components) with other components in the monomer solution. For MIP **2**, which was prepared with DVB, the MIP shows relatively higher binding affinity compared with the MIP **1**, but the amount of rebound diclofenac in the NIP is quite similar to that of the MIP. DVB is also a commonly used cross-linker in the non-covalent imprinting. However, compared with EGDMA, MIP did not show specific binding ability for template diclofenac. Obviously, among these three imprinted polymers, MIP **3** prepared with cross-linker EGDMA shows the best binding performance, because of its best binding affinity demonstrated among the three MIP. Also it showed the largest binding difference between

MIP and NIP. Thus, EGDMA was selected as the cross-linker in molecular imprinting for all the following experiments.

4.6 Binding Characteristics of MIP 3

In the previous binding experiments, MIP **3** showed a good binding affinity to diclofenac in porogen acetonitrile. To further evaluate the binding characteristics of MIP **3**, similar binding tests were performed. A series of diclofenac standard solutions were prepared in organic solvent ACN. Each standard solution (1 mL) was pipetted and mixed with 10 mg of MIP **3** polymer particles in a 1.5 mL glass vial. The mixtures were incubated on a microplate shaker for 30 min, and then rapidly filtrated with a 0.45 µm filter unit. The diclofenac concentration in the filtrate was measured by HPLC/DAD. The amount of diclofenac bound to the imprinted polymer was calculated by subtracting the concentration of free diclofenac from the initial concentration.

4.6.1 Saturation Binding Test

To estimate the binding affinity of MIP **3** for template diclofenac, a saturation binding isotherm experiment and Scatchard plot analysis were carried out [213-218]. The binding isotherm of diclofenac to MIP was measured at various diclofenac concentrations, as shown in curve *a* of Figure 18. The binding of diclofenac to NIP were also measured in the same way (curve *b* of Figure 18). The amount of diclofenac bound to the MIP at binding equilibrium Q increased along with increasing the initial concentration of diclofenac and reached saturation at higher concentration. Comparison of curve *a* and curve *b* shows that the amount of the template bound to the imprinted polymer was much higher than the amount bound to the non-imprinted polymer. This suggested that the imprinted cavities of the MIP may have caused the high affinity binding of the template to the polymer.

The data of the binding isotherm of diclofenac to MIP were also analyzed by Scatchard plot method. The Scatchard analysis equation is:

$$\frac{Q}{[DCF]} = \frac{(Q_{\max} - Q)}{K_D}$$

where Q is the amount of diclofenac bound to MIP at equilibrium, Q_{max} is the apparent maximum number of binding sites, [*DCF*] is the free diclofenac concentration at equilibrium and K_D is the equilibrium dissociation constant of binding sites. Q/[*DCF*] was plotted vs. Q, as shown in Figure 19. The binding characteristics of the MIP can be estimated from the Scatchard analysis (Figure 19a). As can be seen from this figure, the Scatchard plot for MIP is not a single linear curve, but consists of two linear parts with different slopes. The linear regression equation for the left part of the curve in the figure is Q / [*DCF*] = −55.34 Q + 1.28 (r = 0.99; n = 12), the unit of Q is nmol. The K_D and Q_{max} were calculated to be 18.1µmol L^{-1} and 2.3µmol g^{-1} dry polymers, respectively from the slope and the intercept of the Scatchard plot. The linear regression equation for the right part of this curve is Q / [*DCF*] = −1.554 Q + 0.226 (r = 0.98; n = 15).

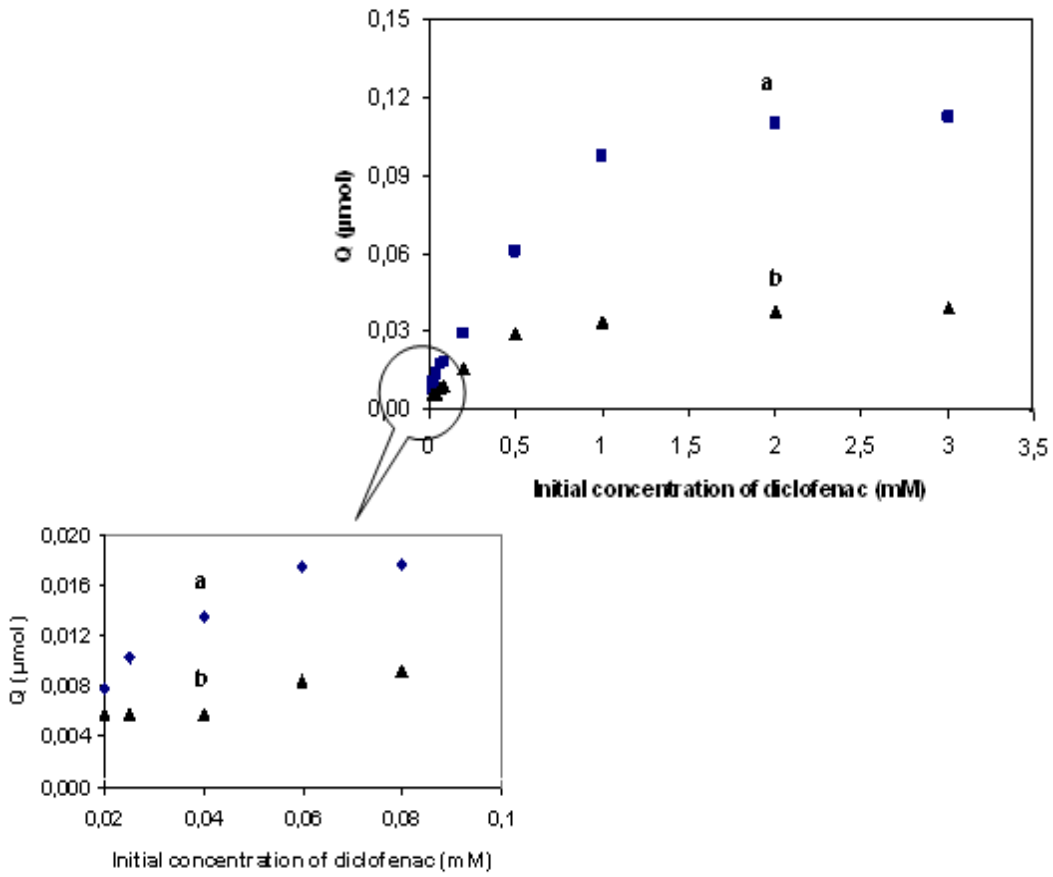


Figure 18: Binging isotherm of diclofenac acid imprinted polymer. Q is the amount of diclofenac acid bound to Polymer (a, MIP; b, NIP). Polymer weight: 10 mg; volume: 1.0 mL; binding time: 30 min.

The K_D and Q_{max} were calculated to be 640 µmol L^{-1} and 14.5 µmol g^{-1} dry polymers, from the Scatchard plot. From Scatchard plot analysis, it may be concluded that the binding site configuration in the MIP are heterogeneous in respect to the affinity for diclofenac and indicated that the binding sites in the imprinted polymer could be classified into two distinct groups with different specific binding properties. In the imprinting process, they would be solidified in the polymer matrix and then form two kinds of binding sites with different binding affinity on imprinted cavities in the polymer bodies. The binding of diclofenac to the NIP was also analyzed by Scatchard plot method. As can be seen from Fig. 19b, the Scatchard plot of the NIP is a single linear curve. The linear regression equation for the right part of this curve is Q / [DCF] = −5.237 Q + 0.212 (r = 0.98; n = 27). The K_D and Q_{max} were calculated to be 190µmol L^{-1} and 14.5 µmol g^{-1} dry polymers, from the Scatchard plot. This suggested that the binding sites on the blank polymer were of similar binding properties. The different characteristic of the binding sites on NIP as compared to those of the MIP proved that without the imprinting process, the binding sites on the polymer would be non-selective to the template analyte.

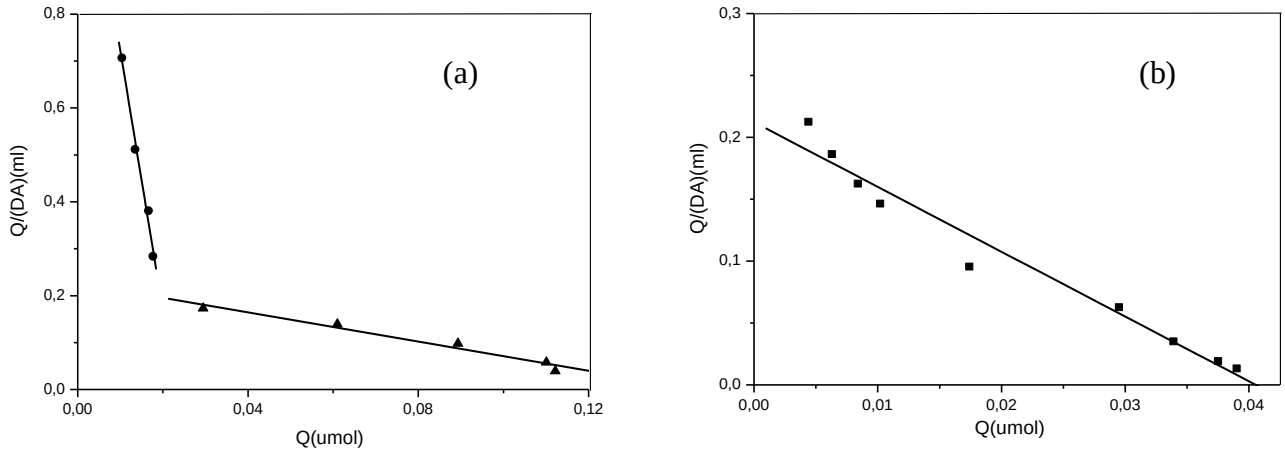


Figure 19 (a): Scatchard plot analysis of the binding of diclofenac to the imprinted polymer. **(b): Scatchard plot analysis of the binding of diclofenac to the non-imprinted polymer.** Q is the amount of diclofenac bound to the polymer (MIP or NIP); [DA] is the concentration of free diclofenac at equilibrium.

4.6.2 Binding Performance of MISPE

The binding characteristics of the imprinted polymer **3** were evaluated by binding isotherm experiments in this work. In the experiments, MIP exhibited high selectivity and affinity for diclofenac, and its molecular recognition mechanism was considered to be perhaps mainly due to the hydrogen bonding interaction between the template and the polymer. These characteristics of the MIP were important for them to be used for MISPE. Different from the isotherm binding tests which were performed in diclofenac standard solution, MISPE binding analysis was done by loading the MIP particles into the SPE cartridge. To prepare the MISPE cartridge, an amount of 100 mg of MIP **3** in 2 mL of MeOH was transferred into an empty 3 mL glass SPE cartridge (Mallinckrodt Baker B.V., Deventer, Holland). PTFE frits (porosity 10 µm, Merck) were placed above and below the polymer particle sorbent bed. As a control, NIP SPE cartridge was also prepared in the same way but was loaded with the non-imprinted polymer particles.

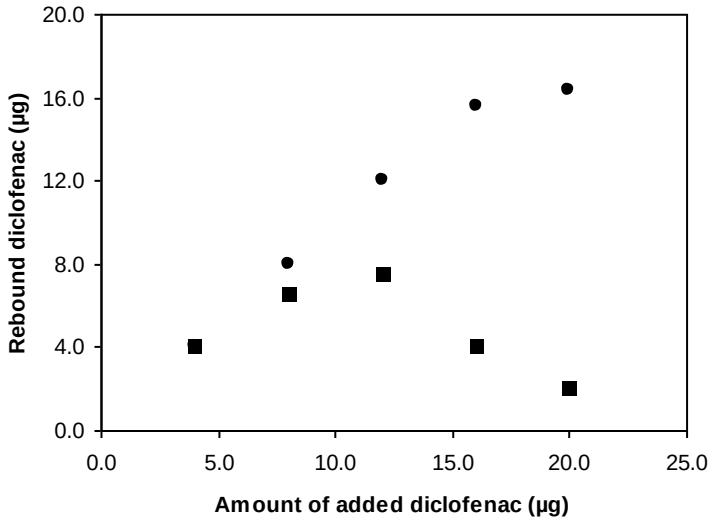


Figure 20: Binding isotherm saturation tests of diclofenac to MISPE and NISPE cartridges. Q is the amount of diclofenac rebound to MIP (●) or NIP (■). Weight of the polymer: 10 mg; volume of diclofenac solution prepared in ACN: 1 mL. Binding saturation time: 30 min.

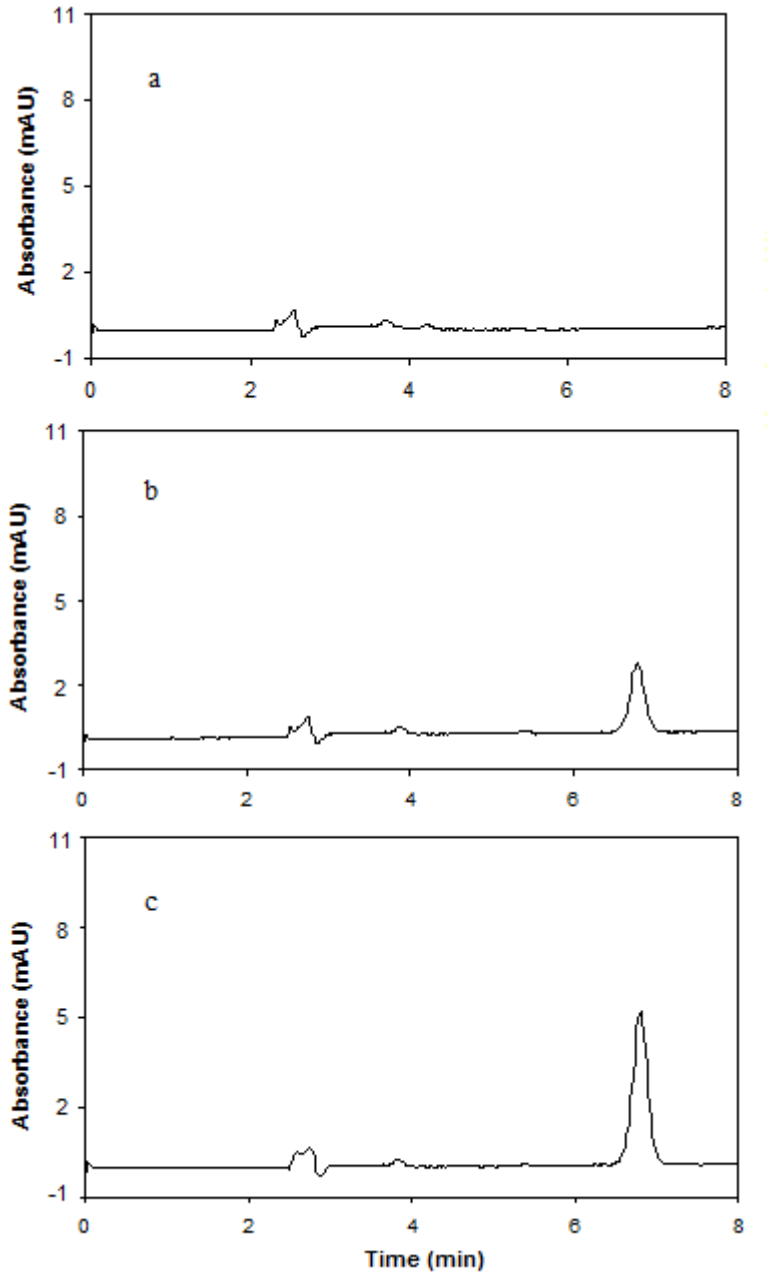


Figure21: Chromatographic analysis of binding isotherm of diclofenac to NISPE. The amount of diclofenac added to the NISPE is: (a) 4.0, (b) 8.0, (c) 12.0, (d) 16.0 and (e) 20.0 µg.

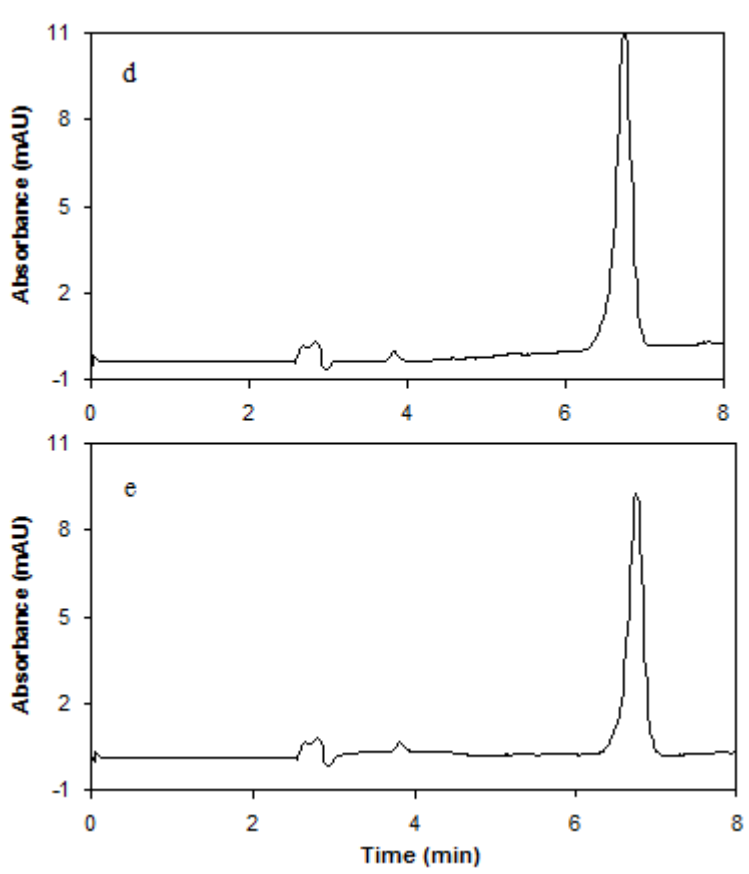


Figure 21 continued

To optimize MISPE binding conditions, 1 mL of 4.0 mg L^{-1} diclofenac solution was loaded into the SPE cartridges, and the eluted solution was collected. Then the 2nd diclofenac solution of identical volume was loaded into the same cartridge, and the eluted solution was also collected. Finally, a volume of 5 mL of the diclofenac solution was loaded into the MISPE cartridge. Each eluted solution from the MISPE cartridge was collected and then analyzed by HPLC/DAD. The binding results of MISPE and NISPE were plotted in Figure 20.

As shown in Figure 20, an increased amount of diclofenac was added into the MISPE and NISPE cartridges to evaluate the binding affinity and capacity of the polymers. During the 1st loading, no diclofenac could be detected in the eluates. This indicated that all the added analytes were rebound in the polymer cartridges (Figure 21 & 22). When 12 µg of diclofenac was loaded into the polymer SPE cartridge, NISPE reached its binding saturation, and the rebound diclofenac in the NIP would begin to decrease if more analyte was added. With MISPE, the amount of the rebound diclofenac still increased along with addition of more

diclofenac until 20 µg of the analyte was loaded where binding saturation was almost reached. From the result of the MISPE binding test, it can be seen that MIP and NIP demonstrated significantly different binding behavior.

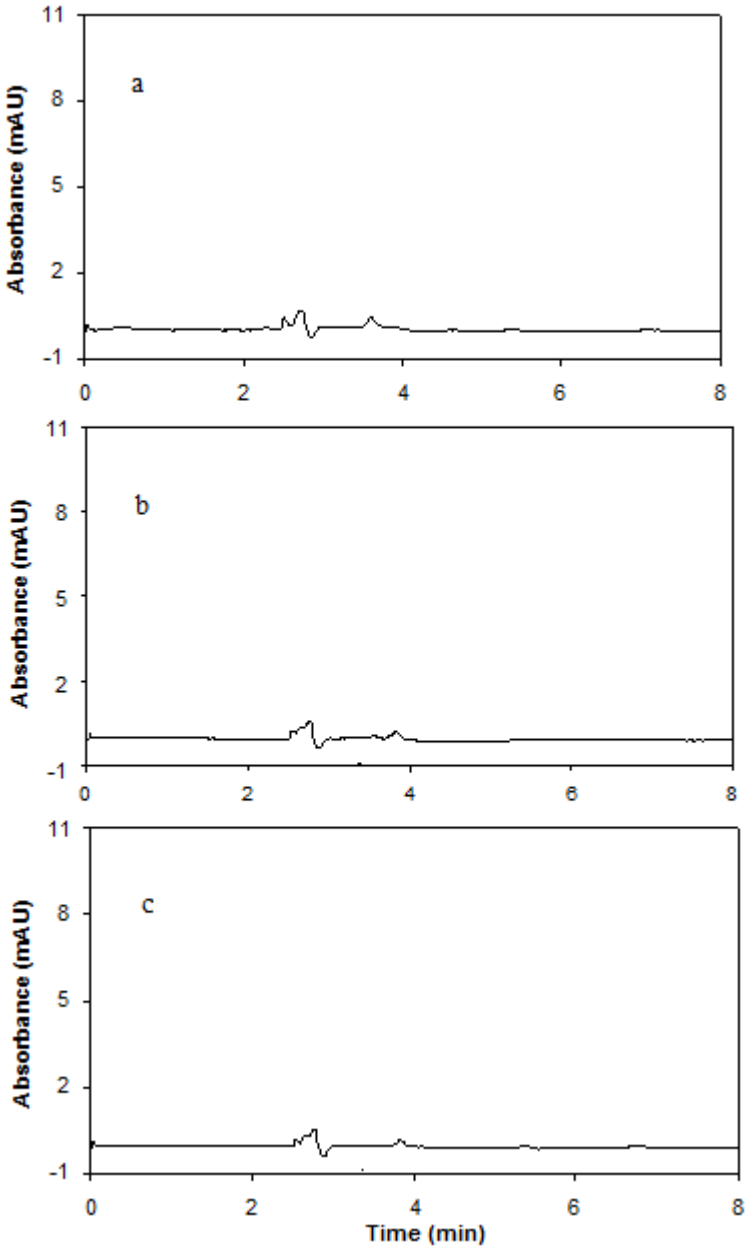


Figure 22: Chromatographic analysis of binding isotherm of diclofenac to MISPE. The amount of diclofenac added to the MISPE is: (a) 4.0, (b) 8.0, (c) 12.0, (d) 16.0 and (e) 20.0 µg.

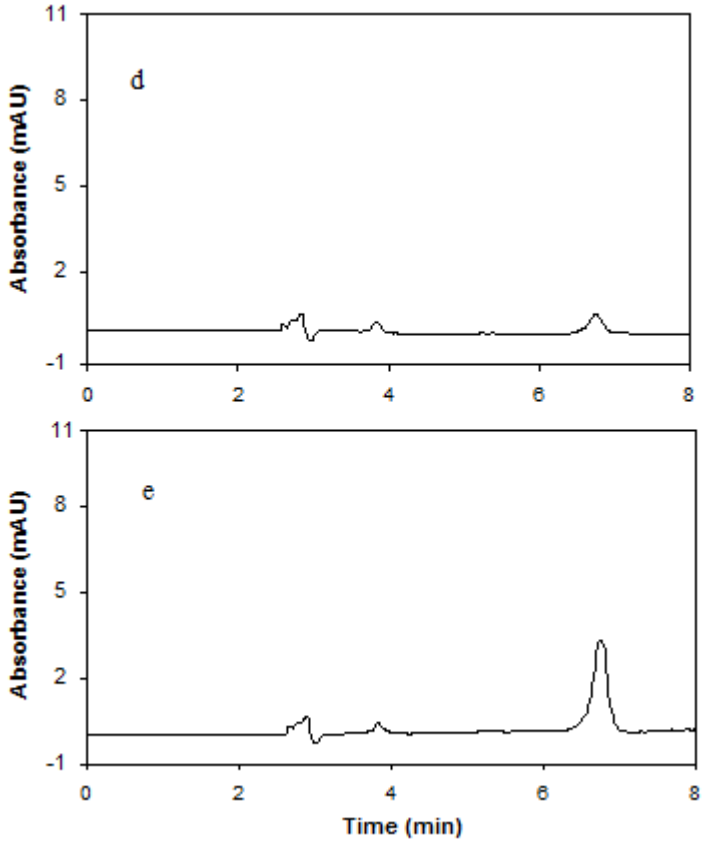


Figure 22 continued

4.7 Preparation of MIP using Different Porogens

It is reported that the effect of solvent (porogen) on the selectivity of the recognition sites was not very significant when covalent imprinting approach was employed in MIP synthesis, although the porogen used also showed a strong influence on the morphology of the polymer network. In contrast, for the non-covalent imprinting approach, the selective rebinding of the template to MIP strongly depended on the solvent used [154, 155]. Some previous studies have also shown that MIP often exhibited higher selectivity in the solvent, which was used as the porogen in the polymerization process. The MIP only rebinds to the template in the solvent used as porogen, and this could be explained by the same way as suggested previously. The imprinted polymers often show different swelling properties in solvents due to the different solvation properties of solvents for a given type of polymer. The varying degrees of swelling in different solvents may considerably change the morphology of the polymer network, as well as the size, shape and relative position of the functional groups.

This would inevitably affect the specific binding of the template molecule to the MIP due to the essential complementary interaction between them.

In diclofenac imprinted polymerization, the porogen solvent effect on the specific recognition properties of the imprinted polymers was investigated through equilibrium binding experiments of the template in different solvents. In the previous polymerization experiments, polar solvent (acetonitrile) was used as porogen in the imprinting process. In another experiment, a porogen mixture consisting of octanol and trichloroethane was tested for the MIP preparation (Table 12). Because one is a "good" solvent and the other one is a "poor" solvent, the mixing of such property-distinguished solvents in the imprinting process may bring high effects on the polymer morphology [219-224]. In addition, polar solvents e.g. H_2O and MeOH were also used in the new polymerization. All the prepared imprinted polymers MIP **4**, **5** and **6** (Tables 11, 13 and 14) will be compared to each other in terms of binding performance.

Table 11: Monomer system composition with MeOH/H_2O as porogen

	MIP 4	**NIP 4**
Diclofenac (mg)	88	/
4-VP (µL)	140	140
MeOH (mL)	1.4	1.4
H_2O (mL)	0.35	0.35
EGDMA (mL)	1.38	1.38
AIBN (mg)	20.0	20.0

Table 12: Some chemical properties of porogens

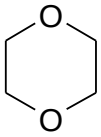
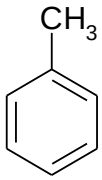
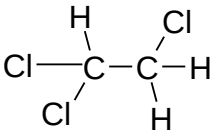
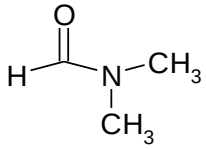
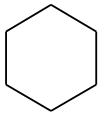
Chemical name	Molecular structure	Boiling point (°C)	Dielectric constant	Density
1,2-Dichloroethane	$ClCH_2CH_2Cl$	83	10.3	1.26
Dioxane		101	2.2	1.034
Toluene		110	2.4	0.865
1,1,2-Trichloroethane		110-115	7.5	1.43
DMF		153	39	0.944
Octanol	$CH_3(CH_2)_6CH_2OH$	196	10.3	0.827
Dodecanol	$CH_3(CH_2)_{10}CH_2OH$	261	6.5	0.833
Octane	$CH_3(CH_2)_6CH_3$	126	2	0.703
Decane	$CH_3(CH_2)_8CH_3$	174	2	0.73
Hexane	$CH_3(CH_2)_4CH_3$	69	2	0.659
Cyclohexane		80.7	2	0.779

Table 13: Monomer system composition with Octanol/Trichloroethane as porogen

	MIP 5	NIP 5
Diclofenac (mg)	88	/
4-VP (µL)	140	140
Octanol(mL)	0.5	0.5
Trichloroethane (mL)	0.5	0.5
EGDMA (mL)	1.38	1.38
AIBN (mg)	20.0	20.0

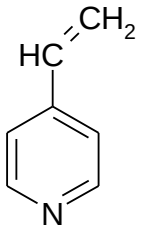
Table 14: Monomer system composition with MeOH as porogen

	MIP 6	NIP 6
Diclofenac (mg)	88	/
4-VP (µL)	140	140
MeOH (mL)	1.5	1.5
EGDMA (mL)	1.38	1.38
AIBN (mg)	19.0	19.0

4.8 Binding Characteristics of MIP 5

For MIP **4**, no binding was observed during the MISPE binding test. So no further experiments were performed with MIP **4**. In the preparation and post-treatment of the imprinted polymers, solvent extraction is a necessary step to wash and extract the template molecules from the polymers. While an often encountered problem of this procedure is that the template molecule can not easily be washed off completely due to the high cross-linking of the polymer, i.e., the polymer body is not sufficiently porous for solvent extraction. This problem will cause template molecules to bleed during the binding experiments from time to

time and it will make a quantitation error in these tests. To solve this problem, the extraction step should be done effectively. However, since the imprinted polymer is prepared to be physically highly cross-linked, normal washing solvent may not easily access the polymer body. This situation could be changed if the prepared polymer is highly porous.



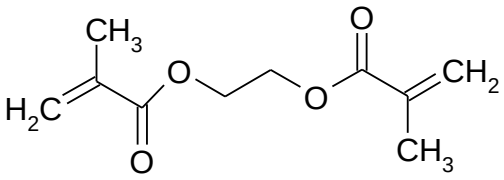
4-VP

CH_3CCl_3

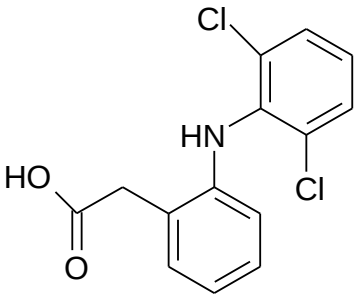
Trichloroethane

$CH_3(CH_2)_6CH_2OH$

Octanol



EGDMA



Diclofenac

Figure 23: Synthesis of diclofenac imprinted polymer MIP 5 using 4-VP as the functional monomer, EGDMA as the cross-linkers and Octanol/Trichloroethane as the porogen solvent.

Therefore, many research works have reported on the synthesis of highly porous polymer monoliths. According to their reports, a solvent mixture consisting of "good" solvents and "poor" solvents could lead to highly porous polymer structure during the polymerization. Based on a study of these research works, an organic solvent mixture composed from trichloroethane/octanol (1:1, v/v) was used as the porogen (Figure 23).

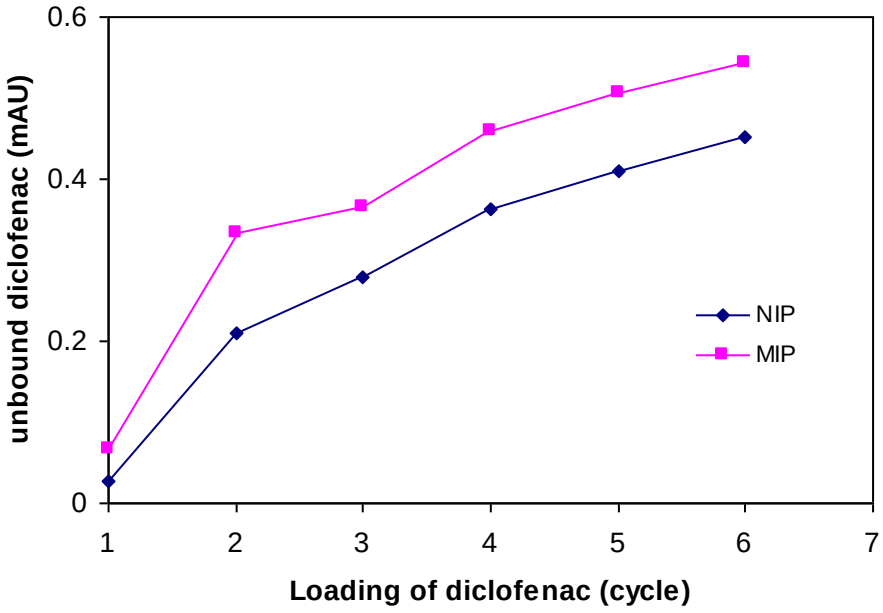


Figure 24: HPLC analysis of diclofenac rebinding tests with MISPE 5 and NISPE 5. Diclofenac standard solution of 0.5 mg L^{-1} was prepared and loaded to the cartridges. Each eluent solution from the cartridges of MIP and NIP were analyzed by HPLC/DAD.

From the rebinding tests of MIP **5** (Figure 24), it can be seen that the binding behavior of MIP **5** and NIP **5** for diclofenac is a little bit of different. The unbound diclofenac recovered in effluent fraction from MIP was higher than that of NIP. This indicates that after 6 cycles of loading of diclofenac standard solution to the polymer cartridges, more diclofenac was dissociated from the MIP cartridge. This could be attributed to the destruction of the binding sites in the MIP, which caused the dissociation of the diclofenac bound in the MIP. However, this diclofenac dissociation was caused by polymer porosity or from other factors is still not fully understood.

4.9 Binding Characteristics of MIP 6

Because MIP **5** imprinted with a solvent mixture did not show a good imprinting effect, a polar porogen was used. In the case where a polar porogen is used in the imprinting, unlike hydrogen bonding, the template-monomer complex formation has to rely on hydrophobic and ionic interactions. It is not, or is at least to a lesser extent disturbed in the presence of high

polar solvents. In the synthesis of MIP **6** (shown in Figure 25), polar solvent methanol was chosen as the porogen.

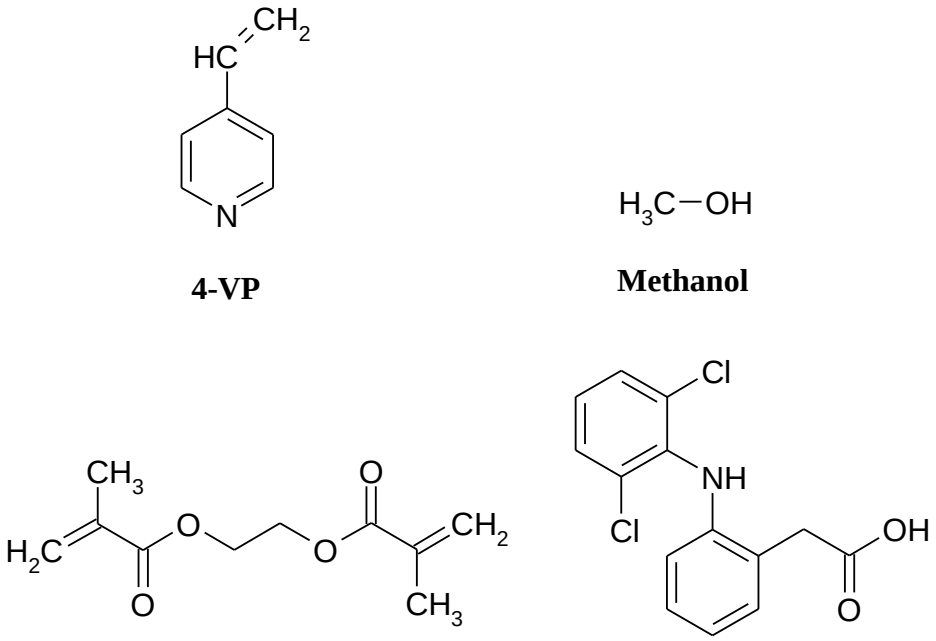


Figure 25: Synthesis of diclofenac imprinted polymer, MIP 6, using 4-VP as the functional monomer, EGDMA as the cross-linkers and polar solvent methanol as the porogen.

The isotherm binding test of NISPE **6** is similar to the binding experiment of NISPE **3**. In this experiment, 1 mL of 1.0 mg L^{-1} diclofenac solution was loaded into the SPE cartridges, and the eluted solution was collected. Then, another diclofenac solution of identical volume was loaded into the same cartridge, and the eluted solution was collected. A total volume of 4 mL of the diclofenac solution (total amount 4 µg) was loaded into the NISPE cartridge. Each eluted solution collected from the NISPE cartridge was analyzed by HPLC/DAD. The chromatograms obtained by loading the diclofenac solution to the NISPE were shown in Figure 26. From the HPLC chromatograms, it is seen that at the beginning of the binding test only a small amount of diclofenac was found in the eluate. In the subsequent addition, most of the loaded diclofenac was then recovered in the eluted solution from the NIP cartridge. It is apparent that NIP cartridge did not show any significant binding affinity to the template analyte.

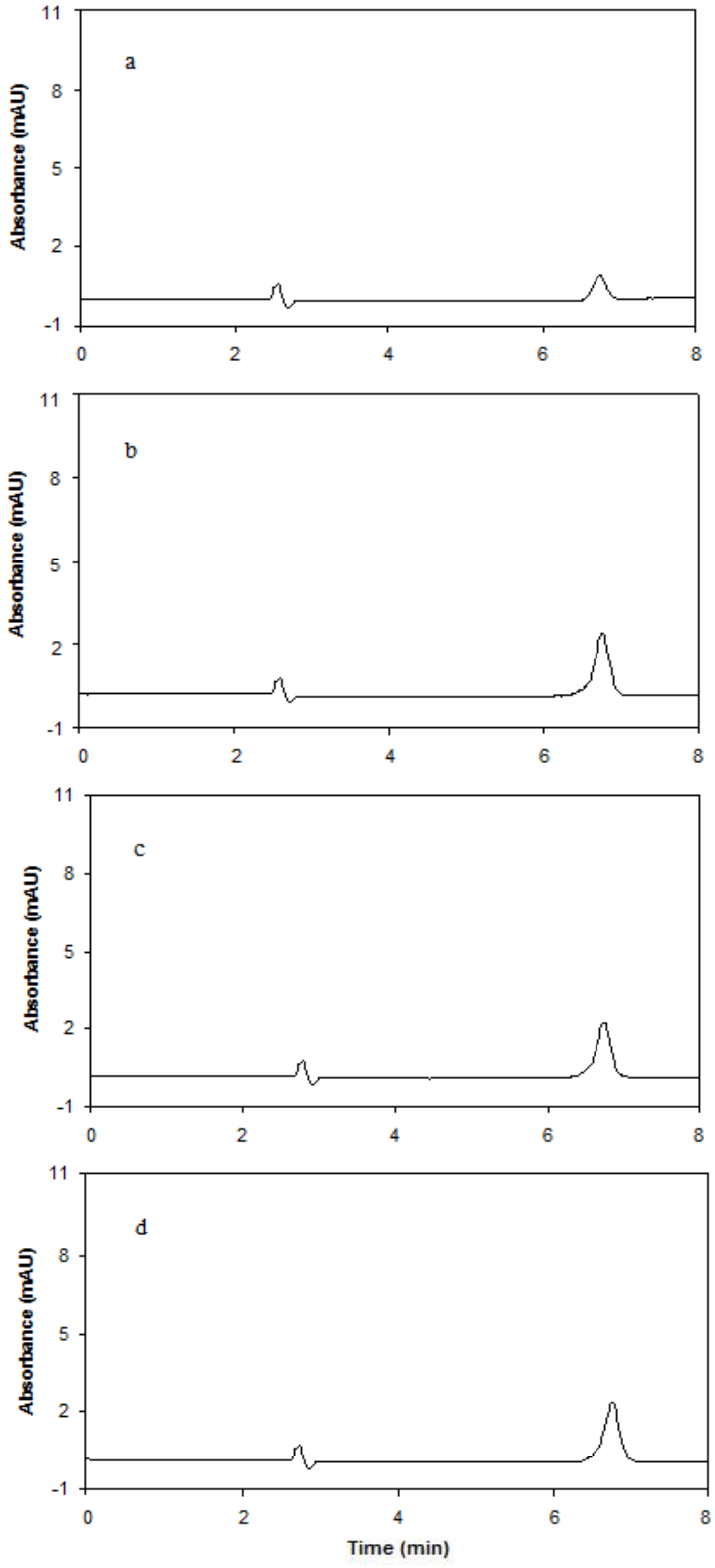


Figure 26: Chromatographic analysis of binding isotherm of diclofenac to NIP 6. The amount of diclofenac added to the NISPE (containing 100 mg polymer) is: (a) 1.0, (b) 2.0, (c) 3.0, and (d) 4.0 µg.

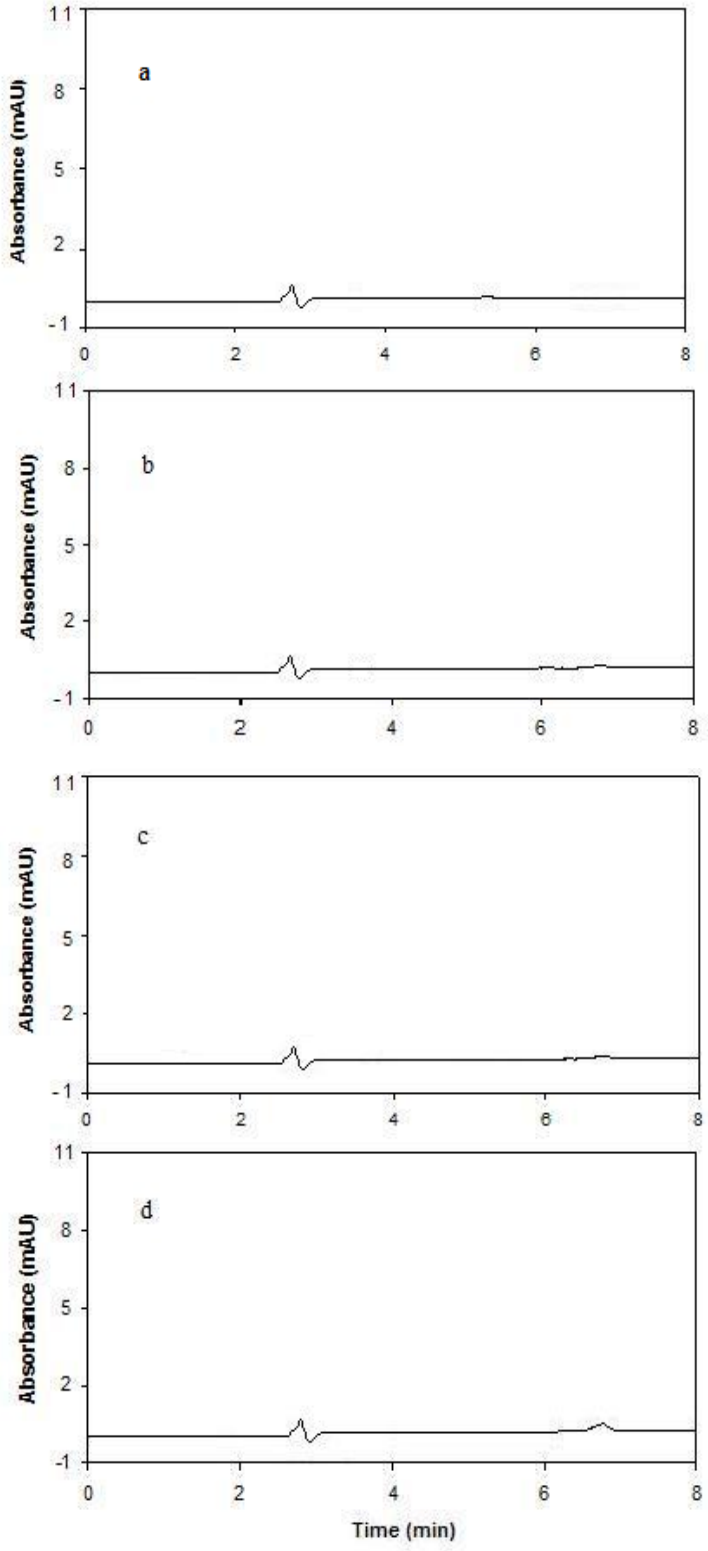


Figure 27: Chromatographic analysis of binding isotherm of diclofenac to MIP 6. The amount of diclofenac added to the MISPE (containing 100 mg polymer) is: (a) 1.0 µg, (b) 2.0 µg, (c) 3.0 µg, and (d) 4.0 µg.

The isotherm binding test of MISPE **6** was performed in the same timeframe and in the same manner. After the binding test was finished, all the collected eluates from the MIP cartridge were analyzed by HPLC/DAD (Figure 27). Upon evaluation of the chromatograms obtained from MISPE **6** binding test, it can be stated that MIP **6** showed a strong binding ability to diclofenac. After loading 1 mL of 1 mg L^{-1} diclofenac solution onto the cartridge three times, only small amount of diclofenac was found in the eluted solution, most of the diclofenac was retained in the MIP cartridge. This MIP cartridge also showed a high capacity to the binding of diclofenac. Comparing the obtained HPLC chromatograms of NIP and MIP, we could conclude that the MIP **6** has a high binding affinity and capacity to the imprinted analyte diclofenac.

5 Synthesis of Imprinted Polymers for Diclofenac using 2-VP as the Functional Monomer

2-VP (pKa 5.06) is another often used functional monomer in molecular imprinting [225-229]. It is similar in physical and chemical properties to 4-VP (pKa 5.51) (shown in Table 15) [230]. It is also a colorless liquid with a distinctively putrid odor, and will be rapidly oxidized to red color when exposed in air. The purification of 2-VP is same as described for 4-VP in Chapter 4. The purified monomer was then stored under inert atmosphere at low temperature, until it was used for imprinted polymerization.

5.1 Synthesis of MIP with Different Porogens

To achieve the best binding performance, polymerization conditions have to be optimized, and these included the use of suitable functional monomers, cross-linkers and the porogen solvent. In our previous work, a different monomer composition with 4-VP as the functional monomer was tested. EGDMA was finally selected as the cross-linker for all the polymerization experiments in this project. Thus, only porogen solvents need to be evaluated and selected for optimization of the polymerization procedure. Previously, polar solvents such as H_2O, MeOH and ACN have been used in the imprinting process. However, to date, these polymers prepared with high polar solvents did not show really high binding selectivity for the template analyte during the binding tests. Thus, several low polar solvents such as DCM and toluene were tested in the MIP synthesis (shown in Tables 16-18) and 2-VP instead of 4-

VP was employed as the functional monomer. In addition, acidic monomer MAA was tested in the monomer mixture (2-VP/MAA).

Table 15: pH and pKa values of substituted pyridines

Compound No.	Substituent in pyridene nucleus	pH of sulfate soln. ± 0.01	pK_a, calculated from pH value ± 0.02	pK_a value according to the literature
1	2	3	4	5
1	Pyridine	3.20	5.14	5.17[4]
2	2-Methyl	3.87	6.12	5.97
3	3-Methyl	3.70	5.87	5.68
4	4-Methyl	3.89	6.15	6.02
5	2, 6-Dimethyl	4.28	6.73	6.75
6	2, 5-Dimethyl	4.09	6.45	6.55[6]
7	2, 4-Dimethyl	4.29	6.74	6.80
8	2-Methyl-5-ethyl	4.06	6.40	—
9	2, 4, 6-Trimethyl	4.89	7.45	7.45[3]
10	2-Chloro	0.61	1.31	—
11	3-Chloro	1.18	2.16	—
12	4-Chloro	1.62	2.80	—
13	2-Bromo	0.80	1.60	—
14	3-Bromo	1.21	2.20	—
15	2-Formyl	2.85	4.63	3.80[10]
16	3-Formyl	2.70	4.40	3.80
17	4-Formyl	3.10	4.99	4.77
18	2-Acetyl	1.17	2.14	—
19	3-Acetyl	1.79	3.06	—
20	2-Methoxycarbonyl	—	—	2.21[11]
21	3-Methoxycarbonyl	—	—	3.13
22	4-Methoxycarbonyl	—	—	3.26
23	3-Ethoxycarbonyl	1.53	2.67	—
24	4-Ethoxycarbonyl	1.80	3.07	—
25	3-Nitro	—	—	0.81[4]
26	3-Hydroxy	3.62	5.75	4.86[12]
27	2-Methyl-3-hydroxy	4.27	6.71	—
28	2-Methyl-5-hydroxy	4.02	6.34	—
29	2, 6-Dimethyl-3-hydroxy	4.92	7.67	—
30	2, 4, 6-Trimethyl-3-hydroxy	5.26	8.17	—
31	2-Propyl-3-hydroxy	4.55	7.13	—
32	2-Ethyl-6-methyl-3-hydroxy	4.74	7.41	—
33	2-Isobutyl-6-methyl-3-hydroxy	4.70	7.35	—
34	2-Isobutyl-3-hydroxy	4.50	7.05	—
35	N-oxide	1.01	1.90	0.79[13]
36	2-Methyl-N-oxide	1.50	2.63	—
37	3-Methyl-N-oxide	1.34	2.39	1.08
38	4-Methyl-N-oxide	1.66	2.86	1.29
39	3-Chloro-N-oxide	0.63	1.34	—
40	4-Nitro-N-oxide	0.47	1.11	(−1.7)
41	2-Vinyl	3.15	5.06	4.92[14]
42	4-Vinyl	3.46	5.51	—
43	2-Methyl-5-vinyl	3.71	5.83	—
44	2-Phenyl	—	—	4.48[15]
45	3-Phenyl	—	—	4.80
46	4-Phenyl	—	—	5.55
47	2-Methoxy	—	—	3.28[12]
48	3-Methoxy	—	—	4.88
49	4-Methoxy	4.51	7.06	6.62
50	2-α-Methylvinyl	3.52	5.62	—
51	2-α-Phenylvinyl	2.88	4.66	—
52	2-(1'-Cyclohexyl)	3.50	5.58	—
53	2-Phenoxy	1.14	2.10	—
54	2-p-Nitrophenyl	—	—	3.63[15]
55	2-o, p-Dinitrobenzyl	2.71	4.41	—
56	2-α-Hydroxybenzyl	2.80	4.55	—
57	2-Benzyl	3.26	5.22	—
58	2-Hydroxymethyl	3.58	5.70	—
59	2-Isobutyl	—	—	5.76[4]

Table 16: 2-VP/MAA functional monomer system using DCM as porogen

	MIP 7	NIP 7
Diclofenac (mg)	86	/
2-VP (µL)	130	130
MAA (µL)	110	110
DCM (mL)	2.0	2.0
EGDMA (mL)	1.2	1.2
AIBN (mg)	20.0	20.0

Table 17: 2-VP functional monomer system using toluene as porogen

	MIP 8	NIP 8
Diclofenac (mg)	89	/
2-VP (µL)	140	140
Toluene (mL)	1.2	1.2
EGDMA (mL)	1.38	1.38
AIBN (mg)	19.0	19.0

Table 18: 2-VP/MAA functional monomer system using ACN as porogen

	MIP 9	NIP 9
Diclofenac (mg)	86	/
2-VP (µL)	140	140
MAA (µL)	100	100
ACN (mL)	1.2	1.2
EGDMA (mL)	2.3	2.3
AIBN (mg)	20.0	20.0

5.2 Binding Characteristics of MIP 7

The first functional monomer employed for non-covalent molecularly imprinted polymer formation was MAA, which formed organic salt complexes with basic templates. Although the use of this monomer has been very successful in the preparation of imprinted polymers, there are many templates for which the use of monomer MAA is not successful. The largest group of templates in this field is those with acidic functionality, the carboxylic and phosphonic acid templates. Thus, basic monomers would provide the highest selectivity performance for carboxylate targets such as diclofenac. Pyridine monomer 2-VP was often examined for binding selectivity; 2-VP in combination with MAA (1:1 ratio) was examined in this experiment for improvement of binding effect. EGDMA was used as the cross-linker and DCM was selected as the porogen (Figure 28).

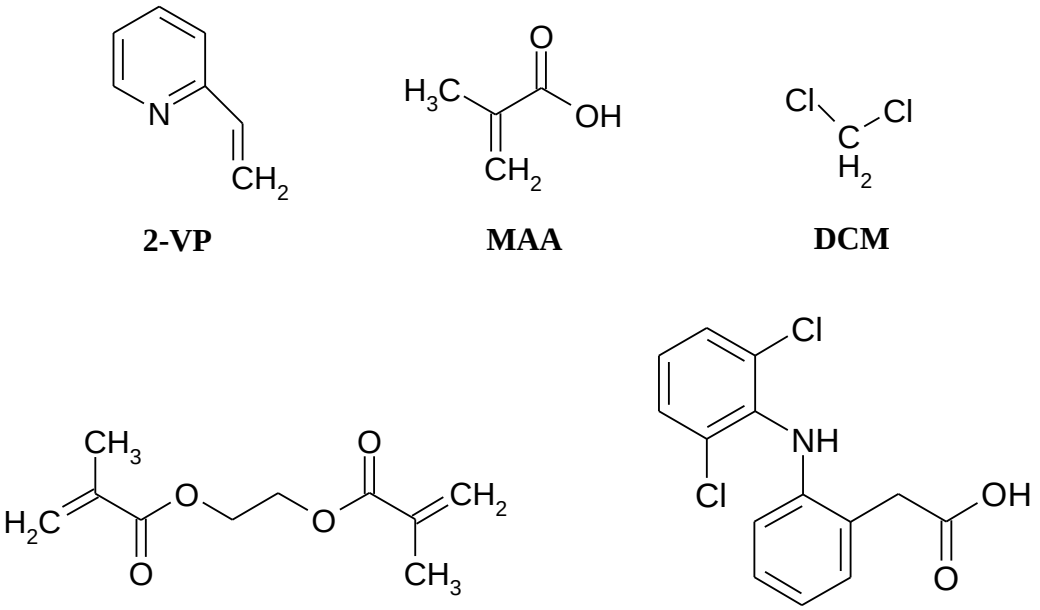


Figure 28: Synthesis of diclofenac imprinted polymers using 2-VP/MAA as the functional monomers, EGDMA as the cross-linker and DCM as the porogen.

After the polymers were extracted by MeOH/HOAc (9:1, v/v) and evaluated by HPLC, 100 mg of MIP **7** and NIP **7** particle with size of 32-63 µm were loaded into SPE cartridges for the binding test. Six diclofenac standard solutions (0.5 mg L^{-1}) with additions of different volumes of acetic acid (v/v) were prepared. The standard solution (1.0 mL) was forced to pass through the MIP and NIP cartridges, simultaneously. The binding affinity differences between MIP and NIP in the binding tests are summarized in Table 19.

Table 19: Effects of acetic acid on the binding of diclofenac (0.5 mg L^{-1}) to MIP and NIP cartridges

Acetic acid in ACN (%, v/v)	MIP 7 (%) retained	NIP 7 (%) retained	Difference (%)
0	100.0	90.5	9.5
0.1	92.6	70.4	22.1
0.2	88.4	62.4	25.9
0.5	80.2	56.1	24.0
1.0	74.8	44.1	30.7
2.0	63.1	38.2	24.9

From the binding results, it can be seen that the percentage of diclofenac retained in MIP **7** and NIP **7** cartridges decreased when the volume ratio of acetic acid in the diclofenac standard solution increased. When diclofenac without acetic acid was loaded to the polymer cartridges, 100% and 90% of diclofenac were retained in MIP and NIP cartridges, respectively. With the addition of acetic acid in the diclofenac solution, more diclofenac was dissociated from the polymers (Figure 29). When 0.5 % of acetic acid (v/v) was added in the diclofenac standard, the percentage of the rebound diclofenac in MIP **7** decreased to ~80%, and it further decreased to ~60% when 2% of acetic acid was present in the diclofenac solution.

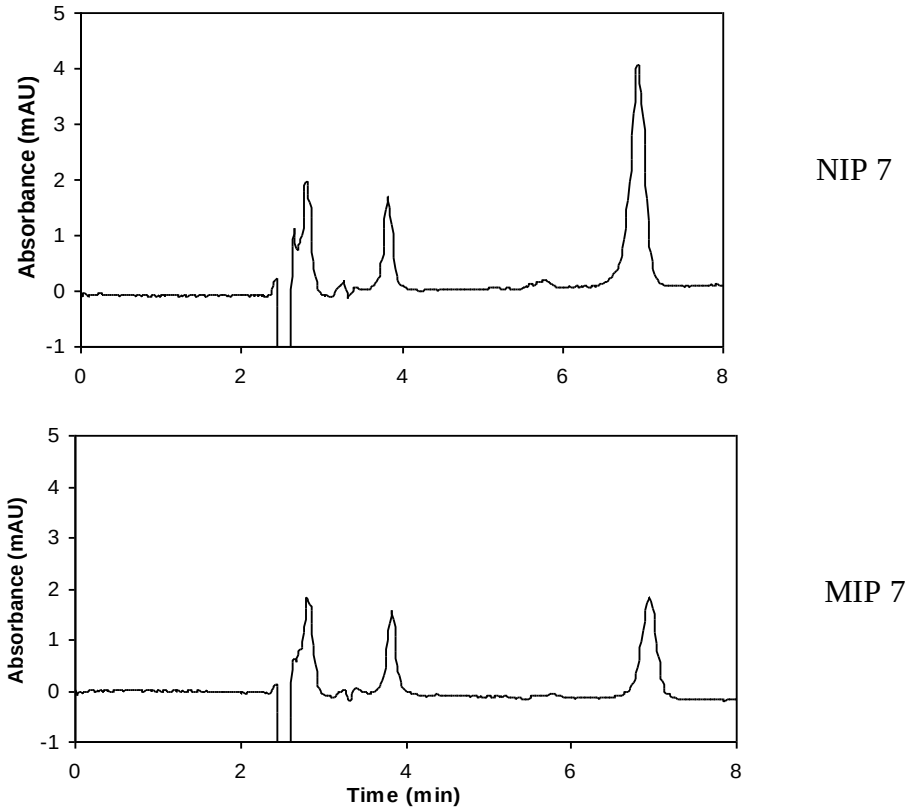


Figure 29: HPLC chromatograms obtained by analysis of flow-through of MIP 7 and NIP 7 cartridges, by binding of 1.0 mL of 0.5 mg L^{-1} of diclofenac solution with the addition of 1.0 % (v/v) acetic acid.

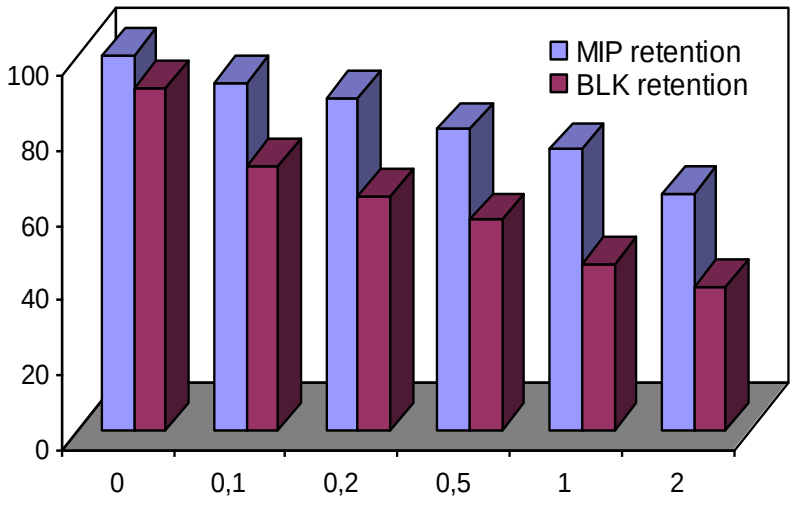


Figure 30: Effect of the increasing concentration of acetic acid on the binding performance of diclofenac to MIP 7 and NIP 7.

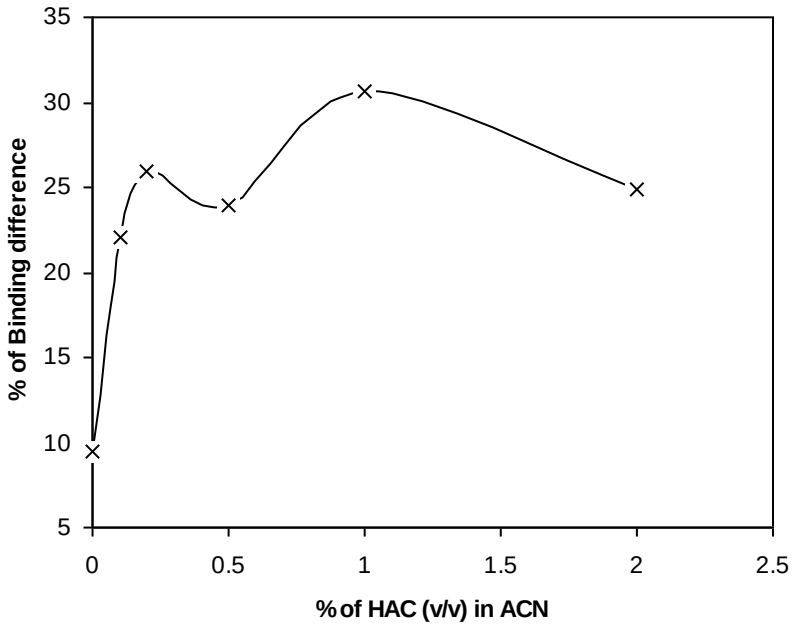


Figure 31: The effect of the volume of acetic acid in the sample on the diclofenac binding difference between MIP 7 and NIP 7 cartridges.

On the other hand, although the addition of acetic acid would increase the dissociation of diclofenac from both MIP **7** and NIP **7** cartridges, the degree of dissociation between MIP and NIP was significantly different. Apparently, the binding difference between MIP and NIP increased slightly when more acetic acid was added in the diclofenac solution (Figure 30). This relationship was demonstrated clearly in Figure 31. With the addition of 1% of acetic acid, the binding difference between MIP and NIP is the largest. It reached 31.7% as compared to binding difference in pure acetonitrile where only an 8.5 % difference was observed. From these results, it may be concluded that the binding mechanism of the MIP **7** is based on a so-called reversed phase interaction between the binding sites present in the polymeric matrix and the diclofenac molecules. This interaction is modulated by the presence of an ion pair between the pyridine ring in the binding site and the carboxyl group of the interacting molecule [231,232]. It follows that the polarity of the solvent directly influences the partition of the template in the MISPE column, so that the polymer MISPE cartridge behaves like a reversed phase system in which an increasing hydrophobicity may cause decease of the binding capacity. The decrease of the polymer binding capacity in the presence of acetic acid is a consequence of the mechanism of the molecular recognition [233-235]. The added acetic acid was able to interfere with the ion pair formation between the pyridine ring and the carboxyl group, and it reduced the retention of the template analyte in the MISPE. Of

course, in this process it also reduced the retention of the template in NISPE, as shown in Table 19.

5.3 Evaluation and Characterization of MIP 8

In previous experiments, different porogens such as MeOH, MeOH/H_2O, ACN and DCM have been used in the synthesis of diclofenac imprinted polymers. Although the polymers obtained have shown binding affinity of the template, a suitable washing solvent which could be used to separate the specific and non-specific binding had not been developed. In order to get an imprinted polymer that can be used for real applications, the non-polar solvent toluene was selected as porogen in the new polymer synthesis (Figure 32). For the preparation of MIP **8**, 89 mg of template diclofenac, and 136 mg of functional monomer 2-VP were dissolved in 1.2 mL of toluene in a 4 mL screw-capped glass vial. An amount of 1.421g of cross-linker EGDMA and 20 mg initiator AIBN were then added to the above solution. The solution was purged with nitrogen for 5 min. The glass vial was then sealed under nitrogen and was placed in a thermoblock TB1 (Biometra, Göttingen, Germany) at 60 °C for 24 h after incubation 20 min at room temperature.

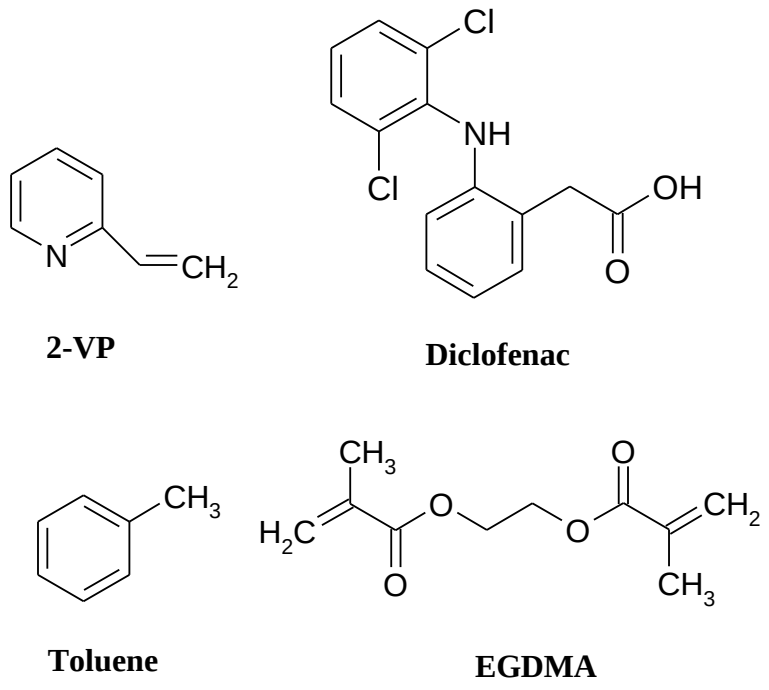


Figure 32: Synthesis of diclofenac imprinted polymer MIP 8 using 2-VP as the functional monomer, EGDMA as the cross-linker and toluene as the porogen solvent.

5.3.1 ^{1}H NMR Study of Interaction between Diclofenac and 2-VP

The UV study of the interaction between diclofenac and 2-VP in ACN showed that functional monomer 2-VP can interact effectively with diclofenac due to the formation of hydrogen bonding. To further evaluate and confirm the template-monomer interaction in the pre-polymerization solution, diclofenac and 2-VP mixed in different porogens was analyzed by ^{1}H NMR. To do so, a series of samples were prepared in corresponding porogen with a fixed concentration of diclofenac (50 mmol L^{-1}) and various concentrations of 2-VP (from 50 to 500 mmol L^{-1}). The ^{1}H NMR spectra were recorded at room temperature with a Bruker AMX 400 MHz (for NMR samples prepared in $CDCl_3$) and 600 MHz (for NMR samples prepared in any solvents beside $CDCl_3$) NMR spectrometers (Bruker, Karlsruhe, Germany).

5.3.1.1 Interaction of diclofenac and 2-VP in ACN-d_3

The interaction between diclofenac and 2-VP in ACN-d_3 (CD_3CN) was studied by ^{1}H NMR. The ^{1}H NMR spectra of diclofenac in the presence of various concentrations of 2-VP in CD_3CN is shown in Figure 33. The concentration of diclofenac was fixed at 50 mmol L^{-1}. Concentration of 2-VP was varied from 100 mmol L^{-1} to 500 mmol L^{-1} (shown in Table 20). There is only one amino proton in diclofenac, and from the ^{1}H NMR spectra it is seen that the chemical shift of the amino proton of NH is at 6.80 ppm in CD_3CN (Table 21). The effect of 2-VP on the chemical shift of the amino proton of diclofenac at room temperature can be seen clearly in Figure 35.

Table 20: Sample preparation of template diclofenac and functional monomer 2-VP for the ^{1}H NMR analysis in ACN-d_3

	Diclofenac (mg)	2-VP (µL)	Concentration of 2-VP (mmol L^{-1})
1	15		50
2	15	12	100
3	15	24	200
4	15	36	300
5	15	60	500

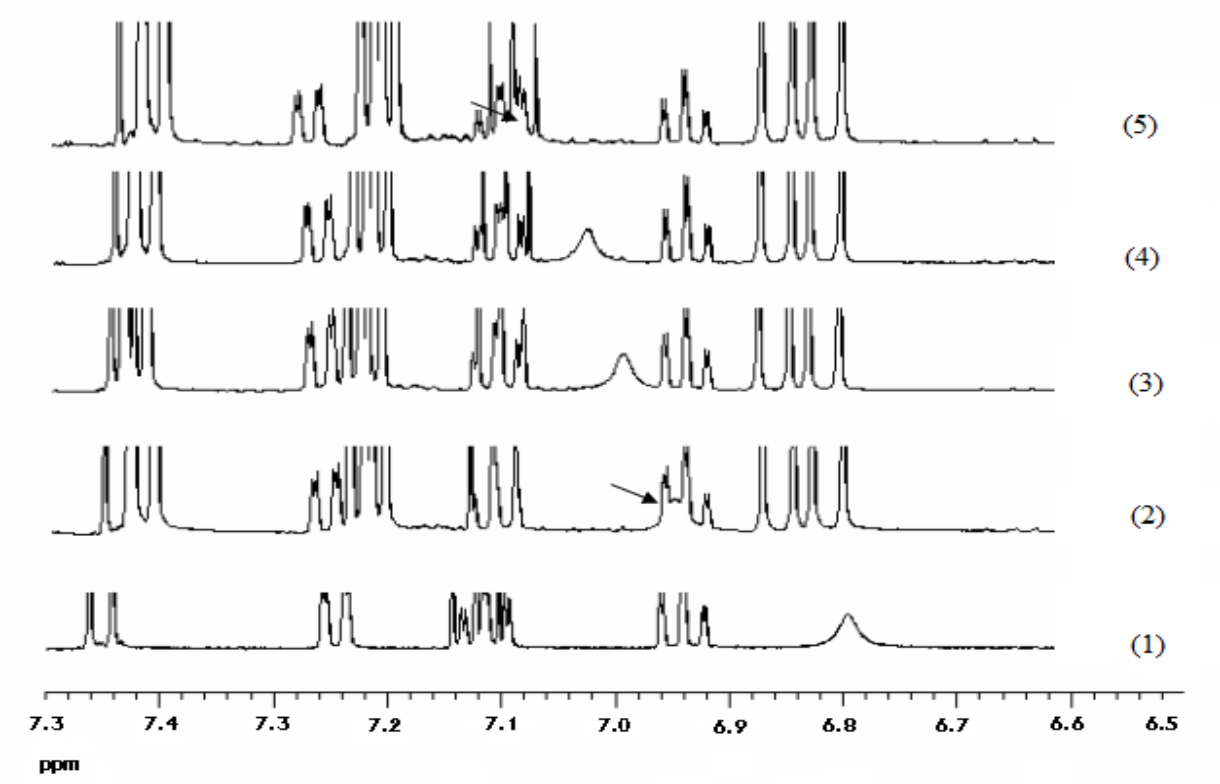


Figure 33: The ^{1}H NMR spectra of diclofenac in the presence of various concentrations of 2-VP in ACN-d_3 at room temperature. Concentration of diclofenac: 50 mmol L^{-1}; Concentration of 2-VP: (1) 0, (2) 100, (3) 200, (4) 300 and (5) 500 mmol L^{-1}. The NMR was recorded at 600 MHz.

Table 21: Chemical shift of the amino proton of diclofenac in ^{1}H NMR in ACN-d_3

Diclofenac (mmol L^{-1})	2-VP (mmol L^{-1})	Chemical shift (ppm)
50	0	6.80
50	100	6.96
50	200	7.00
50	300	7.04
50	500	7.06

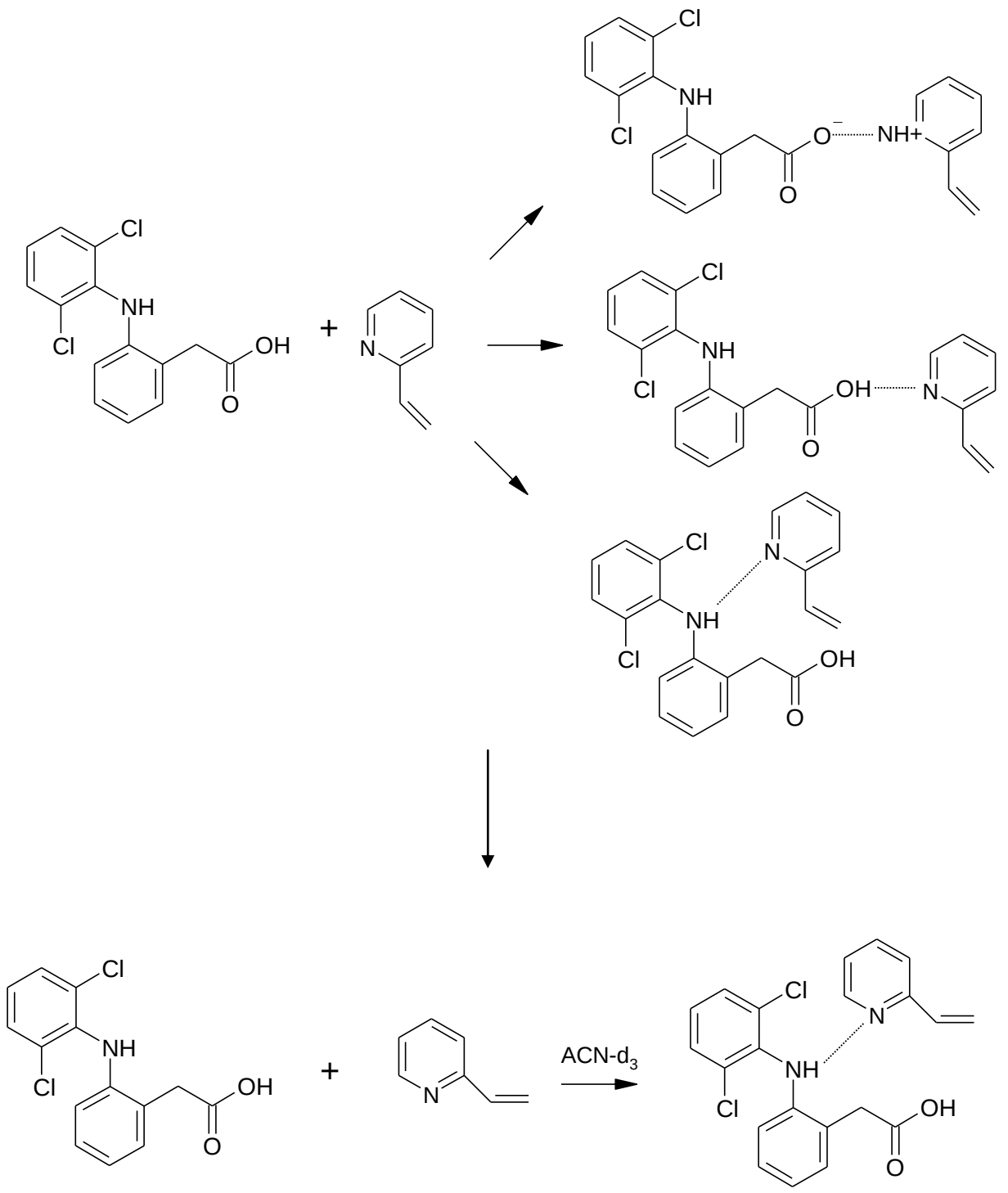


Figure 34: The interaction of diclofenac and 2-VP in ACN-d_3. A hydrogen bond was formed as revealed by the ^{1}H NMR analysis.

In this system, it was expected that the amino group of diclofenac would interact with the N atom in pyridine ring of 2-VP to form hydrogen bond; or the carboxyl group of diclofenac would interact with the N atom in the pyridine ring of 2-VP to form an ion-pair. From the ^{1}H NMR spectra, only a chemical shift of the amino proton of NH was observed. This observation clearly indicated that only hydrogen bonding was formed between diclofenac and 2-VP in acetonitrile (Figure 34).

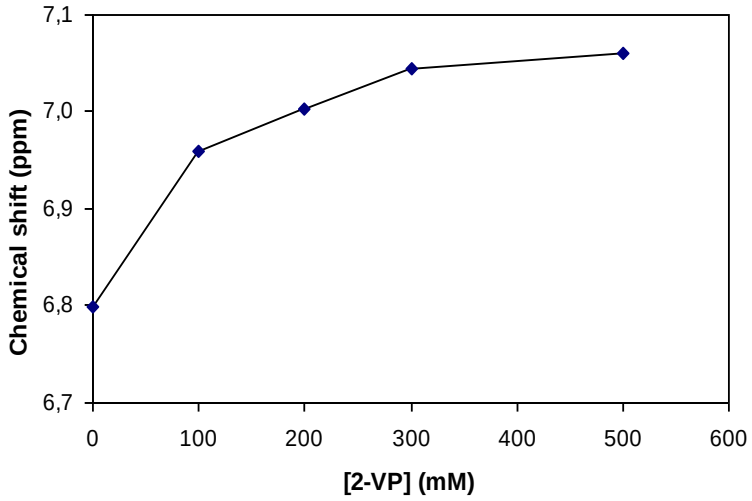


Figure 35: The effect of 2-VP concentration on the chemical shift of the amino protons of diclofenac in ^{1}H NMR in ACN-d3 at room temperature. Concentration of diclofenac: 50 mmol L^{-1}.

5.3.1.2 Interaction of diclofenac and 2-VP in chloroform-d_1

Beside solvent ACN, the interaction between diclofenac and 2-VP in deuterated chloroform ($CDCl_3$) was also studied by ^{1}H NMR. The concentration of diclofenac was fixed at 50 mmol L^{-1}. Concentration of 2-VP was varied from 50 mmol L^{-1} to 500 mmol L^{-1} (Table 22). The ^{1}H NMR spectra of diclofenac in the presence of various concentrations of 2-VP in $CDCl_3$ was shown in Figure 36. There is one carboxyl group in the diclofenac molecule, and the chemical shift of the proton of COOH is at 9.43 ppm in $CDCl_3$ (Table 23).

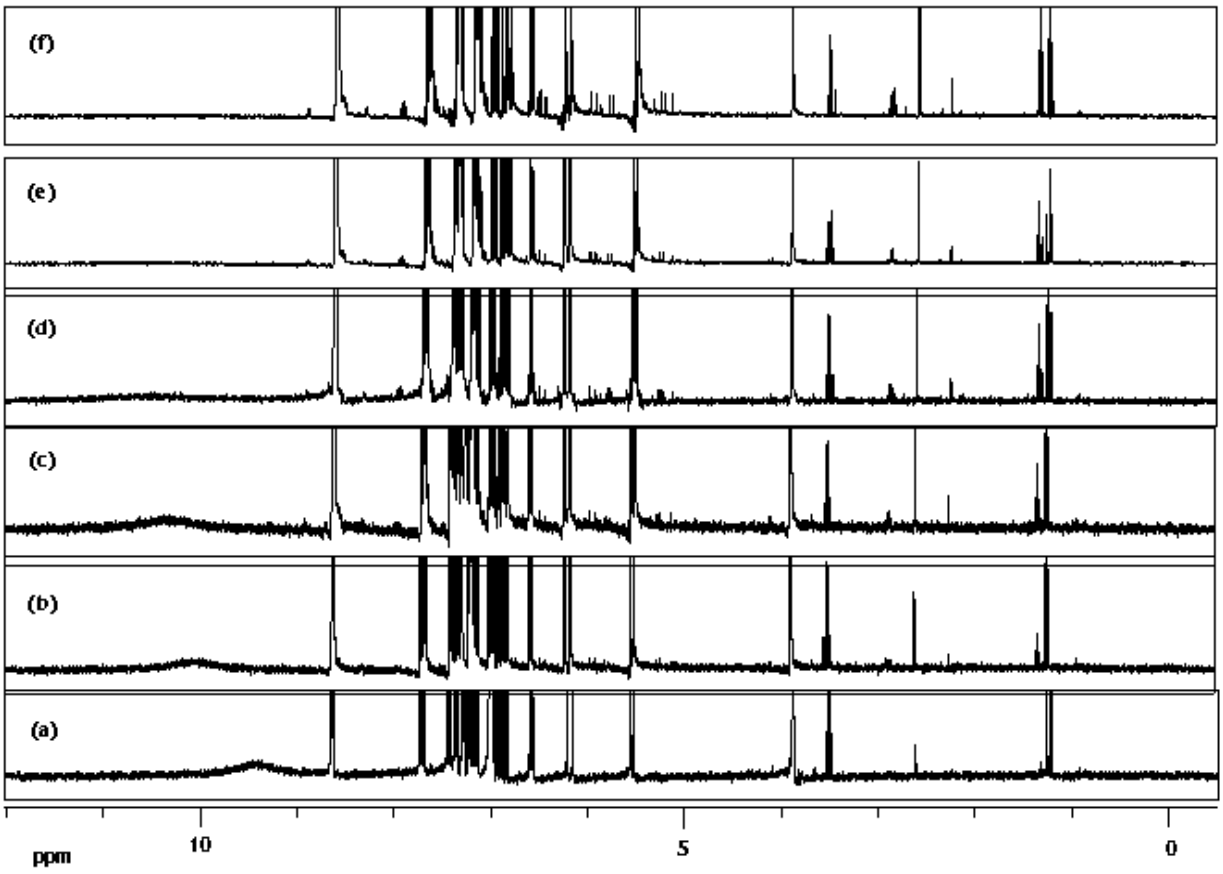


Figure 36: The ^{1}H NMR spectra of diclofenac in the presence of various concentration of 2-VP in $CDCl_3$ at room temperature. Concentration of diclofenac: 50 mmol L^{-1}; Concentration of 2-VP: (1) 50, (2) 100, (3) 200, (4) 300, (4) 400 and (5) 500 mmol L^{-1}. The NMR was recorded at 400 MHz.

Table 22: Sample preparation of template diclofenac and functional monomer 2-VP for the ^{1}H NMR analysis in chloroform-d_1

	Diclofenac (mg)	2-VP (µL)	2-VP concentration (mmol L^{-1})
1	15		50
2		6	50
3	15	12	100
4	15	24	200
5	15	36	300
6	15	48	400
7	15	60	500

Table 23: Summary of chemical shift of the carboxyl proton of diclofenac in ^{1}H NMR in chloroform-d_1

Diclofenac (mmol L^{-1})	2-VP (mmol L^{-1})	Chemical shift (ppm)
50	50	9.43
50	100	10.08
50	200	10.32
50	300	10.50

From the analysis of the ^{1}H NMR spectra, only the chemical shift of the carboxyl proton of COOH was observed. The chemical shift of the proton of the carboxyl group in the template-monomer mixture (1:1) is 9.43 ppm, and the chemical shift of the proton shifted downfield with the addition of more 2-VP in the mixture, until the proton of the mixture (1:6) downshifted to 10.5 ppm (Figure 37). It is well known that hydrogen bonding decreases the electron density around the proton, thus it moves the proton peak to higher frequency in the NMR field. According to this theory, the obtained ^{1}H NMR spectra clearly indicated that only hydrogen bonding was formed between diclofenac and 2-VP molecules in chloroform in the pre-polymerization complex (shown in Figure 38).

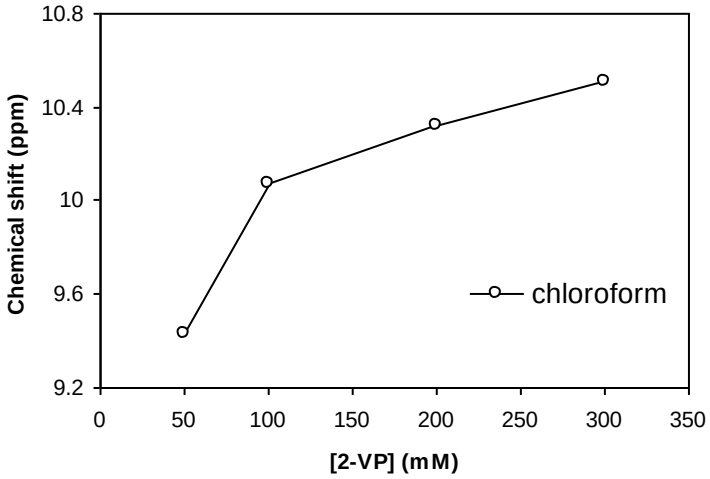


Figure 37: The effect of 2-VP concentration on the chemical shift of the carboxyl protons of diclofenac in ^{1}H NMR in $CDCl_3$ at room temperature. Concentration of diclofenac: 50 mmol L^{-1}.

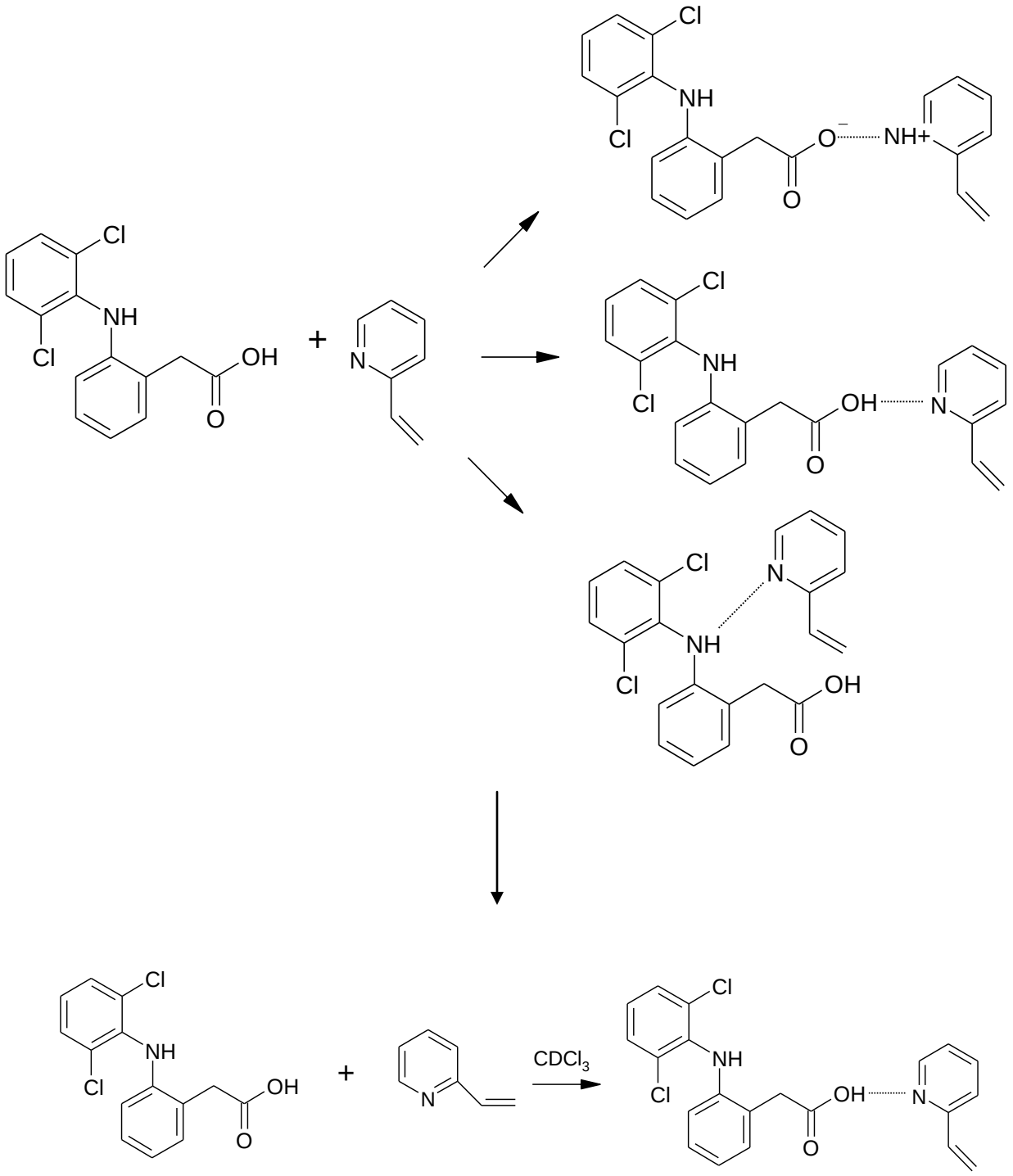


Figure 38: The interaction of diclofenac and 2-VP in $CDCl_3$. A hydrogen bonding was observed by the ^1H NMR analysis.

5.3.1.3 Interaction of diclofenac and 2-VP in toluene-d_8

The interaction between diclofenac and 2-VP in the pre-polymerization mixture in different porogens such as acetonitrile and chloroform were studied by ^{1}H NMR. According to the ^{1}H NMR analysis, with both porogens, the formation of hydrogen bonds was found between the template and the monomer. For imprinted polymer **8**, since the monomer system was prepared in porogen toluene, the template-monomer interaction was studied by ^{1}H NMR in toluene-d_8. The concentration of diclofenac was fixed at 50 mmol L^{-1}. The concentration of 2-VP was varied from 50 mmol L^{-1} to 500 mmol L^{-1} (shown in Table 24). The ^{1}H NMR spectra of diclofenac in the presence of various concentration of 2-VP in toluene-d_8 is shown in Figure 39 and 40.

Based on the results of the previous ^{1}H NMR studies, similar interactions were expected in the toluene. From the analysis of ^{1}H NMR spectra, two types of proton chemical shifts were observed, one is the proton chemical shift of the amino group in diclofenac; the other is the proton chemical shift of the carboxyl group of diclofenac.

Table 24: Sample preparation of template diclofenac and functional monomer 2-VP for the ^{1}H NMR analysis in toluene-d_8

	Diclofenac (mg)	2-VP (µL)	2-VP concentration (mmol L^{-1})
1	15	0	50
2	15	6	50
3	15	12	100
4	15	24	200
5	15	36	300
6	15	48	400
7	15	60	500

The proton chemical shift of amino group in diclofenac is 7.01 ppm in diclofenac solution without the addition of monomer 2-VP. The chemical shift of the proton of the amino group would shift downfield along with the addition of 2-VP in the template-monomer mixture. In the complex mixture of diclofenac/2-VP (1:10, v/v), the proton chemical shift was

downshifted to be 7.82 ppm. According to NMR theory, this observation clearly showed that a hydrogen bond was formed in the template-monomer pre-polymerization complex.

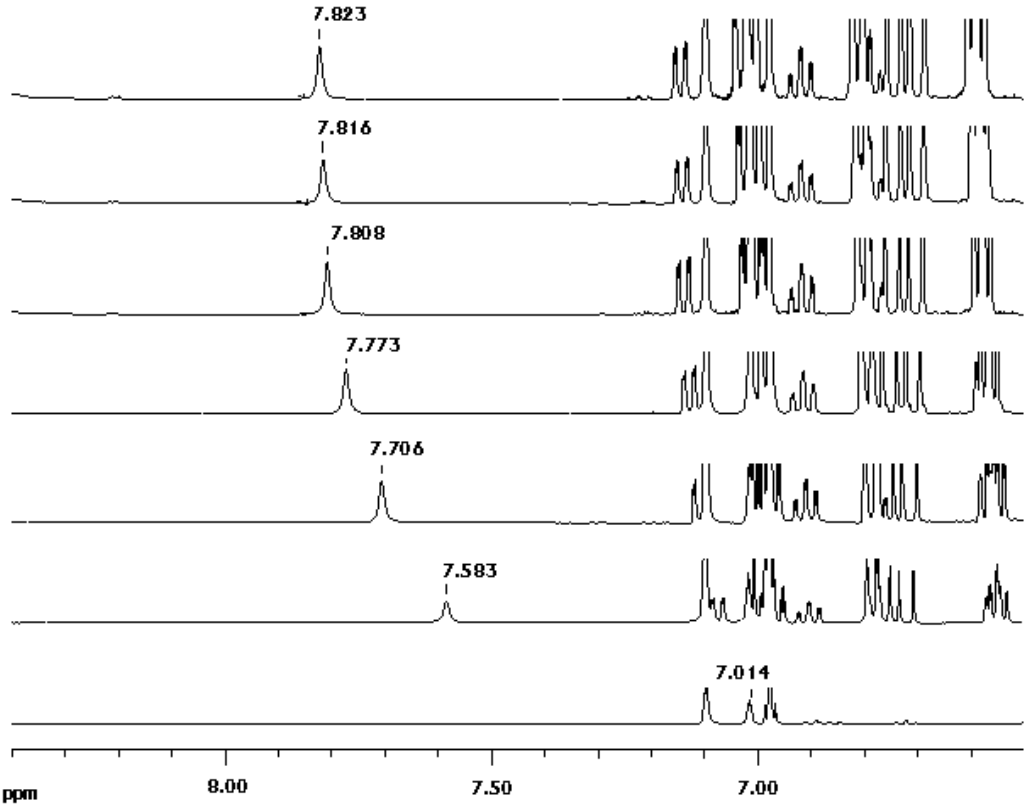


Figure 39: The ^{1}H NMR spectra of diclofenac in the presence of various concentration of 2-VP in the presence of various concentrations of 2-VP in toluene-d_8 at room temperature. Concentrations of diclofenac: 50 mmol L^{-1}; Concentration of 2-VP: (1) 50, (2) 100, (3) 200, (4) 300, (5) 400 and (6) 500 mmol L^{-1}. The NMR was recorded at 400 MHz.

Another interaction in this monomer system is formed due to the interaction of the carboxyl group of diclofenac and the N atom in the pyridine ring of 2-VP. According to our previous ^{1}H NMR study, this interaction force is either a hydrogen bond or an ion-pair. The following analysis proved that the acidic carboxyl group of diclofenac would interact with the N atom of the basic pyridine ring, forming an ionic pair. It was shown that the addition of different amounts of 2-VP to diclofenac solution resulted in an upfield chemical shift of the proton of the carboxyl group from 11.204 (0.05 mol L^{-1} diclofenac in the presence of 0.1 mol L^{-1} 2-VP) to 10.846 ppm (0.05 mol L^{-1} diclofenac in the presence of 0.5 mol L^{-1} 2-VP).

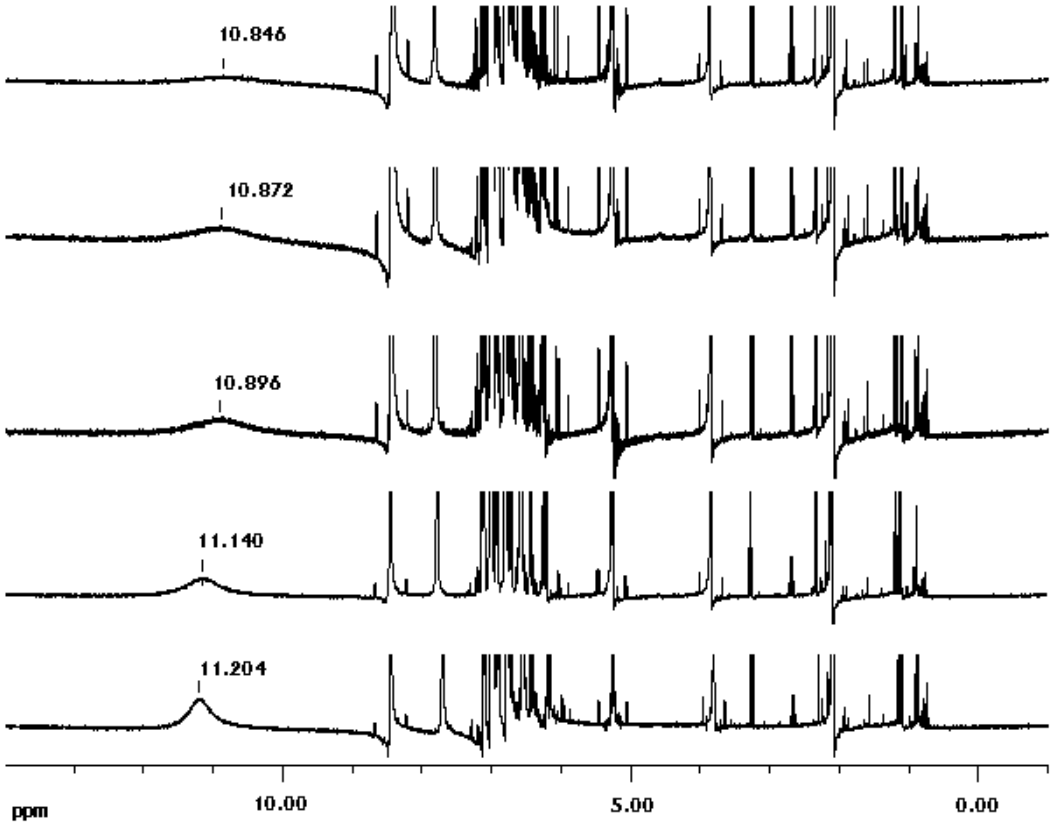


Figure 40: The ^{1}H NMR spectra of diclofenac in the presence of various concentration of 2-VP in toluene-d_8 at room temperature. Concentration of diclofenac: 50 mmol L^{-1}; Concentration of 2-VP: (1) 50, (2) 100, (3) 200, (4) 300, (5) 400 and (6) 500 mmol L^{-1}. The NMR was recorded at 400 MHz.

in solution becomes increasingly protonated. This observation suggests that the proton from the acidic carboxyl group of diclofenac molecule was involved in an ionic interaction. A similar finding was reported by O'Mahony et al. [76, 236, 237] who described the formation of an ion pair between 2,4-dichlorophenoxyacetic acid and 4-VP in deuterated chloroform. Thus, it can be concluded that at the pre-polymerization stage, at least two non-covalent bonds were formed between diclofenac and 2-VP in toluene d_8, one was ionic interaction and another one was hydrogen bonding. For comparison, O'Mahony et al. [176] reported on hydrogen bonding between carboxylic oxygen of naproxen and proton on the 4-vinylpyridine in corresponding NMR studies performed in chloroform. We hypothesize that additional stabilization caused by π-π stacking effects may also occur because of the aromatic nature of both the template and the functional monomer. However, this has to be proved by further

NMR studies. For example, the nature of the complex formation between the NSAID ibuprofen and naproxen with functional monomers 2-VP and 4-VP, respectively, was found to be a π-π stacking arrangement [238-242].

5.3.2 Binding Characterization by HPLC/DAD

The HPLC calibration curve for the quantitative measurement of diclofenac was set up with five diclofenac standards prepared in acetonitrile. The five standard solutions are: (1) 50 ppb, (2) 100 ppb, (3) 250 ppb, (4) 500 ppb and (5) 1000 ppb. Peak areas were used for quantification (Figure 41).

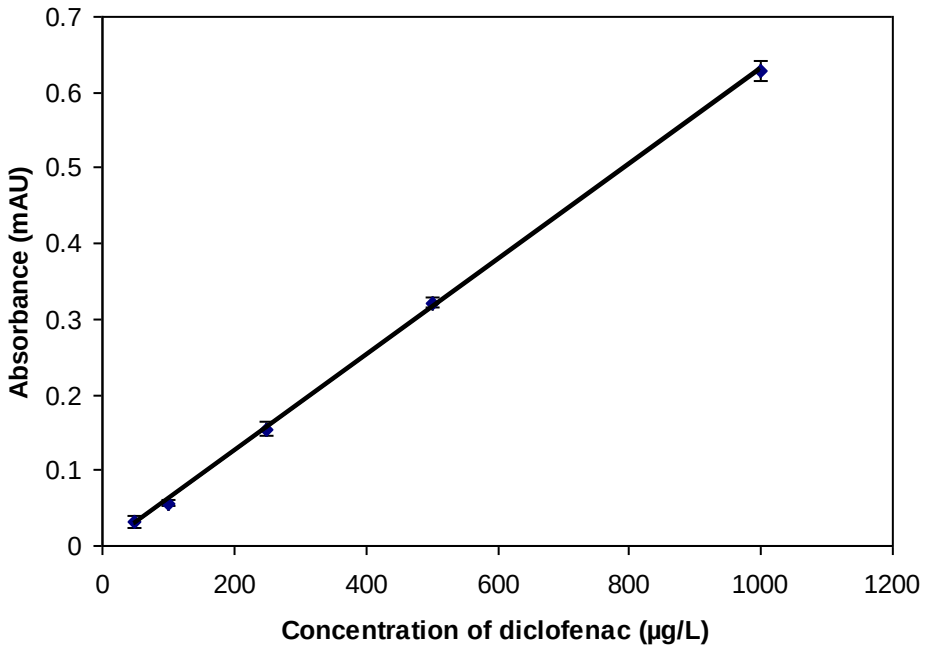


Figure 41: Calibration curve of five diclofenac standards prepared in ACN as obtained by HPLC/DAD. The regression equation of the calibration curve is: y = 0.0006x-0.002, r = 0.99, n = 15.

5.4 Binding Characteristics of MIP 8

As a preliminary examination of the new imprinted polymers and to confirm the equilibration time, a kinetic experiment was carried out to confirm that the template molecule can rebind in the imprinted polymer matrix and to estimate how long it takes to reach the binding equilibrium. The polymers were incubated with the template in ACN for a certain time (30 min) at room temperature, and the amount of diclofenac bound to the polymer was plotted as a function of time, the results are shown in Figure 42. For both high and low concentrations of diclofenac, the room temperature kinetics of the rebinding to MIP were the same. Equilibrium was reached at about 10 min, but it was almost established within 1 min (the amount of diclofenac bound to MIP at 1 min was 88% of that at the equilibrium). For the molecular recognition based on MIP, fast adsorption-desorption kinetics in the host-guest interactions is important. It can directly or indirectly affect the applications of MIP in various fields, such as chemical sensors, catalysis and chromatographic separations. Figure 42 also clearly shows that the adsorption of diclofenac on the non-imprinted polymers under the same experimental conditions was very low.

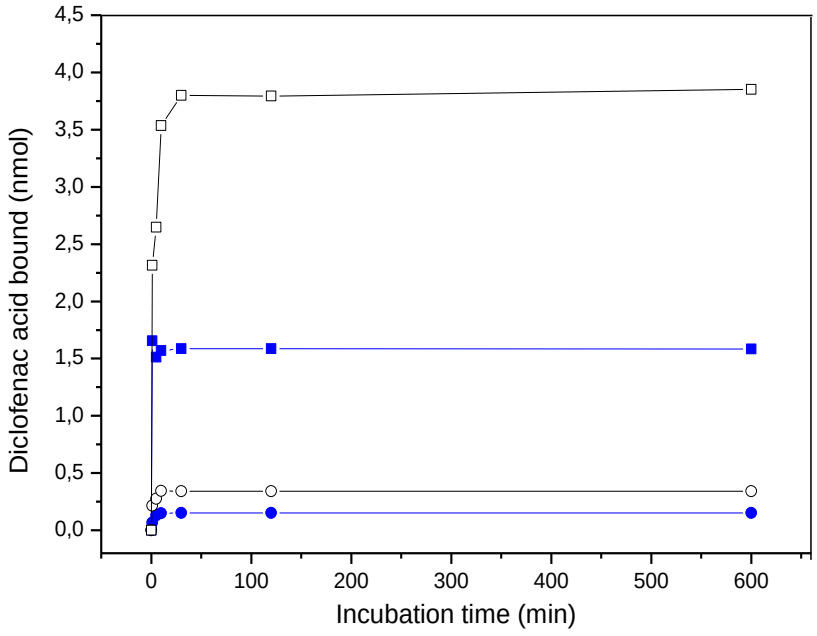


Figure 42: The influence of incubation time for the binding of MIP and diclofenac. The amount of MIP **8** (□, ○) and NIP **8** (■, ●): 10 mg; concentration of diclofenac: (□, ■), 20 µmol L^{-1}; (○, ●), 2 µmol L^{-1}; volume: 1.0 mL.

To study the binding characteristics of MIP **8**, a series of diclofenac standard solutions was prepared in acetonitrile. 1 mL aliquots of each solution were mixed with 10 mg of imprinted polymer particles in a 1.5 mL glass vial. The mixtures were incubated on a microplate shaker (EAS 2/4 from SLT Labinstruments, Crailsheim, Germany) for 30 min, and then rapidly filtrated. The diclofenac concentration in the filtrate was measured by HPLC/DAD. The amount of diclofenac bound to the MIP was calculated by subtracting the concentration of free diclofenac from the initial concentration.

In the attempt to investigate the affinity of the imprinted polymer for diclofenac acid, saturation binding experiments and subsequent Scatchard analysis were carried out. The binding isotherm of diclofenac acid to the imprinted polymer was determined to be in the range of 0-2.0 mmol L^{-1} diclofenac acid (initial concentration), the result are shown in Figure 43. The amount of diclofenac acid bound to the MIP at equilibrium *Q* increased along with increasing the initial concentration of diclofenac and reached saturation at higher diclofenac acid concentration. In many receptor binding isotherm experiments, because of linearly increasing non-specific binding a plot of bound analyte amount versus initial concentrations can not yield a typical saturation profile. However, the obtained binding isotherm in this work showed saturation at higher diclofenac concentration; this suggested that the template bound to MIP was more likely caused by the specific binding to a limited number of binding sites in the polymer network than by the non-specific adsorption.

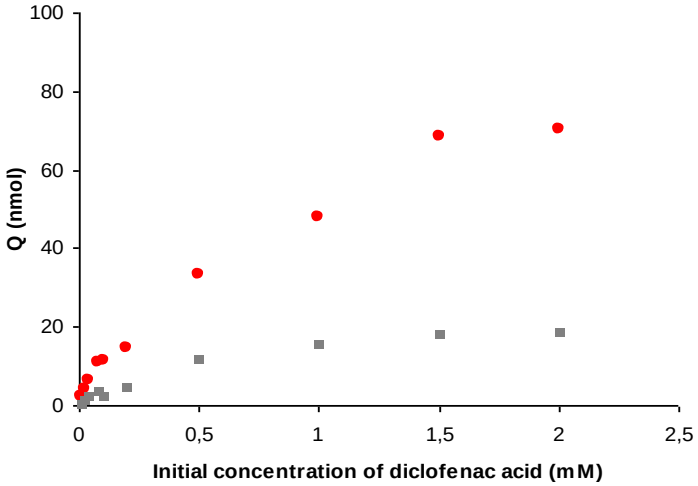


Figure 43: Binding isotherm of diclofenac imprinted polymer. Q is the amount of diclofenac bound to the polymer. Weight of polymer: 10 mg; volume: 1.0 mL; binding time: 30min. (●) MIP and (■) NIP.

The saturation binding data were further processed with Scatchard equation as described before to estimate the binding properties of MIP. As shown in Figure 44 (a), the Scatchard plot for MIP is not a single straight line, but consisted of two linear parts with different slope. The linear regression equations for the two linear regions are:

$$Q / [DCF] = -0.018\ Q + 0.345 \quad (r = 0.98;\ n = 18) \qquad (1)$$

$$Q / [DCF] = -6.99 \times 10^{-4}\ Q + 0.089 \quad (r = 0.95;\ n = 15) \qquad (2)$$

The unit of *Q* is nmol. This suggested that the binding sites in the MIP were heterogeneous in respect to the affinity for diclofenac acid [243,244], and indicated that the binding sites in the imprinted polymer could be classified into two distinct groups with specific binding properties. The K_D and Q_{max} of the higher affinity binding sites can be calculated to be 55.6 µmol $L^{-1}$and 19.1 µmol g^{-1} dry polymers, respectively, from the slope and the intercept of the Scatchard plot.

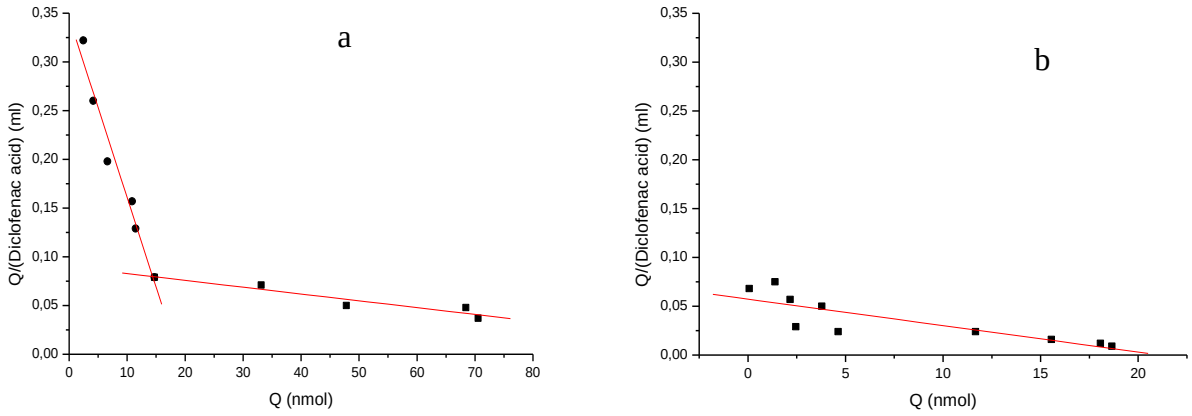


Figure 44: Scatchard plot analysis of the binding of diclofenac acid to the MIP and NIP. Q is the amount of diclofenac bound to polymers; (diclofenac acid) is the concentration of free diclofenac. (a), MIP and (b), NIP.

Similarly, the K_D and Q_{max} of the lower affinity binding sites were found to be 1.43 mmol L^{-1} and 127.2 µmol g^{-1} dry polymers, respectively. Most MIP prepared with non-covalent imprinting approach suffered from a heterogeneous distribution of binding sites, which was

primarily affected by two factors. First, because of amorphous nature of MIP, the binding sites were not identical, somewhat similar to a polyclonal preparation of antibodies. The sites may reside in domains with different cross-linking density and accessibility. The second effect was the incompleteness of the monomer-template association. In most cases only a part of the template associates with the functional monomer to produce selective binding sites due to the major part of the functional monomer existing in a free or dimerized form. The poor yield of binding sites results in a strong dependence of selectivity and binding on the sample load. As shown in Figure 44 (b), the Scatchard plot for NIP is nearly a single straight line. The linear regression equation for the linear regions is:

$$Q / [DCF] = -0.00271\ Q + 0.05725\ (r = 0.82;\ n = 30) \qquad (3)$$

This equation suggests that the binding sites in the NIP were relatively more homogeneous in respect to the affinity for diclofenac acid, compared to that of MIP, and indicated that the binding sites in NIP were caused mainly by non-specific physical absorption with non-specific binding properties. The K_D and Q_{max} of non-specific binding sites in NIP were calculated to be 370.3 µmol L^{-1}, and 21.1 µmol g^{-1} dry polymers, respectively, from the slope and the intercept of the Scatchard plot. The data of K_D and Q_{max} of MIP and NIP are summarized in Table 25.

Table 25: Data of K_D and Q_{max} in Scatchard plot analysis of MIP and NIP, in respect to affinity properties

	Q_{max} (µmol g^{-1})	K_D (µmol L^{-1})	Affinity
MIP [a]	19.1	55.6	high
MIP [b]	127.2	1430.6	low
NIP	21.1	370.3	low

Notes: MIP [a] is the part with higher binding affinity; MIP [b] is the other part with relative low binding affinity in the MIP

It may conclude that the binding site configuration in the MIP are heterogeneous in respect to the affinity for diclofenac and indicates that the binding sites could be classified into two distinct groups with different specific binding properties. We observed a similar finding for MIP prepared with the sulfonylurea herbicide metsulfuron-methyl [129]. The origin of molecular recognition in MIP are generally attributed to both shape selectivity and pre-organization of functional groups, however, the contributions of these effects are difficult to compare. An interesting systematic investigation on shape selectivity versus functional group pre-organization in MIP was reported by Simon et al. recently [245]. The results indicated that MIP prepared with templates with one or two template-functional monomer interactions rely heavily on shape selectivity interactions. Furthermore, a second observation was that greater selectivity is generally found for such templates. Considering the high selectivity of the prepared diclofenac MIP, it can be speculated that it is based on shape selectivity, mainly.

5.5 MISPE Procedure of MIP 8

Prior to any binding tests, the polymer particles of MIP **8** (size in 32-63µm) were washed with MeOH/HOAC (9:1, v/v) until no residual template was present in the polymer. Then a slurry of ~100 mg of MIP in 1 mL of MeOH was placed into an empty glass SPE cartridge (3 mL cartridge from J.T. Baker, Deventer, Holland). PTFE frits (Porosity 10 µm, Merck) were placed above and below the sorbent bed. As a control, a blank SPE column was also prepared in the same manner but with the blank polymer.

For binding tests, standard solutions of diclofenac (in ACN) were prepared in advance and stored in the freezer at temperature of -18 °C. A 1 mL 0.4 mg L^{-1} diclofenac standard solution was passed through the SPE column at a flow rate of ~0.5 mL min^{-1}, the effluent solution was collected. Then the cartridge was dried by attaching it to a vacuum manifold apparatus (model AI 6000, Analytical International, Harbor City, CA), which was connected to an oil vacuum pump, for about 20 min. Then the SPE cartridges (BLK and MIP) were washed with 2-3 mL of a CH_2Cl_2/ACN solution (92:8, v/v). The analytes retained in the MIP cartridge were eluted with 2.0-3.0 mL of MeOH. Both the washing and elution fractions were collected, and the washing fraction was dried using a gentle stream of nitrogen. The residues were redissolved in 1 mL of ACN and analyzed by HPLC/DAD.

As discussed above, the molecular recognition mechanism was considered to be due to both ionic interaction and hydrogen bonding between the template and the polymer. These characteristics of the MIP are important for its use as selective SPE material for trace enrichment of environmental samples. In such applications, an appropriate extraction protocol has to be developed and previously tested on artificial samples to consider the feasibility of the method. There are two different approaches possible. As in the traditional SPE, the extraction column can operate in "normal phase" mode or "reverse phase". With the latter, the elution of the interfering compounds from MIP is obtained by using an optimal washing solvent. This solvent must wash off the non-specifically adsorbed while still keeping the selectively retained fraction on the MIP. To evaluate the usefulness of this clean-up step, and to demonstrate that the polymer synthesized was indeed imprinted, a comparative analysis between NIP and MIP was carried out. Several non-polar solvents (toluene, hexane, and chloroform), polar aprotic solvents (DCM and ACN), and polar protic solvent (methanol) were tested in terms of washing efficiency and ease of sample handling. For optimizing the conditions of the washing step, a standard solution of diclofenac in ACN was applied to the MISPE and NISPE cartridges. After passage of the diclofenac acid standard solution, both the MISPE and the blank polymer columns were submitted to a washing step, which was carried out with 2 mL of either toluene, hexane, chloroform, DCM, ACN, or methanol. Next, the cartridges were eluted with 2 mL of methanol. Both the washing and elution fractions of the solvent were collected and analyzed; the results are shown in Figure 45.

It can be seen that the low polar organic solvents (toluene and hexane) cannot disrupt the non-specific binding between the polymer and diclofenac because all of the diclofenac acid was still retained on the NIP column after it was washed by using 2 mL of either toluene or hexane, while chloroform can only wash off small amount of diclofenac acid from blank column, and no diclofenac acid can be washed off from the MIP cartridge. DCM was proven to be the most effective washing solvent, though diclofenac could not be eluted from NIP completely. Conversely, both methanol and ACN could efficiently remove most non-specifically bound diclofenac from the NIP cartridge, however, the specific interaction between the template and the MIP was also disrupted. Thus, DCM was selected as the washing solvent.

When it was used at a volume of 3 mL, about 45% of diclofenac loaded on the blank cartridge was washed off while diclofenac bound on MIP was still retained. With an increase of the

volume of washing solvent to 6 mL, the amount of diclofenac eluted from the blank cartridge increased along but did not exceed 65%. Therefore, DCM was mixed with different volumes of ACN to increase the polarity stepwise (Figure 46 and 47). Figure 48 shows the recoveries of diclofenac in the washing fractions using 3 mL of washing solvent. As can be seen, the addition of ACN to the wash solution effectively eluted diclofenac from NIP cartridge. At a final ACN concentration of 6% (v/v) in DCM, diclofenac was completely eluted. On the contrary, specifically bound diclofenac on the MIP was retained if the ACN concentration did not exceed 7%. Therefore, 3 mL of DCM/ACN (94:6, v/v) were used as washing solvent. For the quantitative elution, 3 mL of DCM/MeOH (85:15, v/v) were revealed as optimal.

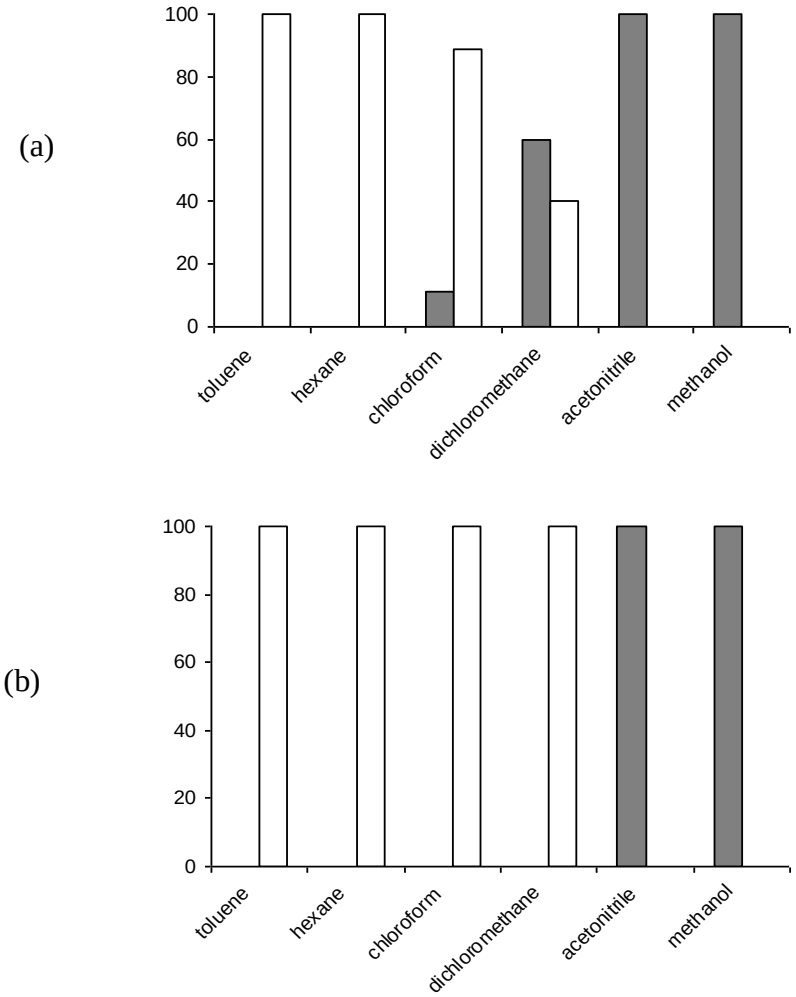


Figure 45: Recovery of diclofenac in the washing (shaded bars) and elution (open bars) fractions after loading 1 mL of 0.4 mg L^{-1} diclofenac solution onto: (a) NISPE and (b) MISPE cartridges; washing steps: 2 mL of each of the solvents. Elution steps: 2 mL of MeOH.

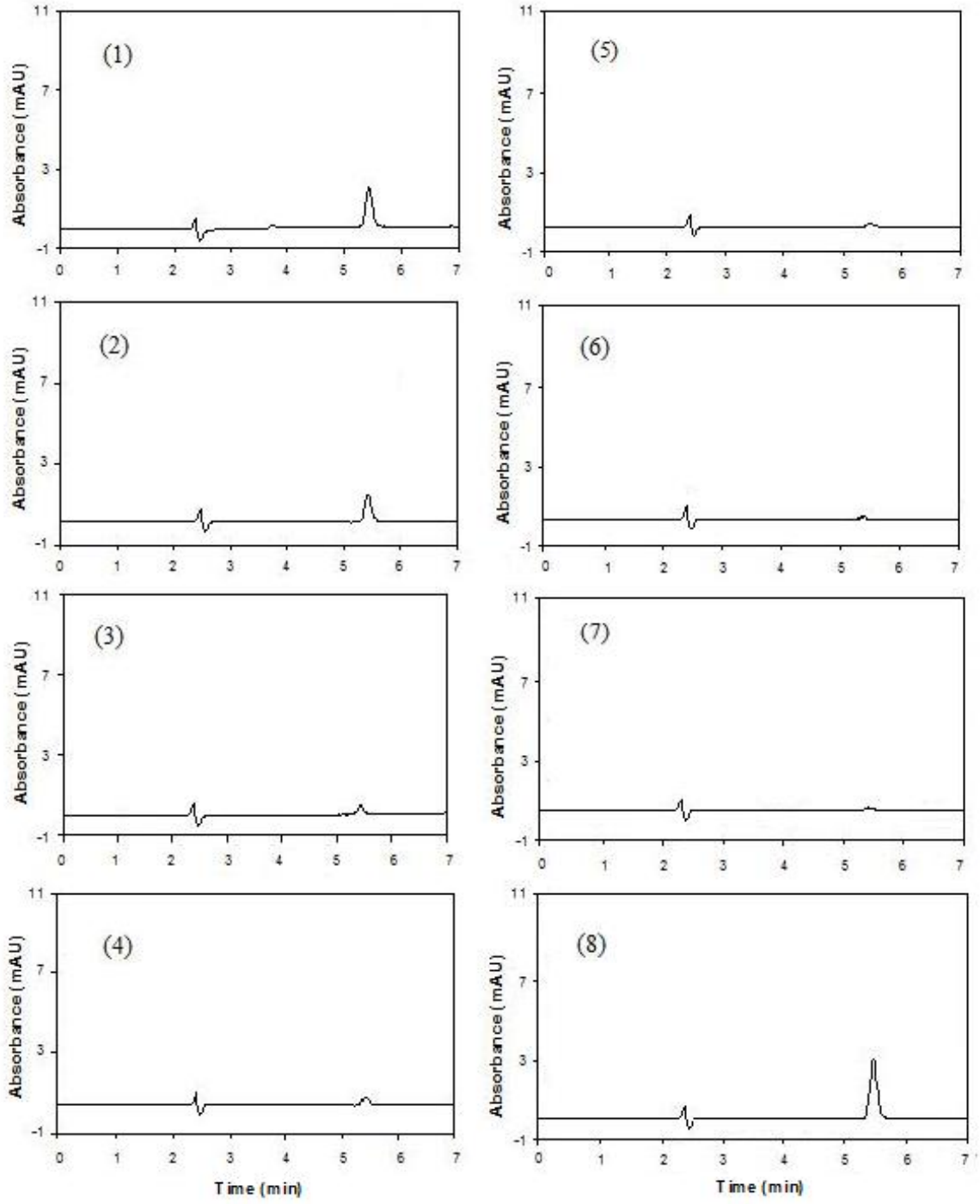


Figure 46: Chromatograms obtained from the washing fractions of MIP 8 and NIP 8 SPE cartridges with different washing solvent mixtures. (1) Loading fraction of 0.4 mg L^{-1} diclofenac from NIP cartridge; (2) 1st washing fraction of NIP by 1 mL DCM solvent; (3) 2nd washing fraction of NIP by 1 mL DCM solvent; (4) 3rd washing fraction of NIP by 2 mL DCM/ACN (98:2) solvent; (5) Loading fraction of 0.4 mg L^{-1} diclofenac from MIP cartridge; (6) 1st washing fraction of MIP by 1 mL DCM solvent; (7) 2nd washing fraction of MIP by 1 mL DCM solvent; (8) 3rd washing fraction of MIP by 2 mL DCM/ACN (98:2) solvent.

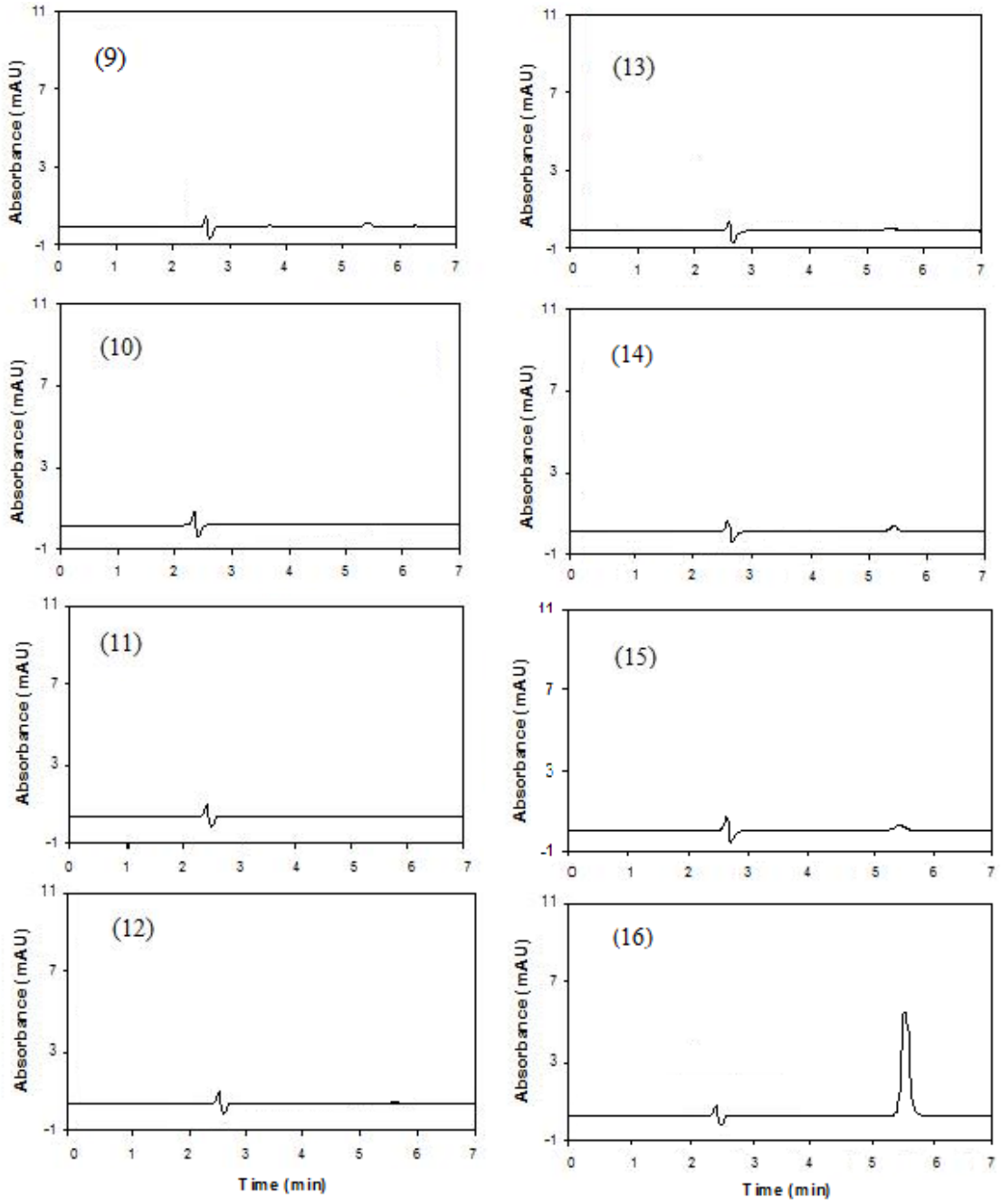


Figure 47: Chromatograms obtained from the washing fractions of MIP and NIP SPE cartridges with different washing solvent mixtures. (9) 4th washing fraction of NIP by 2 mL DCM/ACN (92:8) solvent; (10) 5th washing fraction of NIP by 2 mL DCM/ACN (91:9) solvent; (11) 6th washing fraction of NIP by 2 mL of DCM/ACN (90:10, v/v); (12) elution fraction of NIP cartridge by 2 mL of DCM/MeOH (90:10, v/v); (13) 4th washing fraction of MIP by 2 mL DCM/ACN (92:8) solvent; (14) 5th washing fraction of MIP by 2 mL DCM/ACN (91:9) solvent; (15) 6th washing fraction of MIP by 2 mL of DCM/ACN (90:10, v/v); (16) elution fraction of MIP cartridge by 2 mL of DCM/MeOH (90:10, v/v).

The mixtures of DCM with different concentration of ACN were tested as washing solvent because of the high ability of acetonitrile for disrupting the non-specific interaction of diclofenac and the polymer and its relative low polarity as compared with methanol. Figure 48 shows the recoveries of diclofenac in the washing fractions after pre-concentration on the blank and MIP cartridges by using 3 mL of each of the washing solvents. According to the figure, the amount of diclofenac removed from the NIP cartridge increased along with an increasing in the amount of acetonitrile in dichloromethane. Additionally, diclofenac was totally washed off the NISPE cartridge when the concentration of ACN in DCM is higher than 8% (v/v).

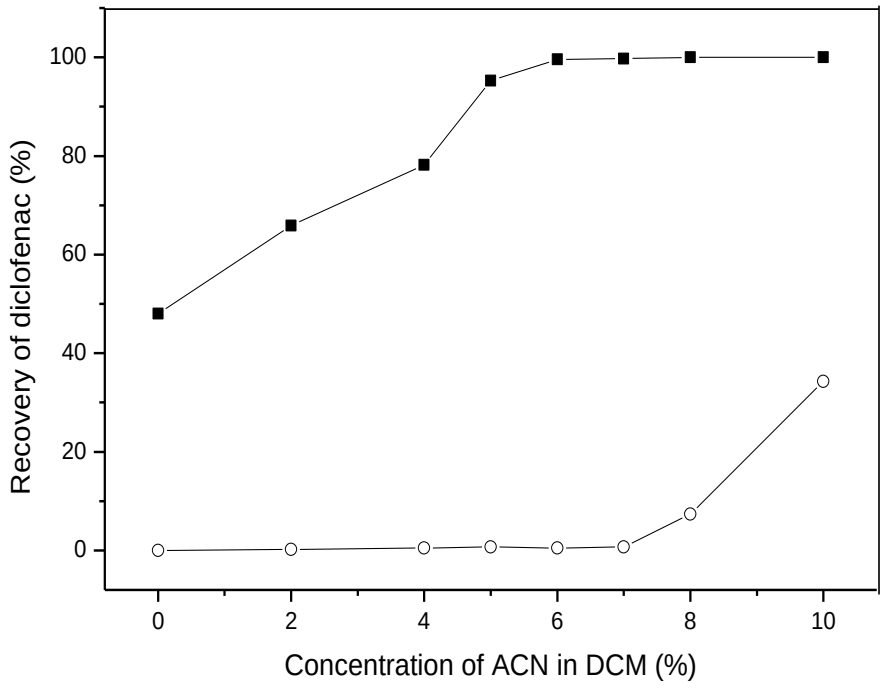


Figure 48: Recovery of diclofenac in the washing fractions after loading 1 mL of 0.4 mg L^{-1} diclofenac solution on the NISPE and MISPE cartridges with different concentration of ACN in DCM washing solution.

5.6 Selectivity Study of MIP 8

To estimate the selectivity of the diclofenac-imprinted polymer, several anti-inflammatory drugs with similar molecular structure such as ketoprofen, mefenamic acid and fenofibrate were evaluated by a comparison of their binding characteristics and diclofenac to the polymer. The molecular structures of these drugs are shown in Figure 49. A total of 1 mL of a mixture prepared in acetonitrile at a concentration of 0.2 mg L^{-1} of each compound was applied to the MIP and NIP cartridges. The washing and elution was performed as described and the compounds in both fractions were analyzed by gradient HPLC/DAD. The chromatograms of NSAID in standard solution, washing fraction, and elution solution are shown in Figure 50.

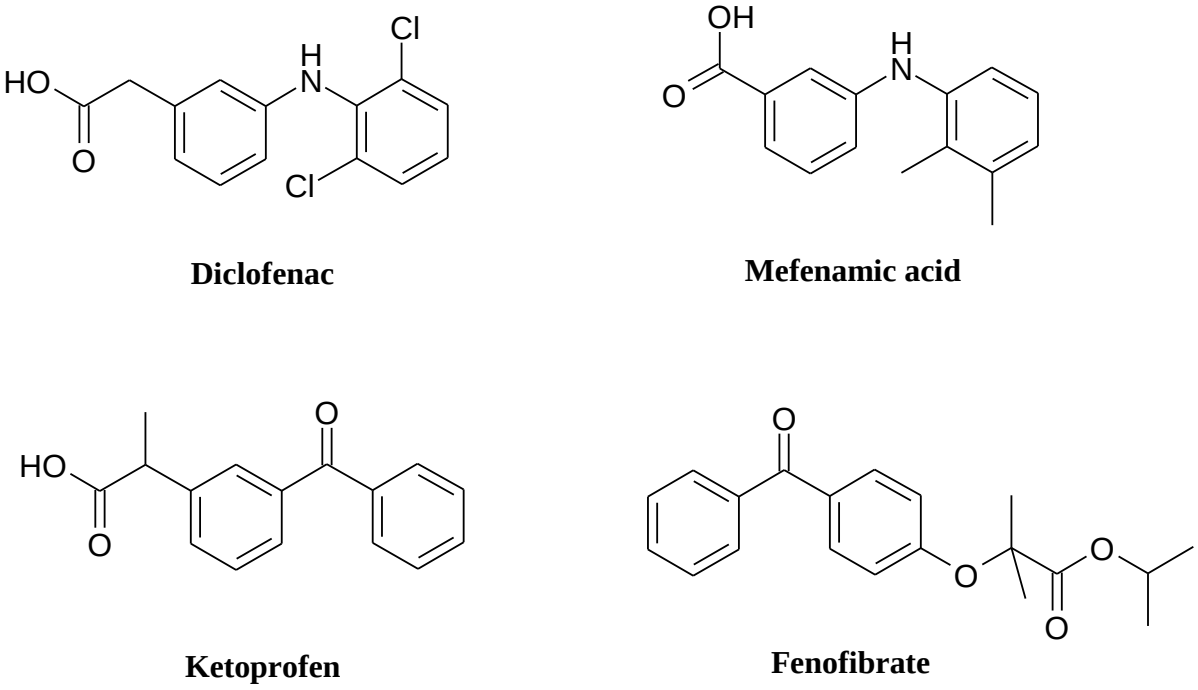


Figure 49: Structures of the NSAID used in the selectivity study, and diclofenac was used as the template molecule in the MIP preparation.

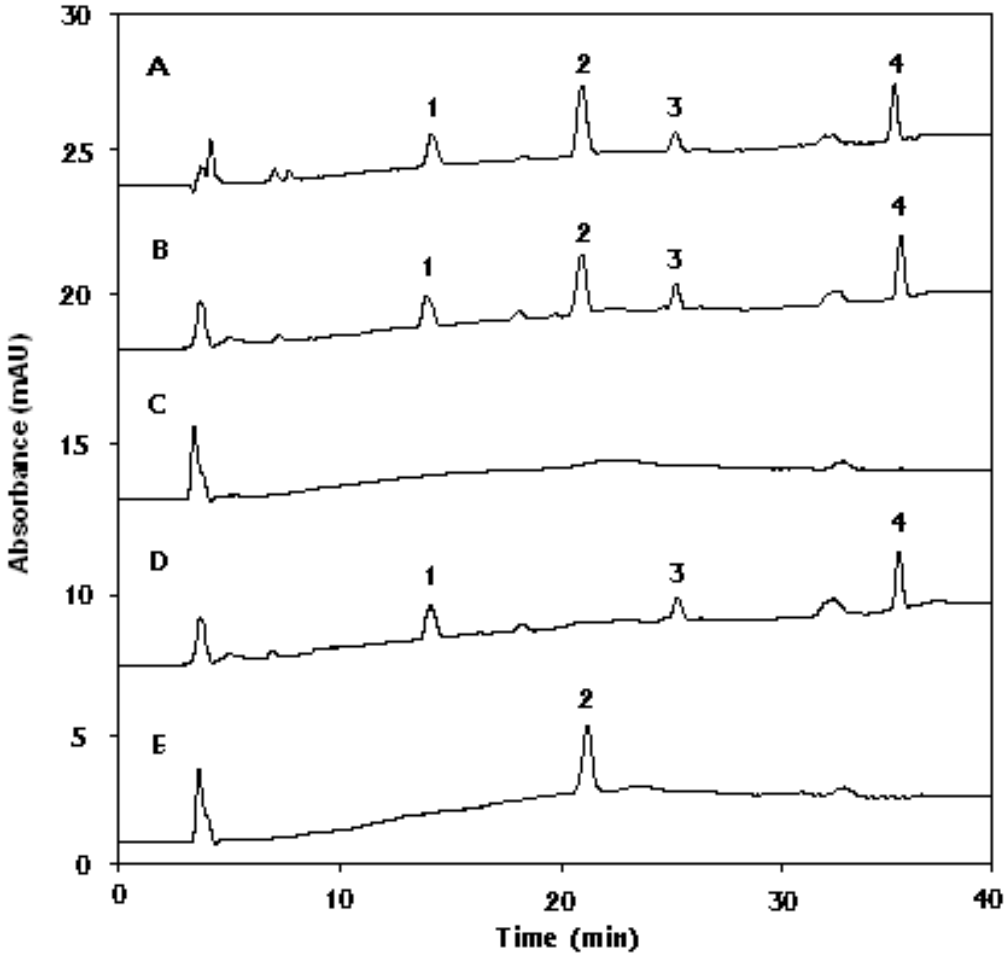


Figure 50: Chromatograms obtained upon percolating 1 mL of a mixture of anti-inflammatory drugs at concentration of 0.2 mg L^{-1} of each compound. (A) standard solution; (B) washing fraction, NIP; (C) elution fraction, NIP; (D) washing fraction, MIP; (E) elution fraction, MIP (1) ketoprofen, (2) diclofenac, (3) mefenamic acid, (4) fenofibrate. Washing solvent: 3 mL of DCM/ACN (94:6, v/v); Elution solvent: 3 mL of DCM/MeOH (85:15, v/v).

It can be seen from the figure that all the compounds including diclofenac were completely washed off from the NIP cartridge, however, a different result was observed for the MIP cartridge. All other compounds were completely washed off the cartridge except diclofenac, which was still selectively retained on the MIP after the washing step. The selectivity of MIP to diclofenac can be further proved from the elution fraction of MIP. Only diclofenac was recovered in the elution fraction; other compounds were removed completely from the polymer in the washing step. The recoveries of these compounds were higher than 98%

(Table 26) in the washing fraction. While, only the recovery of diclofenac was higher than 98% in elution fraction, other compounds cannot be recognized by the MIP and were completely separated from the template analyte even though their structure is similar to that of diclofenac. These results show that the MIP exhibited highly selective binding affinity for diclofenac and demonstrated that the adsorption of diclofenac was due to imprinted binding sites and not due to non-specific binding. From Figure 50, it can be seen that there is only slight difference between the structure of diclofenac and those of NSAID drugs. For mefenamic acid, the structural difference is two chlorines instead of two methyl groups as in diclofenac. For ketoprofen, the difference is a vinyl instead of secondary amino group between the benzene rings. Thus it would be reasonable to assume that the NSAID drugs would not be able to fit into the specific binding sites in the polymer network. This further demonstrates that the imprinting is not only based on the interaction of the functional groups of the analyte with those binding sites in the polymer cavities but also based on the combined effect of shape and size complementarities.

Table 26: Recovery (%) obtained after loading 1 mL of 0.2 mg L^{-1} of the mixture the anti-inflammatory drugs on the MIP and NIP cartridges (n=3)

	NIP (% ± SD)		MIP (% ± SD)	
Analytes	**Washing**	**Elution**	**Washing**	**Elution**
ketoprofen	98 ± 3.1	0	99 ± 2.6	0
diclofenac	99 ± 3.6	0	0	99 ± 2.5
mefenamic acid	98 ± 4.7	0	98 ± 2.5	0
fenofibrate	97 ± 4.2	0	100 ± 3.1	0

6 Analysis of Environmental Water Samples

To estimate the binding ability of the prepared MIP **8** cartridge on water samples, 5 mL of Milli-Q water (ultra-pure) spiked with 20.0 µg L^{-1} of diclofenac (free acid) was loaded onto the NISPE and MISPE cartridges, respectively. Both cartridges were dried under vacuum at room temperature for 15 min to remove water residues thoroughly. After the washing and elution steps, the washing and elution fractions were analyzed by HPLC/DAD analysis. This procedure was done for setting a basis study on the extraction of diclofenac with MISPE from real water samples such as drink water, surface water and wastewater samples.

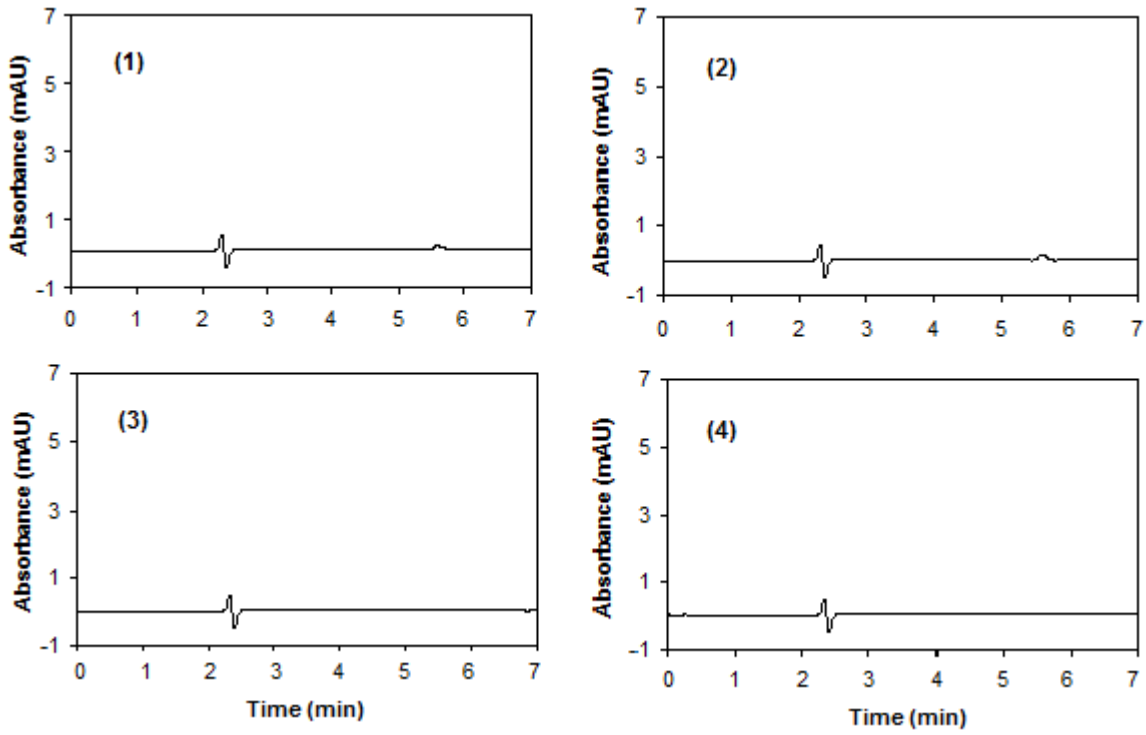


Figure 51: HPLC chromatograms obtained from the washing fractions and elution fraction of the spiked diclofenac extraction from Milli-Q water by the NISPE 8 cartridge. (1) Washing fraction of DCM, (2) washing fraction of DCM/ACN (94:6, v/v), (3) washing fraction of DCM/ACN (90:10, v/v) and (4) DCM/MeOH (85:15, v/v).

As shown in Figures 51, during the washing procedure diclofenac analyte was washed off the NISPE cartridge when DCM was the washing solvent. More diclofenac was recovered in the washing fraction along with increasing the amount of ACN in the washing solvent mixture. The NISPE cartridge was then eluted with 3 mL DCM/MeOH (85:15, v/v) solvent, and no

diclofenac was found in the elution fraction. This indicated that all diclofenac was washed off the cartridge, and it was apparent that the washing solvent effectively disrupted the non-specific interaction between the template and the functional monomer.

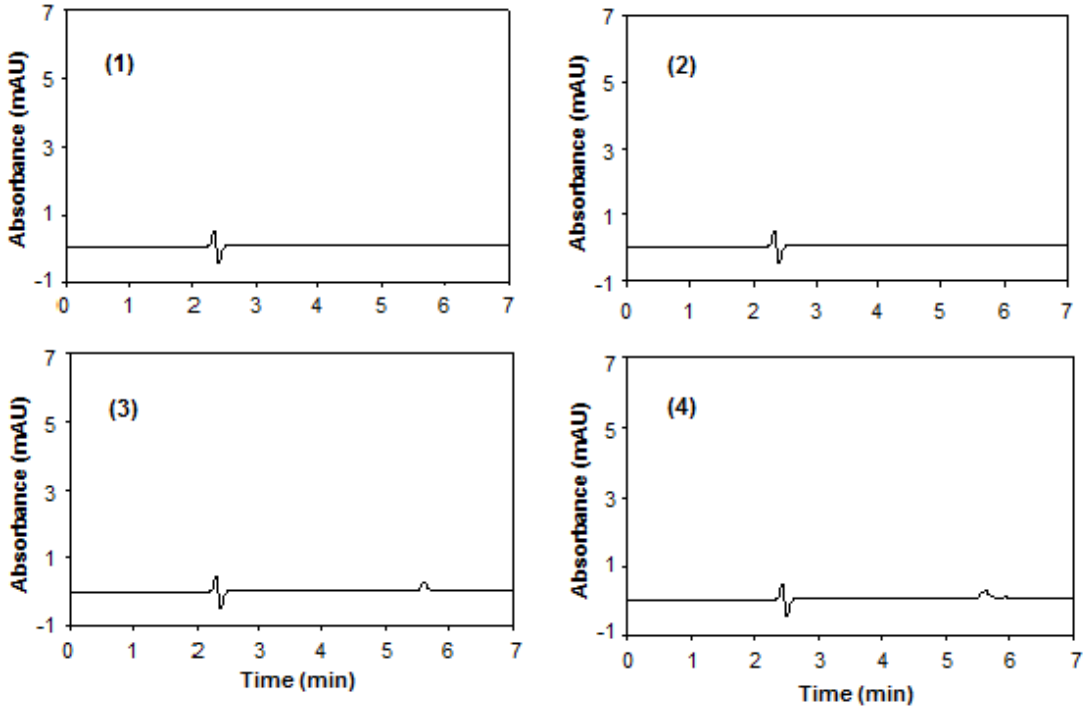


Figure 52: HPLC chromatograms obtained from the washing fractions and elution fraction of the spiked diclofenac extraction from Milli-Q water by the MISPE 8 cartridge. (1) Washing fraction of DCM, (2) washing fraction of DCM/ACN (94:6, v/v), (3) washing fraction of DCM/ACN (90:10, v/v) and (4) DCM/MeOH (85:15, v/v).

From the HPLC analysis shown in Figures 52, as seen under the same washing conditions, MISPE showed a different binding performance on the template diclofenac. In the 1st and 2nd washing steps, no diclofenac was recovered in the washing fraction. Only when acetonitrile in the washing solvent was increased to 10% (v/v), diclofenac was found in the washing fraction. This observation suggested again that the optimized washing solvent DCM/ACN (94:6, v/v) was suitable on the separation of non-specific and specific binding between NISPE and MISPE cartridges. The retained diclofenac in MISPE was eluted out of the cartridge by DCM/MeOH (85:15, v/v) elution solvent.

To demonstrate the applicability and reliability of this method for environmental application, the real environmental samples, such as drinking water (tap water), surface water and wastewater samples were collected and analyzed for diclofenac binding tests. First, tap water was tested by spiking it with diclofenac at the 20 µg L^{-1} concentration level and was pre-concentrated by MISPE. After drying SPE cartridges with vacuum, a clean-up step was done to disrupt the non-specific interaction between NIP and diclofenac, and meanwhile all the analytes were retained in the MIP.

6.1 Matrix Effect

When the MISPE and blank polymer cartridges were directly applied to extract the target analytes from the tap water samples, it was found that the non-specific binding of the analytes on the polymer became so strong that the washing solvent was no longer able to remove the analytes from the blank polymer cartridge. Taking into consideration that the organic compounds in the real water matrix normally showed either no effect or a reduced effect on the performance of MIP, this phenomenon must have been caused by other matrix constituents. Normally, in Munich area, the inorganic ions such as Ca^{2+} are in a high concentration level in the environmental waters, thus the effect of the inorganic ion Ca^{2+} should be the reason. Similar results were obtained in a former study done in our laboratory on MISPE extraction using a metsulfuron-methyl-imprinted polymer [246]. The reason is that the formation of coordinate covalent bonds between functional groups of monomer and template with divalent cations e.g. Ca^{2+} and Mg^{2+} was elucidated. This phenomenon could be explained by the fact that Ca^{2+} ion could form a complex with diclofenac and the monomer 2-vinylpyridine in the polymer body (as shown in Figure 53). It is known from coordination chemistry that -COOH and -NH groups are efficient ligands for divalent metal ions such as Ca^{2+}, Mg^{2+}. They can form coordinate covalent bonds with these metal ions. In this system, the amine group -NH and carboxyl group -COOH on diclofenac can form coordinate bonds with Ca^{2+}. Meanwhile, the Ca^{2+} ion can also form coordinate bonds with the N atom of 2-vinylpyridine in the polymer. Thus, diclofenac was bound to the polymer *via* coordinate bonds with Ca^{2+}, which acted as a "connection bridge".

A coordinate covalent bond is much stronger than a non-covalent bond, which is why the blank polymer cartridge showed a very strong non-specific binding when it was applied to

extract diclofenac in the real water samples. The problem could be solved by masking the metal cations with addition of EDTA to the real water samples.

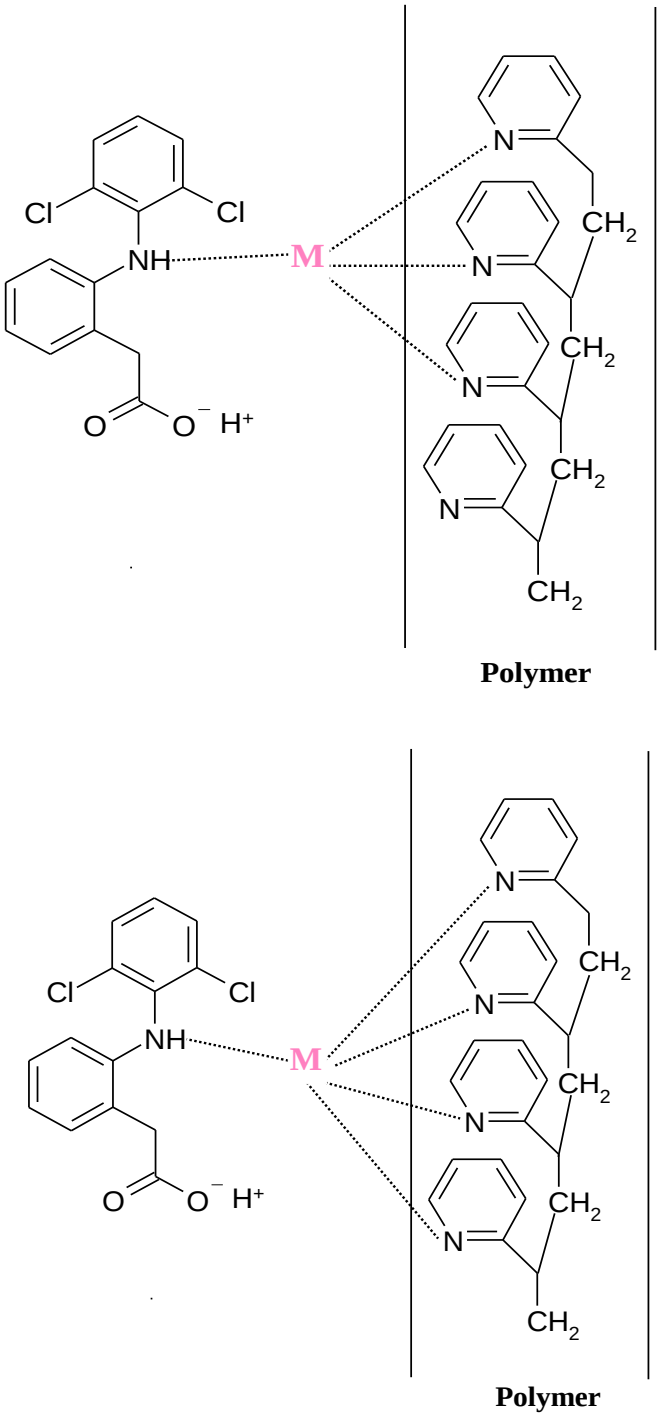


Figure 53: Proposed formation of coordinative bonds between template diclofenac, the divalent metal ions and the polymers. **M** represents divalent metal ions such as Ca^{2+} and Mg^{2+} [247-249].

6.2 Chelating Agent for Masking Divalent Cations in Water

EDTA is an acronym for the widely used chemical compound ethylenediamine tetraacetic acid, which is used as the divalent cation chelating agent with a chemical formula $(HO_2CCH_2)_2NCH_2CH_2N(CH_2CO_2H)_2$. This organic acid is widely applied to sequester di- and trivalent metal ions in water. EDTA binds to metals via four carboxylate and two amine groups. Normally, EDTA forms specially strong complexes with Mn(II), Cu(II), Fe(III), Co(III), Ca (II) and Mg(II) etc metal ions (Figure 54). The EDTA family also includes EDTA disodium and tetrasodium salts, which are easily soluble in water.

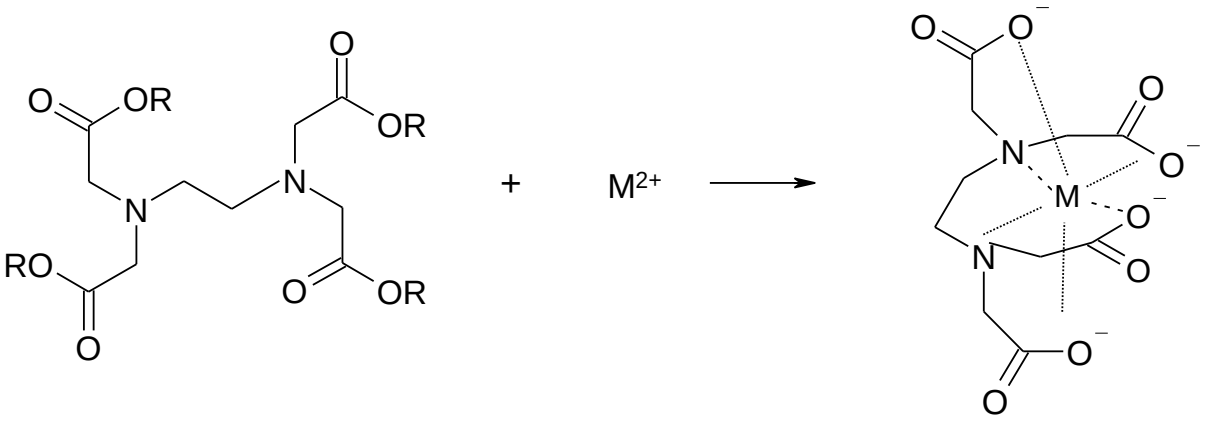


Figure 54: Reaction scheme of divalent metal ions and EDTA. R: represents H or Na^+; M^{2+}: represents divalent metal ions such as Ca^{2+}, Mg^{2+}.

6.3 Evaluation of EDTA as the Chelating Agent

There are several forms of EDTA chemicals which are commercially available to be used as the chelating agent for calcium and magnesium ions in environmental water samples. The solubility of EDTA (free acid) in water is normally very low (~0.5 g L^{-1}). Compared to the EDTA free acid, the disodium salt (100 g L^{-1} of solubility) or tetrasodium salt (550 g L^{-1} of solubility) of EDTA are much more easily soluble in water. To eliminate the matrix effect of real water samples, EDTA disodium/tetrasodium salts were selected and tested as the chelating reagent to mask Ca^{2+} ions in the real water samples. The chemical structures of these different forms of EDTA are demonstrated in Figure 55. From the structures of these chemicals, their solubility in water is apparent.

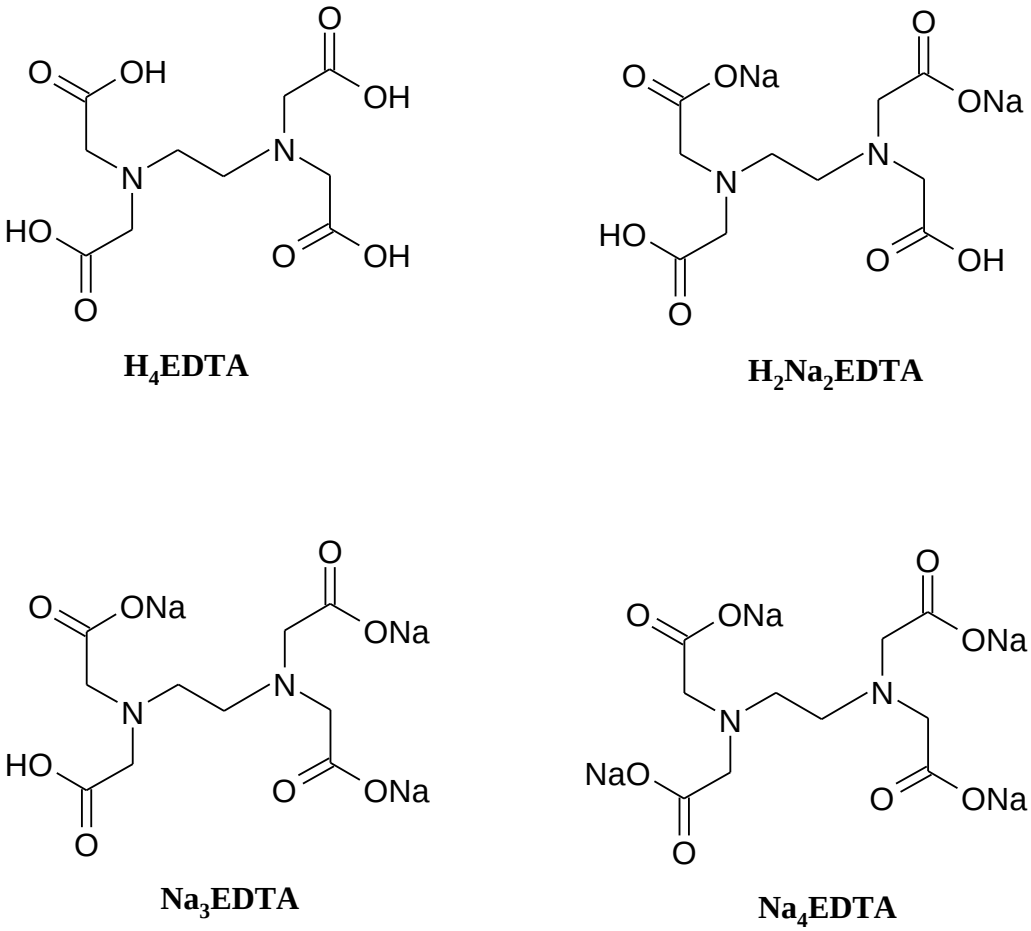


Figure 55: Structures of the EDTA (free acid) and the EDTA disodium and tetrasodium salts.

A highly effective chelating agent, EDTA has been widely used to bind divalent and trivalent metal ions via its four carboxylate and two amine groups. Formation of coordinate covalent bonds also seemed possible for diclofenac and 2-VP with the divalent cation Ca^{2+} as a connection bridge. This assumption was confirmed by an experiment in which EDTA solution was added to the mixture of diclofenac and Ca^{2+} water sample and then loading this solution onto the NISPE cartridge. The results showed that diclofenac was completely recovered in the washing fraction from the NISPE cartridge after the addition of the EDTA solution. This indicated that the presence of EDTA could efficiently mask the free Ca^{2+} ions in the real water samples and completely eliminate its interference with the specificity of the polymer. Even though the binding of diclofenac with NIP and MIP were first affected by high concentration

of Ca^{2+} ions in water samples, the polymer specificity could be completely recovered by washing the cartridges with certain concentrations of the EDTA solution.

Considering the concentration of Ca^{2+} ion in the real waters (i.e. tap water, river water and wastewater) is in the range of approximate 1.5-2.5 mmol L^{-1} in the area nearby Munich, the addition of 10 mmol L^{-1} of EDTA to the water samples was performed to completely mask the free Ca^{2+} and other divalent cations, mainly Mg^{2+} in the real water samples.

To demonstrate the feasibility of EDTA salts on masking the Ca^{2+} ions in tap water sample, 0.2 mg L^{-1} of diclofenac was spiked in the tap water sample, and then 10 mmol L^{-1} of EDTA-disodium was added. The tap water sample was loaded onto the NIP and MIP SPE cartridges. After drying, a washing solvent made of DCM/ACN (94:6; v/v) was used for washing the cartridges. The washing fraction was collected and was then dried up using a gentle N_2 stream. The residue was re-dissolved with the mobile phase solution, and checked by HPLC/DAD.

HPLC chromatograms of the diclofenac binding test of tap water sample with the addition of EDTA (Figure 56) showed that after washing, not only was a high recovery of diclofenac found in the washing fraction of BLK, but a high recovery of diclofenac was found in the MIP washing fraction as well. This result was unexpected prior to this experiment. From previous binding tests, (i.e. the effect of acetic acid on the diclofenac binding performance of MIP **7**), a high recovery of template analyte in the washing fraction of MIP is normally due to the dissociation of diclofenac during the binding procedure, and this dissociation is mostly caused from the binding competition of other acidic compounds. Combined with these previous binding tests, it indicates that EDTA disodium may not be suitable enough as a chelating reagent to mask Ca^{2+} and Mg^{2+} in the real water samples. The pH of EDTA disodium solution was checked by the pH meter, and results showed that the pH value is ~5. This indicated that this EDTA was a relatively acidic solution. So it was assumed that the relative acidic property of EDTA disodium solution may have caused the disruption of the specific interaction between diclofenac and the polymer body. From previous experiments, it was seen that EDTA disodium released diclofenac from being coordinate complexed by masking the Ca^{2+} ions in the water sample. However, in the diclofenac binding process in the MIP, diclofenac analyte rebound in MIP was also washed off after the washing step. It appears that there are other

interfering factors causing this dissociation problem. Another choice for masking Ca^{2+} ions in the water sample is to use EDTA tetrasodium salt.

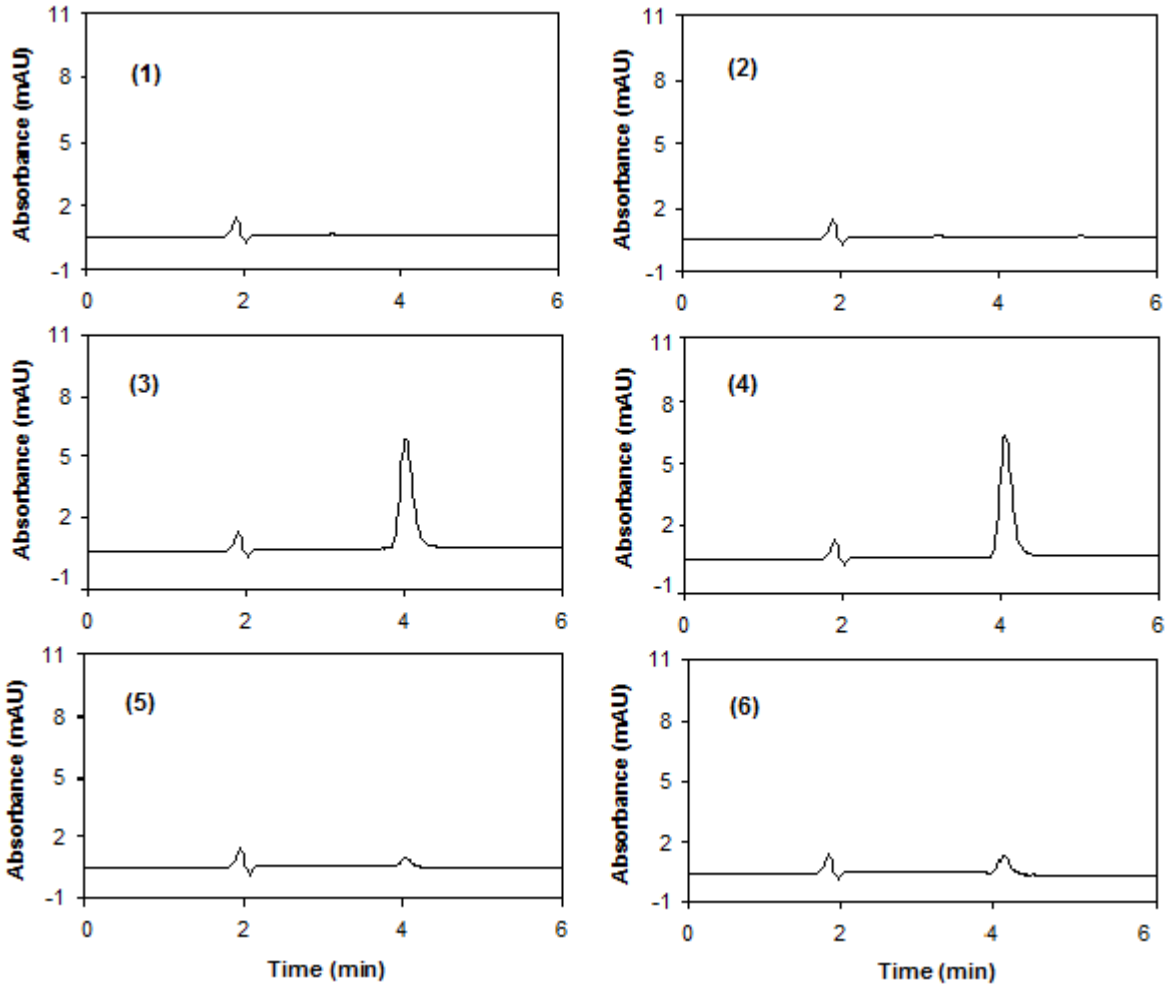


Figure 56: HPLC chromatograms of washing fractions of diclofenac binding test with MIP 8 with the addition of EDTA disodium salt to tap water samples. (1) Washing fractions of DCM/ACN (94:6, v/v); (2) washing fractions of DCM/ACN (94:6, v/v), with the addition of 10 mmol L^{-1} EDTA; (3) elution fractions of DCM/MeOH (94:6, v/v).

EDTA tetrasodium solution with concentration of 10 mmol L^{-1} was prepared for the purpose of testing its capacity on masking divalent cations and at the same time, to see if it could disrupt the extraction behavior of the MIP on real water samples or not. The testing procedure is similar to that of EDTA disodium solution and was repeated for the same purpose (shown in Figure 57).

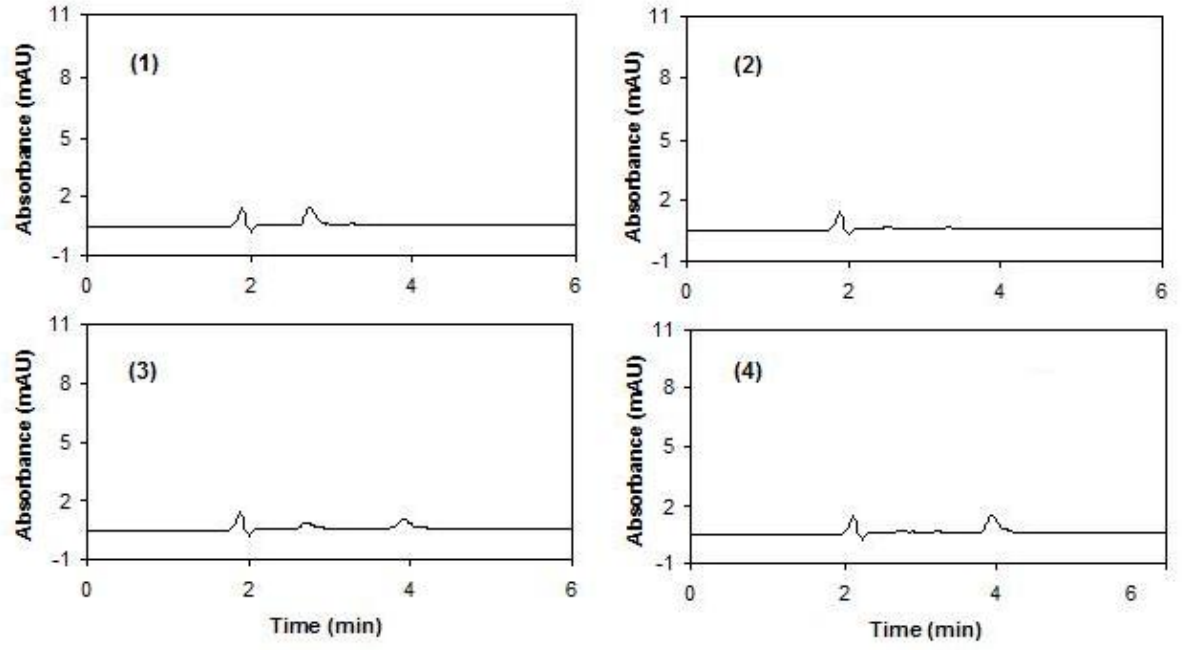


Figure 57: HPLC analysis of the influence of EDTA *disodium* and *tetrasodium* on the binding of diclofenac on the MIP. (1), NIP **8** washing fraction (100% DCM) with the addition of EDTA tetrasodium in the water sample; (2), MIP **8** washing fraction (100% DCM) with the addition of EDTA tetrasodium in the water sample; (3), NIP **8** washing fraction (100% DCM) with the addition of EDTA disodium in the water sample; (4), MIP **8** washing fraction (100% DCM) with the addition of EDTA disodium in the water sample.

HPLC chromatograms showed that almost no diclofenac acid was found in both the washing fractions of NISPE and MISPE cartridges which were loaded with water sample with the addition of 10 mmol L^{-1} of EDTA tetrasodium, and this indicated a high nonspecific binding in the NIP cartridge. The pH of EDTA tetrasodium solution was determined to be about 11, a very high basic solution. On the other hand, diclofenac was recovered in both the NIP and MIP washing fractions which were added with EDTA disodium solution. These observations further confirmed the assumption that the pH of EDTA solution has a strong influence on the diclofenac binding performance. Thus the effect of pH on the polymer binding must be considered when using EDTA disodium or tetrasodium to mask the Ca^{2+} ions in the water sample

6.4 Use of Ion Exchange Resin to Remove Divalent Cations in Water

Since the concentration of Ca^{2+} was high (1.5-2.5 mmol L^{-1}) in the natural water in the Munich area in Germany, and this high concentration Ca^{2+} has been proven to affect the MIP enrichment of diclofenac from the water samples, it is necessary to remove these effects by using chemicals to mask the metal cations. EDTA is a good chelating agent for masking Ca^{2+} and other divalent metal ions, i.e. Mg^{2+}. However, the testing experiments have shown that although EDTA could efficiently mask the Ca^{2+} ions in the water samples, this approach is still not satisfactory for this project due to the influence of pH on the binding behavior of MIP. It is well known that in nature water is hardened by the passage of rainwater containing dissolved carbon dioxide through layers of stone such as calcite, gypsum, or limestone. Hard water contains multiply charged ions such as calcium, magnesium, and heavy metal ions. The concentrations of Mg^{2+} and Ca^{2+} are much higher than any other ions responsible for hardness, and total water hardness is defined as the sum of the calcium and magnesium concentrations. Thus in the water industry, hard water can be softened by an ion exchange method. The working mechanism is: polymer resins exchange dissolved salts (cations and anions) for hydrogen ions (H^+) and hydroxyl ions (OH^-). The H^+ and OH^- subsequently combine to form pure water.

An ion exchange resin is an insoluble matrix normally in the form of small (1-2 mm diameter) beads, usually white or yellowish or black fabricated from an organic polymer substrate. The material has highly developed structure of pores on the surface, which have sites where ions are easily trapped and released. The trapping of ions takes place only with a simultaneous releasing of other ions; thus the process is called ion exchange. There are different types of ion exchange resin which are fabricated to selectively prefer one or several different types of ions.

In this experiment, a polymer resin MB 400 (black color) was used to remove the divalent cations by ion exchange chromatography. As shown in Table 27, Ca^{2+}, Mg^{2+} have been almost completely removed.

Table 27: Atomic absorption spectroscopic measurements of the concentrations of Mg^{2+} and Ca^{2+} ions from river water samples before and after ion exchange chromatography using MB 400 resin. Additionally the concentration of the Mg^{2+} and Ca^{2+} ions in untreated tap water was determined

	Windach river water untreated	Ion exchange	Tap water untreated
Mg^{2+} (mg L^{-1})	23.9	0.03	14.1
Ca^{2+} (mg L^{-1})	94.8	0.32	61.8

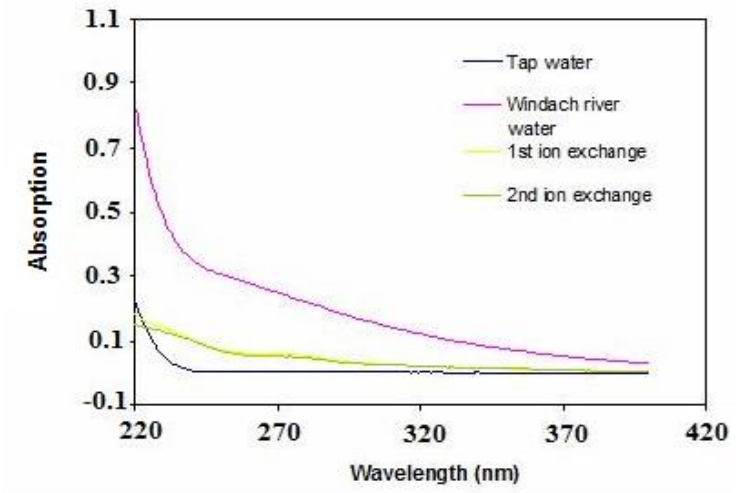


Figure 58: UV spectra of tap water and river water samples before and after treatment by the ion exchange resin MB 400.

UV-Vis spectroscopy is a useful tool to estimate the content of organic substances in water samples which is quite different in real water samples such as tap water, river water and wastewater. From Figure 58, it can be seen that surface water has much higher UV absorbance than tap water which is quite normal. After ion exchange chromatography it was observed that large part of organic compounds was also removed by unspecific adsorption to ion exchange resin. However, content of organic substances was still higher than that in tap water. In addition, effluent and influent wastewater samples were also compared (Figures 59

and 60). As expected, considerable amount of organic matter was also contained in these samples which were partially removed by unspecific adsorption to the ion exchange polymer.

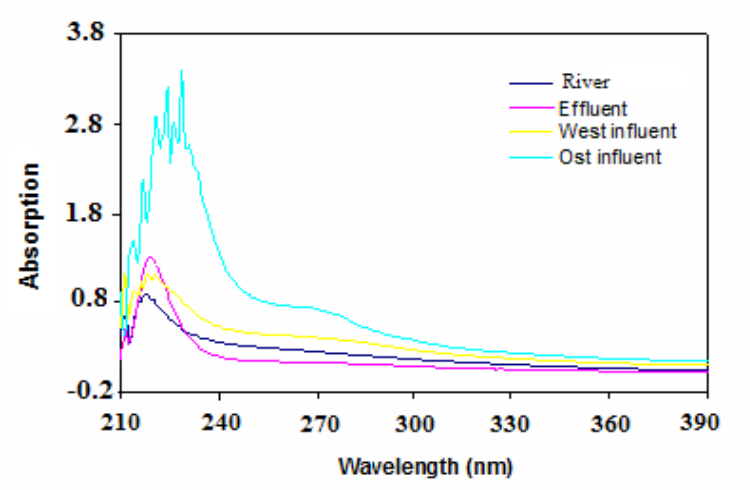


Figure 59: UV spectra comparison of different water samples before the treatment (top figure) by the ion exchange resin MB 400.

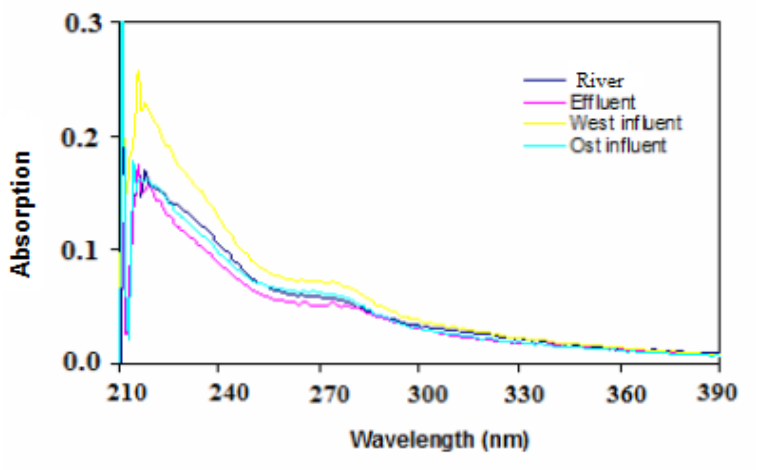


Figure 60: UV spectra comparison of different water samples after the treatment by the ion exchange resin MB 400.

6.5 Effect of pH on MISPE Extraction

To study the binding performance of MIP **8** on diclofenac in water samples, tap water was selected as the first real sample. A high non-specific binding on the NIP was observed which was so strong that the used washing solvent could not remove the rebound analyte from the blank polymer. The following investigation proved that a high concentration of divalent metal ions (mainly $Ca^{2+}$and Mg^{2+}) in the water caused this non-specific binding. In order to eliminate the influence of these cations, first the commonly used chelating agent EDTA was introduced into the water samples to mask the cations. However, it was found that as a weak acid (EDTA disodium salt) or base (EDTA tetrasodium salt) different pH will affect its chemical properties. Concurrently, the pH of EDTA solution will also affect the template binding performance of the MIP. Therefore, another approach was tested i.e., to remove Mg^{2+} and Ca^{2+} ions in the water sample by ion exchange chromatography.

During the testing of ion exchange resins to remove Mg^{2+} and Ca^{2+} ions from water samples, analysis by UV spectroscopy and atomic absorption spectroscopy (AAS) proved the effectiveness of this approach. However, it was observed that the resin could bind non-specifically organic materials such as humic acids and, unfortunately, also the target analyte diclofenac. This would greatly affect the accuracy of MISPE. Based on this finding, the approach of using ion exchange resin was considered to be not applicable and instead, EDTA was the final choice for masking Mg^{2+} and Ca^{2+} ions in real waters. Because of the pH dependence, the effect of different pH values on the diclofenac binding performance had to be tested.

6.5.1 Binding Performance of MIP at Different pH

In order to favour the binding interactions between MIP and diclofenac, and to further understand the influence of the chelating agent EDTA on the template binding performance, the pH of the sample should be studied [250-252]. Thus, the pH of different water samples and EDTA solutions were summarized in Table 28. To study the pH effect, acidified Milli-Q water spiked with diclofenac was analyzed first. In this experiment, 5 mL of Milli-Q water, acidified to pH 3 with diluted HCl, was spiked with 20 µg L^{-1} of diclofenac standard and loaded to the NIP and MIP cartridges.

Table 28: The pH values of different water samples and EDTA solutions

	Milli-Q water	Tap water	EDTA disodium (10 mmol L^{-1})	EDTA tetrasodium (10 mmol L^{-1})
pH value	5.6	6.2	5.2	10.5
	River water	**Wastewater effluent**	**Wastewater influent 1**	**Wastewater influent 2**
pH value	8.1	7	7.3	7.5

When the water samples were loaded onto the MIP and NIP cartridges, all the template analytes in the water samples would be completely retained in the cartridges mainly because of the strong nonspecific hydrophobic interaction. After drying the SPE cartridges, a clean up step with 3 mL of DCM/ACN (94:6, v/v) was performed to disrupt the nonspecific interaction established between diclofenac and the NIP. As was expected, all of the template molecules were washed off from the blank polymer. However, when checking the MIP washing fraction by HPLC, the same amount of diclofenac was found. Comparing the amounts of diclofenac in both NIP and MIP washing fractions, almost no difference was observed. Concluding, no specific interaction was established between diclofenac and the MIP after the application of Milli-Q water sample which was acidified at pH 3.0. From the chromatograms shown in Figure 61, it was seen that pH 3.0 is not applicable for MISPE.

Next, the effect of neutral pH (7.0) using a 0.8 mol L^{-1} phosphate buffer solution (PBS) on the template binding was investigated. It was prepared as follows:

i. Take 41.6 mmol of $NaH_2PO_4 \cdot 2H_2O$
ii. Take 38.3 mmol of $Na_2HPO_4 \cdot 2H_2O$
iii. Make up to 100 mL with pure water
iv. Ionic strength = 1.566 M
v. Thermodynamic pKa = 7.2

The binding test procedure in buffer was as follows:

1. Weigh EDTA· 2Na 37.4 mg in a beaker, add in 8 mL tap water, stir the solution
2. Add 2 mL of the prepared PBS to the above solution, so the final concentration of EDTA· 2Na is 10 mmol L^{-1}
3. Spike with 20 µg L^{-1} diclofenac acid standard
4. Load the sample to MIP and NIP cartridges

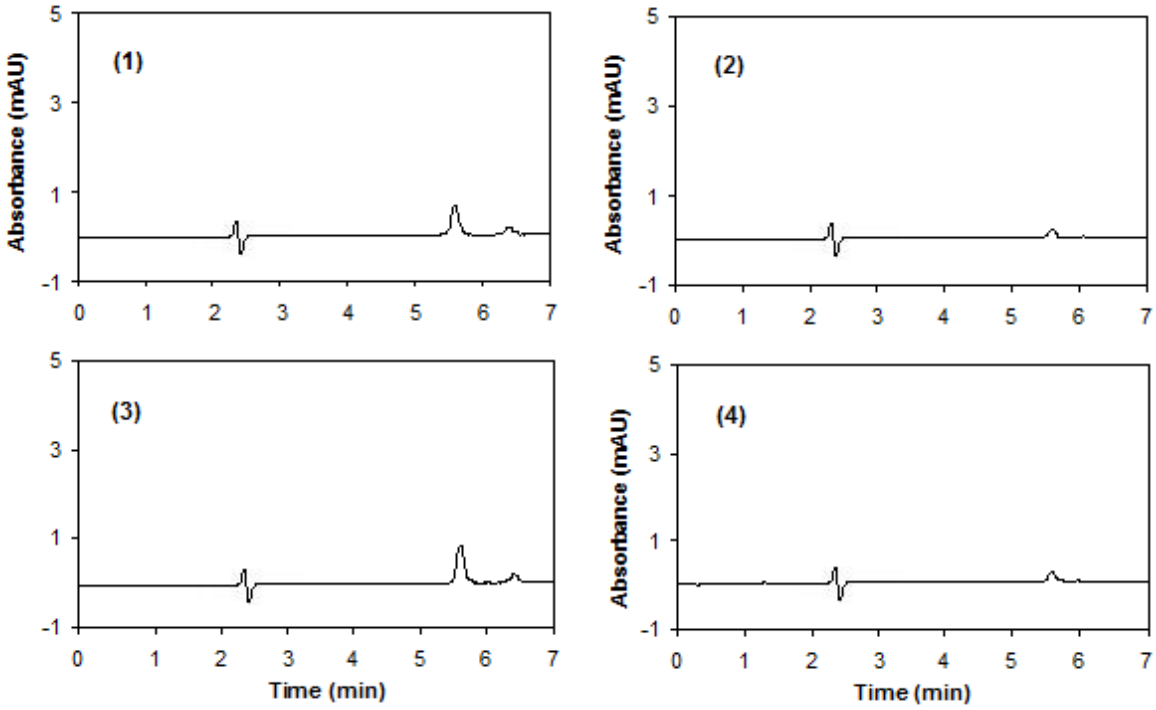


Figure 61: HPLC chromatograms of washing fractions obtained upon loading 5 mL of acidified Milli-Q water (pH 3.0) spiked at 20 µg L^{-1} with diclofenac. (1) NIP 1st washing fraction; (2) NIP 2nd washing fraction; (3) MIP 1st washing fraction; (4) MIP 2nd washing fraction.

After drying the cartridges under vacuum for 25 min, they were washed with 2 mL DCM/ACN (94:6, v/v) and fractions analyzed by HPLC/DAD. HPLC analysis shows in the washing fraction of the 1st wash of 1.0 mL DCM/ACN (94:6, v/v), there are small amounts of diclofenac in both MIP and NIP washing fraction. After the 2nd DCM/ACN (94:6, v/v) wash, there is a high recovery of diclofenac in both washing fractions. This result indicates also that the pH 7.0 PBS buffer may not be suitable for the purpose of pH adjustment. Further consider

the reason why MIP still cannot extract diclofenac from the PBS buffer adjusted tap water sample must be considered further. A possible reason is that although pH 7.0 PBS can maintain the solution to be neutral, the acidic component ($NaH_2PO_4 \cdot 2H_2O$) in the buffer solution can still give out enough protons, which will affect the protonation/deprotonation state of the low concentration diclofenac acid spiked in the tap water, finally affecting its binding in the MIP.

In order to fully understand the pH effect on the binding performance of MIP, a series of buffer solutions with different pH values (pH 3-9) were prepared and spiked with diclofenac at a concentration of 20 µg L^{-1}. A 5 mL volume of each solution was loaded both onto the NIP and MIP cartridges and washing fractions DCM/ACN (94:6, v/v) were analyzed by HPLC/DAD. Data were plotted in Figure 62. In this figure, it can be seen that when sample pH was 5 or lower, diclofenac was increasingly eluted from the MIP and NIP columns in the washing fraction. At a pH of 3, more than 96% of added diclofenac was washed off.

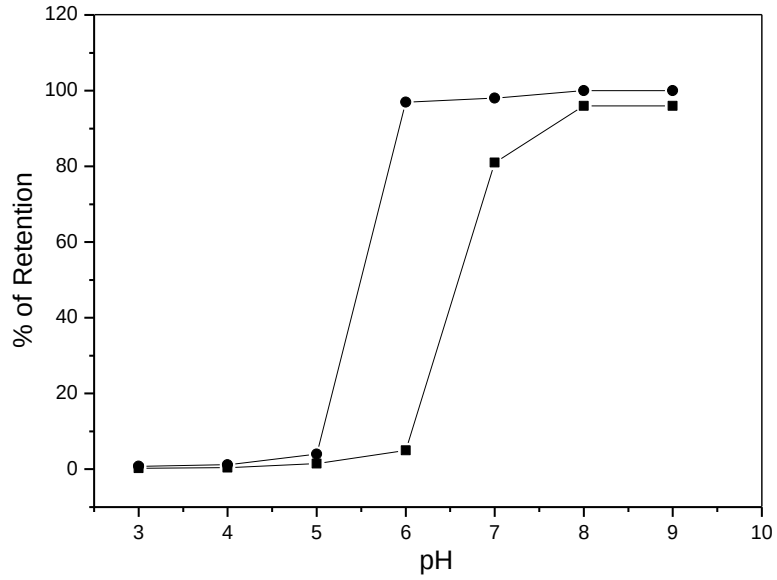


Figure 62: Binding of diclofenac to NIP and MIP cartridges as a function of pH of the applied aqueous buffer solution. 5 mL aliquots (concentration of the diclofenac was 20 µg L^{-1}) were applied.

Considering the importance of the ionic interaction mechanism in the binding process of diclofenac to the MIP, the optimum pH of the water sample should be in the range where a

positively charged pyridinium group at 2-VP and a negatively charged carboxyl group at the diclofenac should coexist to achieve the maximum binding interaction between the analyte and polymers.

Both diclofenac (pKa 4.1) and 2-VP (pKa 5.06) are protonated below pH 3.0 and thus, the ionic interaction can not be developed. However, at approximately pH 6.0, maximal selectivity was observed. At this pH, almost 100% of the added diclofenac could be found in the washing fraction of the NIP, but it was almost fully retained (~ 95%) on the MIP. Therefore, the best results were found in the pH range where a certain portion of the positively charged pyridinium group was existed**,** but well above the pH where diclofenac was considerably deprotonated, maintaining the complementary negatively charged diclofenac anion. This indicated that retention was mainly controlled by an ion-interaction process, as already was concluded from the NMR studies. Therefore, this pH value was selected in subsequent experiments for water sample analysis i.e. pH of water samples was adjusted to 6 by the addition of appropriate amounts of PBS buffer solution.

6.6 MISPE Extraction of River Water Sample

To further study the binding capability of the MIP for water samples, a surface water sample was collected from river *Windach* in south Bavaria. The surface water samples were filtered using glass fiber filter MN85/90 BF (Macherey-Nagel, Düren, Germany) to remove particles larger than 1.2 µm, and were kept at 4°C in the dark until analysis. MISPE was spiked with diclofenac analyte, at the same time, free Ca^{2+} and Mg^{2+} ions in the water sample were complexed by the addition of EDTA disodium salt at a concentration of 10 mmol L^{-1}. Then, PBS was added to adjust the pH to be 6.0. For recovery studies, the surface water samples were spiked with diclofenac at a 0.5 µg L^{-1} concentration level and a total of 200 mL of river water sample was extracted by MISPE. NISPE extraction of the river water sample was done in the same way for binding performance comparison.

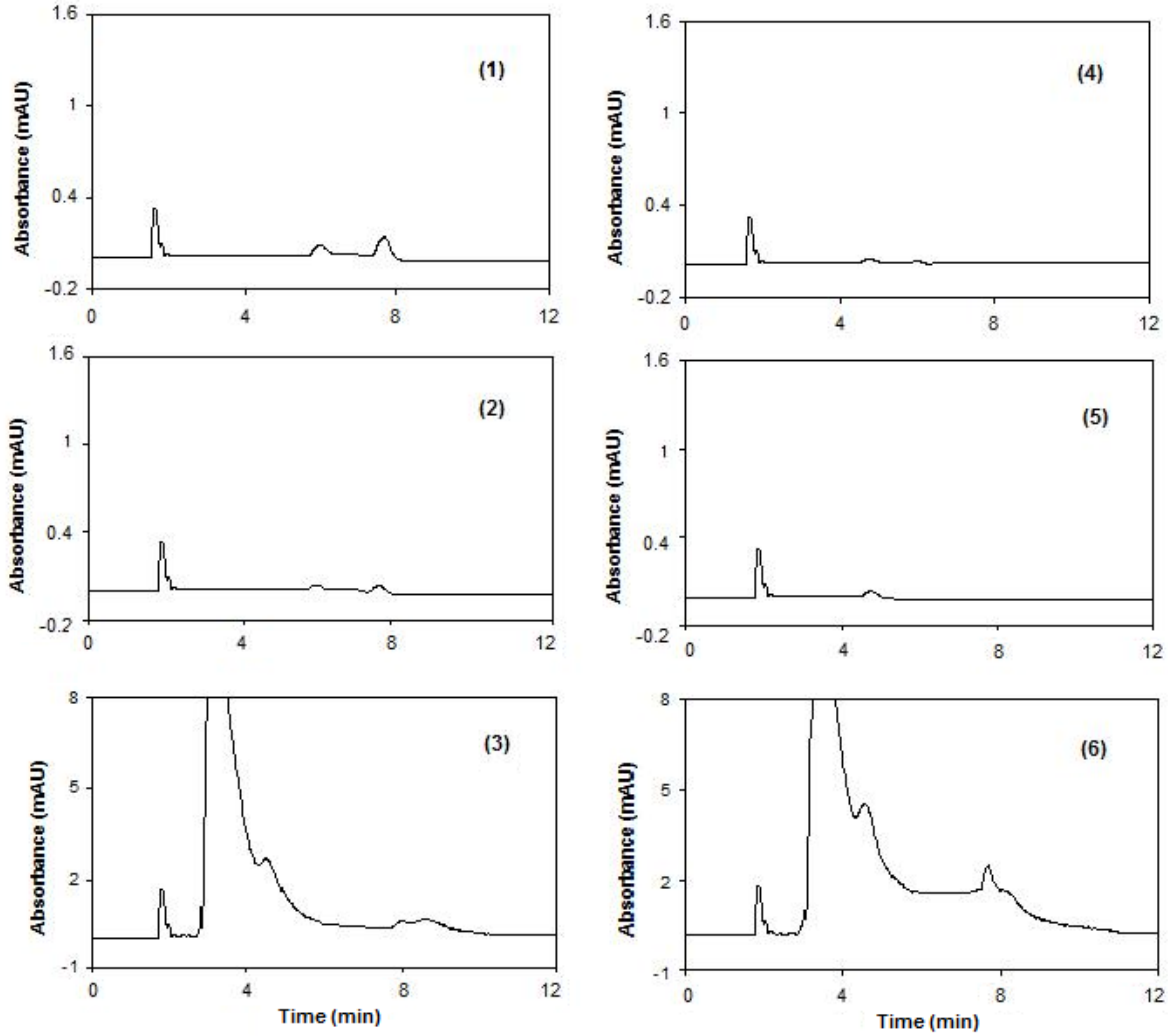


Figure 63: HPLC chromatograms obtained from washing fractions and eluates of NIP and MIP cartridges after extraction of 200 mL of river water sample spiked with diclofenac at a concentration of 0.5 µg L^{-1}. (1) 1st washing fraction from NISPE; (2) 2nd washing fraction from NISPE; (3) eluate from NISPE; (4) 1st washing fraction from MISPE; (5) 2nd washing fraction from MISPE; (6) eluate from MISPE.

In HPLC analysis (Figure 63), a modified HPLC gradient method was developed to shorten the time of analysis. The composition of the mobile phase was increased linearly from an initial 40% ACN to 66.5% in 10 min and then to 75% in 5 min. To clean the column, the composition was kept constant for 2 min. The initial mobile phase composition was restored and the column was equilibrated for 10 min. The recovery of the analyte in the washing fractions of NIP was higher than 95% (97% ± 4.1, n=3). With MIP, no diclofenac was

detectable in the washing fraction but in the eluate. Corresponding recovery was 96% ± 3.3% (n=3).

6.7 MISPE Extraction of Wastewater Samples

According to the earlier investigation [69], diclofenac at low concentrations (µg L^{-1} level) has been detected in several wastewater samples from wastewater treatment plants in Bavaria and Austria. These analyses were performed using ELISA and GC-MS. In the present project, to prove the usability of the MIP cartridge for extracting diclofenac from more complex samples such as wastewater was investigated. At the beginning, 200 mL of an effluent sample, after filtration with a glass microfiber filter, were spiked with diclofenac at a concentration of 0.5 µg L^{-1} and then passed through the MISPE cartridge by negative pressure. After drying the cartridge, diclofenac in washing and elution fractions were analyzed by HPLC/DAD. Meanwhile, template extraction with the NISPE cartridge was performed in the same manner.

From the HPLC analysis of the washing fractions and eluates, it was known that after washing, all the nonspecifically bound diclofenac on the NIP cartridge was recovered in the washing fractions, no diclofenac was found in the eluate. It was different for the MIP, and no diclofenac was found in the washing fractions. However, by increasing the percentage of acetonitrile in the washing solvent mixture to 8% (v/v), diclofenac could partially be recovered (about 12%). The rest of the bound diclofenac in MIP was recovered in the elution fraction by DCM/MeOH (85:15, v/v). This observation indicated that the MISPE extraction protocol used in the previous experiments were also applicable to wastewater samples. In the eluates, although considerable amounts of unknown substances were also eluted both from NIP and MIP cartridges, however, these substances did not show any interference with quantification of diclofenac.

6.7.1 Direct Extraction of Diclofenac from Non-spiked Wastewater Samples

To get better separation of diclofenac in more complex influent wastewater, the HPLC analysis method was modified using a longer gradient running time (30 min) compared to the previous effluent wastewater analysis (12 min). In this experiment, the influent wastewater

sample was directly extracted by NISPE and MISPE, e.g. without preliminary diclofenac spiking, and then further analyzed by HPLC/DAD (Figure 64).

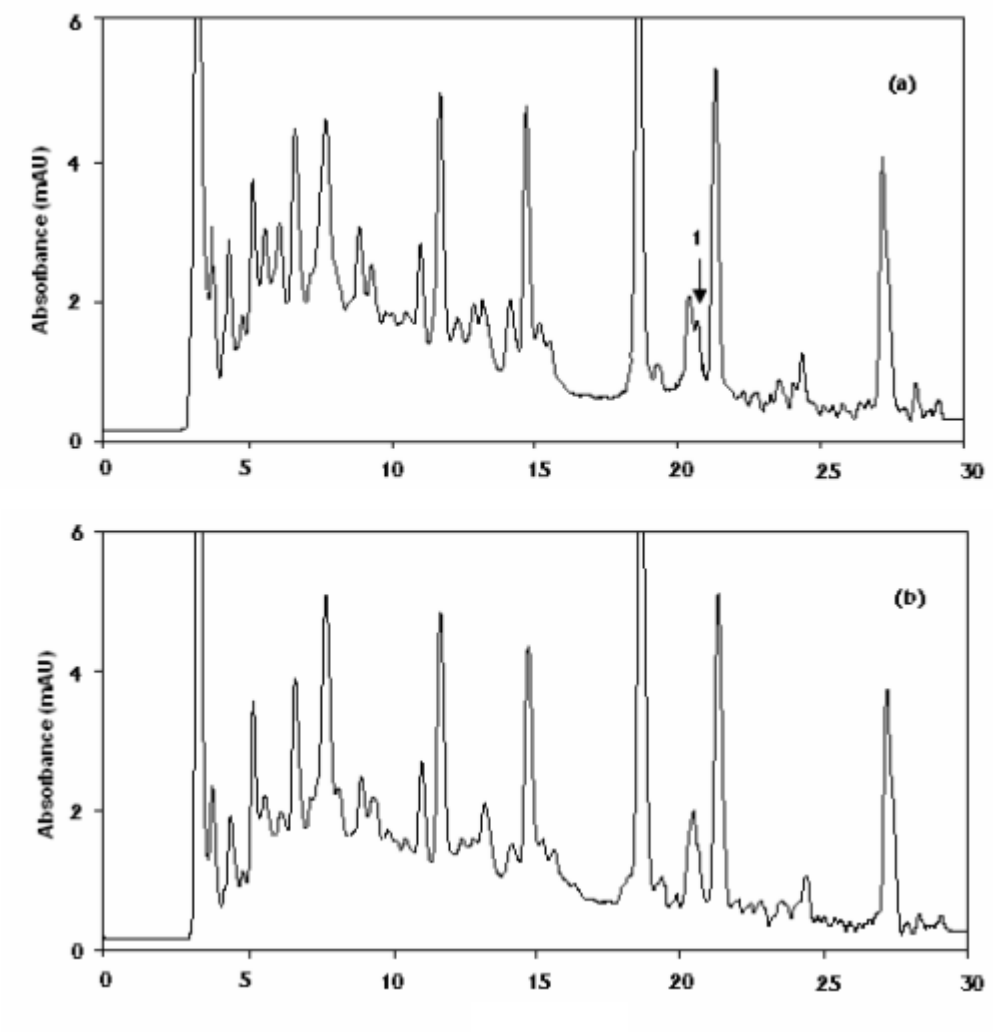


Figure 64: HPLC chromatograms obtained by direct extraction of diclofenac from 200 mL of raw influent wastewater with NIP and MIP cartridges. (a) washing fraction, NIP; (b) washing fraction, MIP (c) eluate, NIP; (d) eluate, MIP. **1**, diclofenac; **2** and **3**, unknown compounds.

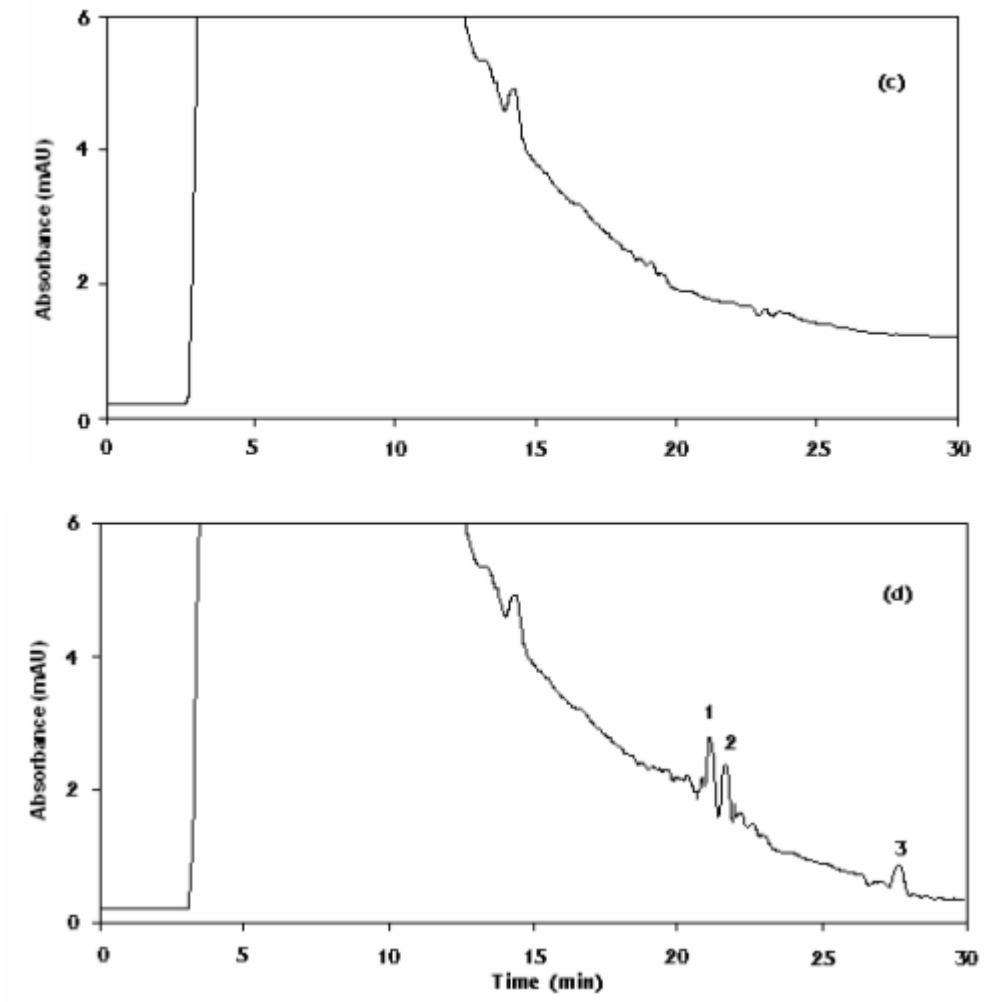


Figure 64 continued

While no diclofenac was found in the eluate of NISPE cartridge (Figure 64c), it was only detected in the MISPE eluate (Figure 64d). In the latter, beside diclofenac, two unknown compounds (designated **2** and **3**) were co-extracted. So far it is not clear whether or not they are diclofenac metabolites. The estimated concentration of diclofenac in the influent wastewater sample was 1.31 ± 0.055 µg L^{-1} (n = 3).

6.7.2 Method Comparison

Further, a comparison of NISPE, MISPE and a commercial SPE C18 cartridge (Strata C18-E of Phenomenex, Aschaffenburg, Germany) with regard to selectivity and analyte recovery was performed correspondingly using a final effluent wastewater (Figure 65). As expected, considerable numbers of analytes were trapped on the C18 column based on non-specific hydrophobic interaction. At least two unknown compounds, A and B, could not be separated from diclofenac without an additional washing step by the solvent mixture DCM/ACN (94:6, v/v). The overlay of their peaks made a quantification of diclofenac impossible.

On the other hand, MISPE exhibited high binding selectivity for diclofenac after the selective washing step. The concentration of diclofenac in effluent water was 1.60 ± 0.049 µg L^{-1} (n=3) which is slightly higher than in raw influent water. This indicated that no degradation of the parent compound occurred. This clearly pointed to the fact that the degradation rate of pharmaceuticals is also subject to seasonal influences, e.g., temperature and solar irradiation. For comparison, a degradation rate of ~ 25% was observed for samples taken from this STP not in December but in July [69].

In addition, the Strata C-18 SPE cartridge was also applied for the extraction of diclofenac with three additional wastewater samples (effluent NKB, West influent and Ost influent). The eluates of these wastewater samples were analyzed by HPLC/DAD (Figure 66).

It can be seen from all HPLC chromatograms that the C-18 cartridge can not separate diclofenac from other unknown co-extracted components in the water samples, as was already described above. This indicated that MISPE prepared in this work was much more efficient on the extraction of diclofenac from the wastewater samples than the commercial C-18 cartridges.

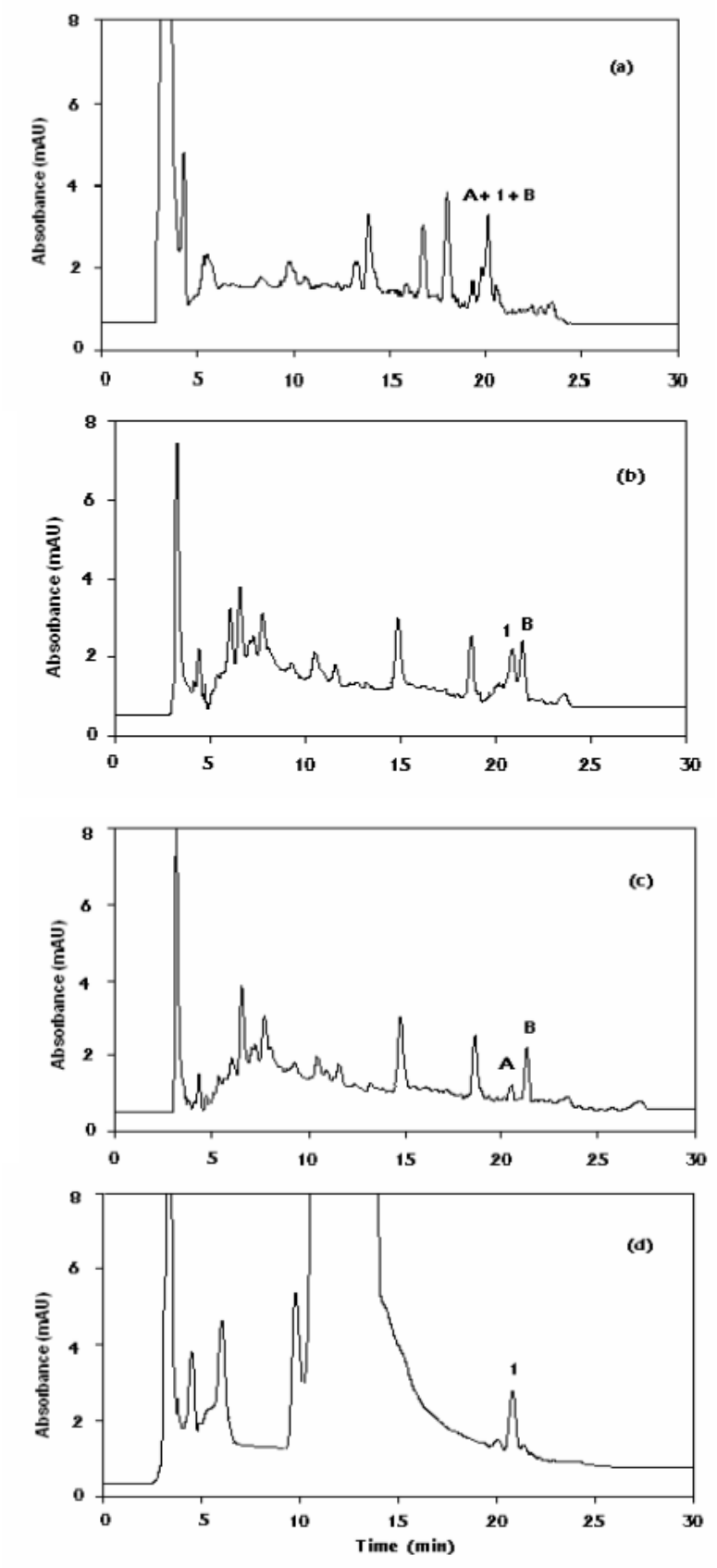


Figure 65 HPLC chromatograms obtained by direct extraction of diclofenac from 200 mL of final effluent wastewater with SPE C18, NISPE and MISPE cartridges. (a) eluate of SPE C18; (b) washing fraction, NIP; (c) washing fraction, MIP; (d) eluate, MIP. (1) diclofenac; (A) and (B) unknown compounds.

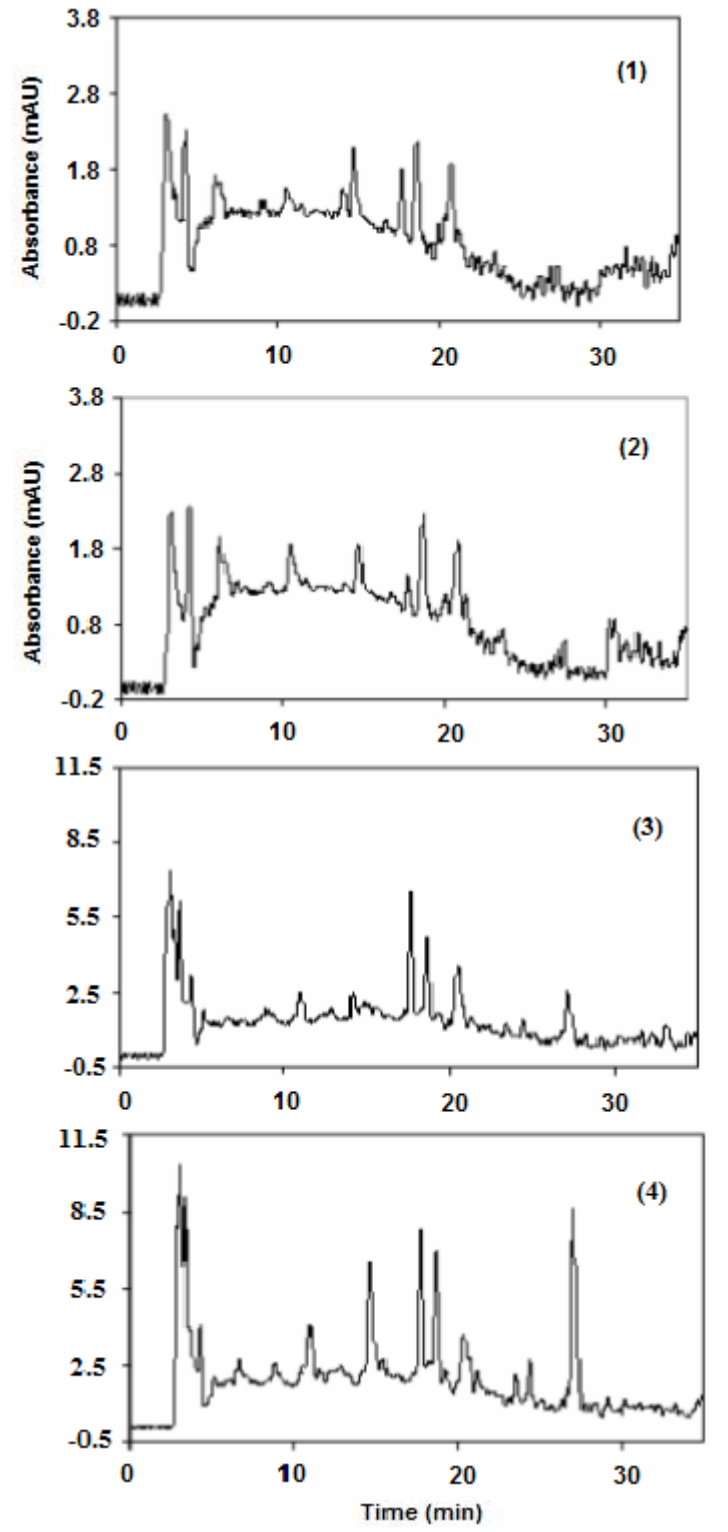


Figure 66: HPLC chromatograms obtained with eluates after extraction of 200 mL of different wastewater samples with SPE C-18 cartridge (1) eluate of final effluent; (2) eluate of NKB effluent; (3) eluate of West influent; (4) eluate of Ost influent.

6.7.3 LC/MS/MS Measurement of Wastewater Samples

Analysis of wastewater samples using LC/MS/MS was performed in parallel by another laboratory (LfU-Bavarian Office for the Environment). LC/MS/MS analyses of diclofenac and 4-hydroxydiclofenac were carried out as the following procedure: a Sciex 4000 Q-Trap (Applied Biosystems, Foster City, CA) was equipped with a Jasco low-pressure HPLC system. The chromatographic separation was performed using a Phenomenex Synergy Polar column (150mm×2.0mm i.d., 4.0µm) with a guard column of the same packing as the column (4.0mm×2.0mm, 4.0µm) purchased from Phenomenex (Torrance, CA). The mobile phases were constituted by solvent A, methanol/HPLC-water (95:5, v/v) and solvent B, HPLC-water/methanol (95:5, v/v), with addition of 0.05mL formic acid to both solvents. The linear gradient program was 0-18 min 10% A, 18-23 min up to 90% A, 3-24 min held at 90% A, 24-31 min back to 10% A, running at a constant flow rate of 0.3 mL min^{-1}. An identical injection volume of 10-100 µL was applied. Ionization of analytes was performed using electrospray ionization in the positive ion mode. The ion source 1/2 and curtain gas flow rates and collision energy were set at the optimized conditions. The ion source temperature was maintained at 450°C with a capillary voltage of 4.5 kV. Diclofenac-d4 was used as the internal standard in diclofenac quantitation. The first SRM (SRM1) monitored to quantify diclofenac was m/z 296→215, while the second SRM (SRM2) for analyte confirmation was m/z 296→250 (m/z 300→219, 300→254 for diclofenac-d4 and 312→266, 312→231 for 4-hydroxydiclofenac, respectively). Data processing was performed using Analyst 1.4.2 software. LC/MS/MS revealed the concentrations of template diclofenac of 1.40 µg L^{-1} and 1.50 µg L^{-1} in influent and effluent samples, respectively. Data are in good agreement with MISPE-HPLC/DAD as discussed above. In addition, 4-hydroxydiclofenac, as a main metabolite of diclofenac, was identified at a concentration of 61 ng L^{-1} and 58 ng L^{-1}, respectively. Further experiments might elaborate on the enrichment by MISPE on this and other metabolites.

III Summary and Conclusion

Molecularly imprinted polymers have been thoroughly investigated over the past decade due to their promising and innovative biological and environmental applications. The use of imprinted polymers includes several analytical techniques, such as liquid chromatography, capillary electrophoresis, capillary electrochromatography, immunoassay and solid phase

extraction. Current research interests on imprinted polymers focus on the highly selective solid phase extraction of trace chemical pollutants in the environment. Various template analytes, including several types of drug compounds and related substances, were imprinted for such applications. Non-steroidal anti-inflammatory drugs (NSAID) is a type of drugs which were imprinted for the detection of this type of drug residue e.g. ibuprofen, naproxen and diclofenac. Diclofenac as a member of the NSAID family belongs to the most frequently detected pharmaceuticals in the European water-cycle. However, to date, only a few papers from Tuñón-Blanco and coworkers [178-180] reported on the use of diclofenac as the template molecule, focused on the development of voltammetric sensors. This detection method did not show a very low detection limit, i.e., diclofenac could only be quantified at mg L^{-1} concentration level. Thus, in this thesis, different approaches were investigated for imprinting diclofenac. The interactions between template and functional monomer in pre-polymerization complex,and the role of the porogenic solvent on the resulting polymer were studied in detail.

With 4-VP (pKa 5.51) which is one of the most commonly used basic functional monomers and acetonitrile as porogen, several commonly used cross-linkers (PEGDA, EGDMA, DVB) were tested. The binding performance to template analyte diclofenac was compared with that of the corresponding non-imprinted polymers prepared without the addition of template. A 33% difference in binding between MISPE and NISPE of Polymer 3 (cross-linker EGDMA) was observed. In contrast, a difference of only 18% or less was found for Polymers 1 and 2, which were prepared with cross-linkers PEGDA and DVB Thus, EGDMA was selected as the cross-linker for synthesis of all subsequent imprinted polymers. Great efforts were devoted to the removal of the template from prepared MIPs using different solvents and extraction procedures such as Soxhlet extraction, microwave-assisted extraction and ultrasonication. Ultrasonication combined with solvent MeOH/HAc (90:10, v/v) was proven to effectively remove the template molecule from the highly cross-linked imprinted polymer, although the washing procedure was time-consuming.

Besides the comparison of cross-linkers, some porogens of different polarity such as methanol, methanol/water, acetonitrile, and octanol/trichloroethane were also compared in terms of their influence on the binding properties of the synthesized polymers. Methanol and acetonitrile proved most advantageous.

As the second important functional monomer, which is similar in structure and chemical properties to 4-VP, 2-VP (pKa 5.06), was examined in the thesis. A series of binding characterization measurements was performed on the 2-VP or 2-VP/MAA polymers. With polymer **7**, which was prepared with porogen DCM and cross-linker EGDMA, it was found that the addition of acetic acid to diclofenac standard solutions strongly affected the binding of the template to the polymer. Thus, the binding was strongly dependend on the pH value i.e., the presence of an ion pair between the pyridine ring of 2-VP and the carboxyl group of the template molecule.

A further imprinted polymer (MIP 8) was synthesized using toluene as porogen and 2-VP was employed as the functional monomer. The binding characteristics of MIP 8 were examined extensively by saturation tests and Scatchard plot analysis. The saturation isotherm plot showed that saturation of template binding to the MIP compared to the blank polymer was at higher diclofenac concentration which suggested that diclofenac bound to MIP was more likely caused by the specific binding to imprinted binding sites of the polymer than by the non-specific adsorption. Further, the resulting Scatchard plot of the imprinted polymer was not a single straight line, but consisted of two lines with different slopes. This strongly indicated that the binding sites in the polymer were heterogeneous in respect to the affinity for diclofenac, and could be classified into roughly two groups, lower affinity binding sites and higher affinity binding sites. K_D and Q_{max} of the higher affinity binding sites were calculated to be 55.6 µmol L^{-1} and 19.1 µmol g^{-1} dry polymers, respectively, from the slope and the intercept of the Scatchard plot. Similarly, the K_D and Q_{max} of the lower affinity binding sites were found to be 1430 µmol L^{-1} and 127.2 µmol g^{-1} of dry polymer, respectively. In contrast, the Scatchard plot for NIP was nearly a single straight line. Thus, Scatchard plot analysis confirmed the presence of high affine binding sites of MIP **8**.

To further evaluate the binding properties of the imprinted polymer, MISPE extraction is normally used to separate specific from non-specific retention. Using this procedure, a certain amount of polymer particles is packed in a SPE cartridge. After the sample (containing the analyte) was loaded into the cartridge, an optimized washing solvent is used to wash off non-specific bound template analyte from the polymer. Meanwhile, the specific bound analyte is retained. To develop the MISPE extraction procedure for MIP **8**, several non-polar solvents, i.e., toluene, hexane and chloroform, polar aprotic solvents, i.e., dichloromethane (DCM) and acetonitrile, and polar protic solvent methanol were tested in terms of washing efficiency and

ease of sample handling. The test results showed that DCM was the most effective washing solvent although diclofenac could not be washed off completely. On the other hand, methanol and acetonitrile (ACN) efficiently removed most non-specifically bound diclofenac from the NIP cartridge. However, the specific interaction between the template and the MIP was also disrupted. Thus, DCM mixed with different concentrations of ACN was tested as the washing solvent due to the high ability of ACN for disrupting the non-specific interaction of diclofenac with the imprinted polymer. Finally, 3 mL of DCM/ACN (94:6, v/v) was successfully selected as a washing solvent. For the quantitative elution, 3 mL of DCM/MeOH (85:15, v/v) revealed optimal.

To estimate the applicability and reliability of MISPE extraction for environmental application, water samples such as tap water, river water and wastewater were collected and characterized. During testing, the major problem encountered was a strong non-specific binding of diclofenac to the NIP, caused by matrix constituents. The washing solvent was not able to remove the bound analytes. Further investigation revealed that the high concentration of Ca^{2+} and Mg^{2+} ions was the reason for this finding. It could be explained as follows: the ions could form a complex with diclofenac and the functional monomer 2-VP of the polymer body. In this complex, the amine group and carboxyl group of diclofenac can form coordinate bonds with divalent ions and the nitrogen atom of 2-VP of the polymer. Thus, diclofenac could be bound to the polymer via coordinate bonds with the ions, which acted as a "connection bridge". Coordinate covalent bond is much stronger than a non-covalent bond, thus explaining why the NIP cartridge showed a strong non-specific binding when it was applied to real water samples. After adding the chelating agent EDTA to the water sample before loading it to the NIP cartridge led to complete recovery of diclofenac in the washing solution. Accordingly, 10 mmol L^{-1} EDTA was used to mask the divalent ions in the water for sample extraction by MISPE. Further, the effect of sample pH value was also studied. It was found that at low pH (e.g. pH < 5), diclofenac was increasingly recovered in the washing fraction. At pH 3, approximately 96% of diclofenac was washed off. By contrast, in a neutral solution (pH 7) or above, diclofenac was increasingly retained in the cartridge after the washing step. Considering the pKa of both diclofenac (pKa 4.1) and 2-VP (pKa 5.06), the optimum pH should be in the range where the positively charged pyridinium group is fully protonated and diclofenac is deprotonated and thus maintaining complementary charged ions. This indicated that the retention of diclofenac was controlled by an ionic interaction, mainly.

Concluding, pH 6 was selected as optimal and sample pH adjusted through the addition of appropriate amounts of sodium phosphate salt.

^{1}H NMR was utilized for investigation of the template-functional monomer interaction of pre-polymerization complex of polymer **8**. To do this, a series of samples was prepared in different deuterated porogenic solvents (ACN, chloroform and toluene) with a fixed concentration of diclofenac and various concentrations of 2-VP. The ^{1}H NMR spectra were recorded at room temperature with a NMR spectrometer of 400 MHz (for NMR samples prepared in $CDCl_3$) and 600 MHz (for NMR samples prepared in the other solvents). By analyzing the ^{1}H NMR spectra, it was concluded that only hydrogen bonding was formed between the amine group of diclofenac and pyridine ring of 2-VP in ACN. For the template-functional monomer interaction in chloroform, the ^{1}H NMR spectra clearly indicated that a hydrogen bond between carboxyl group of diclofenac and pyridine ring of 2-VP was formed, and no other interactions were found in the NMR analysis. On the contrary, ^{1}H NMR study in toluene confirmed in addition to hydrogen bonding also an ionic interaction, i.e. the formation of at least two non-convalent interactions. The results are in agreement with data from literature which report e.g. on the formation of an ion pair between the acidic herbicide 2,4-D and 4-VP in deuterated chloroform and additional stabilization by pi-pi stacking in aqueous solution.

While ^{1}H NMR was used for analysis of the interactions that exist in the pre-polymerization complex, for evaluation and characterization of polymer binding performance, HPLC/DAD was utilized in the entire project. Depending on the application of samples, isocratic or gradient elution mode was used during the HPLC analysis. In MISPE application of wastewater samples, the estimated concentration of diclofenac in influent and effluent water was 1.31 ± 0.055 µg L^{-1} (n = 3) and 1.60 ± 0.049 µg L^{-1} (n = 3), respectively. Parallel analysis of samples by LC/MS/MS revealed that the diclofenac concentrations were of 1.40 and 1.50 µg L^{-1} in influent and effluent samples, respectively. Data are in good agreement with MISPE-LC/DAD. In conclusion, a series of diclofenac imprinted polymers using 4-VP and 2-VP as functional monomers, PEGDA, DVB, and EGDMA as cross-linkers and ACN, MeOH, DCM and toluene as porogens were prepared and characterized in this project. Among these polymers, MIP **8** prepared with monomer 2-VP, cross-linker EGDMA and porogen toluene has shown the best binding affinity to template analyte diclofenac compared to the other imprinted polymers. At the pre-polymerization stage, at least two non-covalent bonds were

formed between diclofenac and 2-VP in toluene: ionic interaction and hydrogen bonding. It could be speculated that in aqueous solution π-π stacking may occur, additionally. The successful application of MIP **8** to real wastewater samples has further proven the applicability and reliability of this polymer.

IV Experimental Section

1. Synthesis

1.1 Preparation of diclofenac acid

The 6-step conversion procedure was performed as follows:

(1) Diclofenac sodium salt (1.0 g) was weighed in a clean beaker, and then 60 mL milli-Q water was added in the beaker, the solution was stirred with a stirring bar to fully dissolve the salt.

(2) HCl solution (1.2 M) was prepared by diluting concentrated hydrochloric acid. Then the diluted HCl solution was added drop by drop to the diclofenac salt solution. Immediately, white color precipitates appeared in the solution. This precipitate was the non-soluble diclofenac free acid.

(3) HCl solution was added continually until no precipitates were generated any more. The pH of supernatant solution was checked with a pH meter. When the pH was 2-3, titration was stopped.

(4) Diclofenac solution was then transferred into a clean separation funnel and mixed with 20-30 mL of chloroform solvent.

(5) After shaking, the separation funnel was put on an iron stand and liquids allowed to settle for 5 min. The solution was separated into organic and aqueous phases. The aqueous solution in the lower part was discarded.

(6) The chloroform part in the funnel was evaporated in vacuum and white colored crystals of diclofenac acid were obtained.

1.2 Synthesis of MIP 1

For the synthesis of diclofenac imprinted polymer MIP **1**, 87 mg (0.3 mmol) of diclofenac were mixed with 140 mg (1.2 mmol) of functional monomer 4-VP in a 4 mL screw-capped glass vial followed by addition of 1.2 mL of porogen ACN. An amount of 2.48 g of cross-linker PEGDA and 19 mg initiator AIBN were then added to the above solution. The solution was sonicated and spurged with N_2 for 5 min. The test tube was sealed under nitrogen and was then placed in a thermoblock TB1 (Biometra, Göttingen, Germany) at 60 °C for 24 h. As a blank, the non-imprinted polymer NIP **1** was simultaneously prepared in the same way but without the addition of the diclofenac template.

1.3 Synthesis of MIP 2

87 mg (0.3 mmol) of diclofenac were mixed with 140 mg (1.2 mmol) of functional monomer 4-VP in a 4 mL screw-capped glass vial followed by addition of 1.5 mL of porogen ACN. An amount of 1.28 g of cross-linker DVB (80%) and 19 mg initiator AIBN were then added to the above solution. The solution was sonicated and spurged with N_2 for 5 min. The test tube was sealed under nitrogen and was then placed in a thermoblock TB1 (Biometra, Göttingen, Germany) at 60 °C for 24 h. NIP **2** was simultaneously prepared in the same way but without the addition of the diclofenac template.

1.4 Synthesis of MIP 3

87 mg (0.3 mmol) of diclofenac were mixed with 140 mg (1.2 mmol) of functional monomer 4-VP in a 4 mL screw-capped glass vial followed by addition of 1.2 mL of porogen ACN. An amount of 1.38 g of cross-linker EGDMA and 19 mg initiator AIBN were then added to the above solution. The solution was sonicated and spurged with N_2 for 5 min. The test tube was sealed under nitrogen and was then placed in a thermoblock TB1 (Biometra, Göttingen, Germany) at 60 °C for 24 h. NIP **3** was simultaneously prepared in the same way but without the addition of the diclofenac template.

1.5 Synthesis of MIP 4

88 mg (0.3 mmol) of diclofenac were mixed with 140 mg (1.2 mmol) of functional monomer 4-VP in a 4 mL screw-capped glass vial followed by addition of 1.4 mL of MeOH and 0.35 mL H_2O . An amount of 1.38 g of cross-linker EGDMA and 20 mg initiator AIBN were then added to the above solution. The solution was sonicated and spurged with N_2 for 5 min. The test tube was sealed under nitrogen and was then placed in a thermoblock TB1 (Biometra, Göttingen, Germany) at 60 °C for 24 h. NIP **4** was simultaneously prepared in the same way but without the addition of the diclofenac template.

1.6 Synthesis of MIP 5

88 mg (0.3 mmol) of diclofenac were mixed with 140 mg (1.2 mmol) of functional monomer 4-VP in a 4 mL screw-capped glass vial followed by addition of 0.5 mL of octanol and 0.5 mL of trichloroethane. An amount of 1.38 g of cross-linker EGDMA and 20 mg initiator AIBN were then added to the above solution. The solution was sonicated and spurged with N_2 for 5 min. The test tube was sealed under nitrogen and was then placed in a thermoblock TB1 (Biometra, Göttingen, Germany) at 60 °C for 24 h. NIP **5** was simultaneously prepared in the same way but without the addition of the diclofenac template.

1.7 Synthesis of MIP 6

88 mg (0.3 mmol) of diclofenac were mixed with 140 mg (1.2 mmol) of functional monomer 4-VP in a 4 mL screw-capped glass vial followed by addition of 1.5 mL of MeOH. An amount of 1.38 g of cross-linker EGDMA and 19 mg initiator AIBN were then added to the above solution. The solution was sonicated and spurged with N_2 for 5 min. The test tube was sealed under nitrogen and was then placed in a thermoblock TB1 (Biometra, Göttingen, Germany) at 60 °C for 24 h. NIP **6** was simultaneously prepared in the same way but without the addition of the diclofenac template.

1.8 Synthesis of MIP 7

86 mg (0.3 mmol) of diclofenac were mixed with 130 mg (1.2 mmol) of functional monomer 2-VP and 110 mg of MAA in a 4 mL screw-capped glass vial followed by addition of 2 mL of DCM. An amount of 1.2 g of cross-linker EGDMA and 20 mg initiator AIBN were then added to the above solution. The solution was sonicated and spurged with N_2 for 5 min. The test tube was sealed under nitrogen and was then placed in a thermoblock TB1 (Biometra,

Göttingen, Germany) at 60 °C for 24 h. NIP **7** was simultaneously prepared in the same way but without the addition of the diclofenac template.

1.9 Synthesis of MIP 8

89 mg (0.3 mmol) of diclofenac were mixed with 140 mg (1.2 mmol) of functional monomer 2-VP in a 4 mL screw-capped glass vial followed by addition of 1.2 mL of toluene. An amount of 1.38 g of cross-linker EGDMA and 19 mg initiator AIBN were then added to the above solution. The solution was sonicated and spurged with N_2 for 5 min. The test tube was sealed under nitrogen and was then placed in a thermoblock TB1 (Biometra, Göttingen, Germany) at 60 °C for 24 h. NIP **8** was simultaneously prepared in the same way but without the addition of the diclofenac template.

1.10 Synthesis of MIP 9

86 mg (0.3 mmol) of diclofenac were mixed with 140 mg (1.2 mmol) of functional monomer 2-VP and 100 mg of MAA in a 4 mL screw-capped glass vial followed by addition of 1.2 mL of ACN. An amount of 2.3 g of cross-linker EGDMA and 20 mg initiator AIBN were then added to the above solution. The solution was sonicated and spurged with N_2 for 5 min. The test tube was sealed under nitrogen and was then placed in a thermoblock TB1 (Biometra, Göttingen, Germany) at 60 °C for 24 h. NIP **9** was simultaneously prepared in the same way but without the addition of the diclofenac template.

The resultant MIP monolith was crushed, ground mechanically and wet-sieved using acetone. The particle size fraction of 32-63 µm was collected. These particles were then sonicated in methanol/acetic acid solution (9:1, v/v) for 15 min, followed by centrifugation to remove supernatant solvent. This procedure was repeated a few of times until the template molecule could not be detected in the supernatant by HPLC. Then, the particles were sonicated again in methanol three times for 15 min per cycle to remove residual acetic acid. Finally, the extraction solvent was removed by centrifugation and the particles were dried under vacuum. As a blank, NIP was similarly prepared, though in the absence of the diclofenac template.

2 Instrumentation and Accessories

2.1 Preparation and characterization of samples

Baker Bond Speedisk Extraction Disk	DVB-8068-06, Baker, Deventer, NL
Elemental Analyzer Perkin Elmer 240 C	Perkin Elmer, USA
Envi-Carb ® SPE cartridge	57088, Supelco, Bad Homburg
Fluorescence Spectrometer, LS 50B	Perkin Elmer, USA
Ganzglas-Vakuumfiltrationsgerät	Sartorius, Göttingen
^{1}H NMR Spectrometer, Bruker AMX 400	Bruker, Karlsruhe
^{1}H NMR Spectrometer, Bruker AMX 600	Bruker, Karlsruhe
Incubator/Shaker HT iEMS	Labsystems, Helsinki, Finland
LiChrolut ® Fritten (PTFE), Porosität 10 µm	Merck, Darmstadt
LiChrolut ® Glassäulen 3, 5 and 8 mL	Merck, Darmstadt
LiChrolut ® SPE cartridge	1.02023, Merck, Darmstadt
Mikropräzisionssieb, 32 µm	Fritsch GmbH, Idar- Oberstein
Mikropräzisionssieb, 63 µm	Fritsch GmbH, Idar-Oberstein
Oasis ® HLB SPE cartridge, 1, 3 mL	WAT094225/6, Waters, Eschborn
Oasis ® MAX SPE cartridge, 1mL	186000366, Waters, Eschborn
Orbital-Schüttler for MTP, EAS 2/4	SLT Labinstruments, Crailsheim
Parafilm M, 4 x 250 ft	American Nat. Can, Greenwich, USA
Peristaltikpumpe ISM 726	Ismatec, Wertheim-Mondfeld
pH-Meter, Piccolo plus, HI 1295	Neolab, Heidelberg
Reinstwasseranlage Milli Q plus 185	Millipore, Eschborn
Rotationsverdampfer Rotavapor RE 111	Buchi, Flawil, Schweiz
SPE-Arbeitsstation Visiprep	Supelco, Bad Homburg
Strata X ™ SPE cartridge	8BS100, Phenomenex, Aschaffenburg
Strata X ™ C18-E SPE cartridge	S201-52, Phenomenex, Aschaffenburg
ThermalSeal Film	Sigma-Aldrich, Steinheim
Thermoblock TB1	Biometra, Göttingen
Ultra Turrax ® T25 basic	IKA, Staufen
UV-Vis Spectrophotometer DU 650	Beckman Instruments, Fullerton, USA
Whatman Glasfaserfilter GF/C, ∅ 4.7cm	516F2311, Merck, Darmstadt
Zentrifuge EBA 3S	Hettich, Tuttlingen

2.2 HPLC-UV/DAD

Autosampler AS-50	Dionex, Germering
Column Guard-cartridge (2 µm)	Alltech, Unterhaching
Degasser DG-2410	Dionex, Germering
HPLC Column, 250x4.6 mm,	Phenomenex, Aschaffenburg
Synergi Max-RP, 4 µm	Phenomenex, Aschaffenburg
HPLC Column, 250x3.0 mm,	Phenomenex, Aschaffenburg
Synergi Max-RP, 4 µm	Phenomenex, Aschaffenburg
Polypure II 4 mm Spritzenfilter 0.45 µm	Alltech, Unterhaching
Pump P580	Phenomenex, Aschaffenburg
Software CHROMELEON 6.50	Dionex, Germering
Spritzenfilter 25 mm 0.2 µm PTFE	Roth, Karlsruhe
UV-Vis Detector PDA 100	Dionex, Germering
Vorsäule C18 (4 mm L x 3 mm ID)	Phenomenex, Aschaffenburg

2.3 LC/MS

Agilent µLC-MSD/SL 1100-System	Agilent, Waldbronn
HPLC column Inertsil C18 (150 x 3.0 mm)	MZ-Analytical, Mainz
MassLynx ™ 4.0 Software	Micromass, Milford, USA
Quadrupole MS/MS Ultima ™ Pt	Micromass, Milford, USA
Waters Alliance ® HT 2795 LC	Waters, Eschborn
Zorbax SB-Phenyl Spezial Column (1 x 250 mm), 5 µm	Agilent, Waldbronn

3 Chemicals and Reagents

Acetic acid, 100%, p.a.	1.00063, Merck, Darmstadt
Acetonitrile, Chromasolv®, gradient grade	34851 Riedel de Haen, Seelze
Acetonitrile-d_3, min. 99.95%	1.13753, Merck, Darmstadt
2,2'-Azobisisobutyronitrile	Merck, Darmstadt
Chloroform	Sigma-Aldrich, Steinheim

Chloroform-d, min. 99.8%	Sigma-Aldrich, Steinheim
Cyclohexane, p.a.	1.09666, Merck, Darmstadt
Dicalciumhydrogenphosphate, 99%	04248, Riedel de Haen, Seelze
Diclofenac sodium salt	Sigma-Aldrich, Steinheim
Ethylene glycol dimethacrylate	Sigma-Aldrich, Steinheim
N-(3-dimethylaminopropyl)-N ethyl-Carbodiimide-hydrochloride	8.00907, Merck, Darmstadt
Decane	Sigma-Aldrich, Steinheim
Deuterated water D_2O	Sigma-Aldrich, Steinheim
Dichloromethane	Sigma-Aldrich, Steinheim
N,N-dimethylformamide, p.a.	1.03053, Merck, Darmstadt
Dimethylsulfoxide, p.a.	1.16743, Merck, Darmstadt
Di-(N-succinimidyl)-carbonate	43720, Sigma-Aldrich, Steinheim
Dioxane, dry	1.03110, Merck, Darmstadt
Divinylbenzene	Sigma-Aldrich, Steinheim
Dodecanol	Sigma-Aldrich, Steinheim
Ethanol, p.a.	1.00983, Merck, Darmstadt
Ethylacetate	Sigma-Aldrich, Steinheim
1,2-Dichloroethane	Sigma-Aldrich, Steinheim
Dimethyl sulfoxide	Sigma-Aldrich, Steinheim
Ethylenedinitrilotetraacetic acid-disodium salt	Sigma-Aldrich, Steinheim
Ethylenedinitrilotetraacetic acid-tetrasodium salt	Sigma-Aldrich, Steinheim
Fenofibrate	Sigma-Aldrich, Steinheim
Formic acid, p.a.	8.22254, Merck, Darmstadt
n-Hexane, LiChrosolv®	1.04391, Merck, Darmstadt
Hydrochloric acid, 37%, p.a.	84422, Fluka, Buchs, Schweiz
Ketoprofen	Sigma-Aldrich, Steinheim
Mefenamic acid	Sigma-Aldrich, Steinheim
Methacrylic acid	Sigma-Aldrich, Steinheim
Methanol, p.a.	1.06009, Merck, Darmstadt
Methanol, LiChrosolv®	1.04391, Merck, Darmstadt
Octanol	Sigma-Aldrich, Steinheim

Poly(ethylene glycol)dimethacrylate	Sigma-Aldrich, Steinheim
Potassium dihydrogen citrate	60214, Fluka, Buchs, Schweiz
Potassium dihydrogen phosphate	60219, Fluka, Buchs, Schweiz
di-Potassium hydrogen phosphate	04248, Riedel de Haen, Seelze
Potassium hydroxide, p.a.	30603, Sigma-Aldrich, Steinheim
Sodium acetate, p.a.	32319, Riedel de Haen, Seelze
Sodium carbonate, p.a.	6395, Merck, Darmstadt
Sodium chloride	71376, Fluka, Buchs, Schweiz
Sodium citrate, p.a.	1.106448, Merck, Darmstadt
Sodium dihydrogenphosphate dihydrate	1.06345, Merck, Darmstadt
Sodium dodecylsulfate	8.17034, Merck, Darmstadt
Sodium hydrogencarbonate, p.a.	1.06329, Merck, Darmstadt
Sodium hydroxide, p.a.	1.06495, Merck, Darmstadt
Sodium sulphate, dry, p.a.	1.06649, Merck, Darmstadt
Sulphuric acid, 98%, p.a.	1.00732, Merck, Darmstadt
Tetrahydrofuran	Sigma-Aldrich, Steinheim
Tetramethoxysilane, p.a.	87680, Fluka, Buchs, Schweiz
Toluene	Riedel de Haen, Seelze
Toluene-d_8	Sigma-Aldrich, Steinheim
1,1,2-Trichloroethane	Sigma-Aldrich, Steinheim
2-Vinylpyridine	Sigma-Aldrich, Steinheim
4-Vinylpyridine	Sigma-Aldrich, Steinheim

4 Buffer solution

Phosphate buffer solution (PBS, pH 2.0)	Roth, Karlsruhe
Phosphate buffer solution (PBS, pH 3.0)	Roth, Karlsruhe
Phosphate buffer solution (PBS, pH 4.0)	Roth, Karlsruhe
Phosphate buffer solution (PBS, pH 5.0)	Roth, Karlsruhe
Phosphate buffer solution (PBS, pH 6.0)	Roth, Karlsruhe
Phosphate buffer solution (PBS, pH 7.0)	Roth, Karlsruhe
Phosphate buffer solution (PBS, pH 8.0)	Roth, Karlsruhe
Phosphate buffer solution (PBS, pH 9.0)	Roth, Karlsruhe
Phosphate buffer solution (PBS, pH 10.0)	Roth, Karlsruhe

Phosphate buffer solution (PBS, pH 11.0)	Roth, Karlsruhe
Phosphate buffer pH 6.0	1.56 g NaH_2PO_4, dihydrate
(for water sample pH adjustment)	12.46 g Na_2HPO_4, dihydrate
	In 1 L water

5 HPLC

5.1 LC-UV/DAD

Analysis was performed on a Dionex BioLC system equipped with a DG-2410 degasser, ternary HPLC pump, PDA-100 photodiode array detector, and an AS-50 thermal compartment column oven (Dionex, Germering, Germany). Chromatographic separations were carried out with a Synergi Max-RP column 250 x 4.6 mm i.d. (4.0 µm particle size) (Phenomenex). Injection was performed with an AS-50 autosampler connected with a 25 µL sample loop. The UV detector wavelength was set at 278 nm. The column oven temperature was set at 35 °C, and the temperature of autosampler tray was set at 24 °C.

Isocratic

Mobile Phase:	A: ACN, 0.1 M HOAc (5.7 mL HOAc in 1L ACN)
	B: distilled H_2O, 0.1 M HOAc (5.7 mL HOAc/1L H_2O)
	A/B, 60:40 (v/v)
Flow Rate:	0.8 mL/min

Gradient Elution 1

Mobile Phase:	A: distilled H_2O, 0.1 M HOAc (5.7 mL HOAc/1L H_2O)
	B: ACN, 0.1 M HOAc (5.7 mL HOAc in 1L ACN)
Flow Rate:	0.6 mL/min
Gradient:	40% B linear to 66.5% B in 16 min
	66.5% B linear to 85% B in 10 min

85% B constant for 10 min
85% B linear to 40% B in 15 min

Gradient Elution 2

Mobile Phase:	A: distilled H_2O, 0.1 M HOAc (5.7 mL HOAc/1L H_2O)
	B: ACN, 0.1 M HOAc (5.7 mL HOAc in 1L ACN)
Flow Rate:	0.6 mL/min
Gradient:	40% B linear to 66.5% B in 10 min
	66.5% B linear to 75% B in 5 min
	75% B constant for 2 min
	75% B linear to 40% B in 10 min

5.2 LC-MS

For the LC-MS (Waters micromass LCT) measurement, a Zorbax SB-Phenyl special-HPLC column (1 x 250 mm) with 5 µm particle size (Agilent) was used for separation. The injection volume was 20 µL.

Gradient Elution 3

Mobile Phase:	A: distilled H_2O, 0.1 M HOAc (5.7 mL HOAc/1L H_2O)
	B: ACN, 0.1 M HOAc (5.7 mL HOAc in 1L ACN)
Flow Rate:	1.0 mL/min
Gradient:	40% B linear to 66.5% B in 16 min
	66.5% B linear to 85% B in 10 min
	85% B constant for 10 min
	85% B linear to 40% B in 14 min

V Glossary of Abbreviations

A	Absorption
AAS	Atomic absorption spectroscopy
AIBN	Azobisisobutyronitrile
AU	Absorption Unit
ACN	Acetonitrile
BLK	Blank polymer
Bp	Boiling point
C	Concentration
CE	Capillary Electrophoresis
CR	Cross-Reactivity
DAD	Diode Array Detector
DCM	Dichloromethane
δ	Chemical shift
DCF	Diclofenac
DMF	Dimethylformamide
DMSO	Dimethylsulfoxide
DVB	Divinylbenzene
EDTA	Ethylenediaminetetraacetic acid
EGDMA	Ethylene glycol dimethacrylate
ELISA	Enzyme Linked ImmunoSorbent Assay
ESI	Electrospray Ionization
g	Gram
GC	Gas Chromatography
h	Hour
HPLC	High Performance Liquid Chromatography
IS	Internal standard
K	Constant
K_d	Dissociation constant of binding sites
LC	Liquid Chromatography
LOD	Limit of Detection
LOQ	Limit of Quantitation

M	Molar
m	Mole
MAA	Methacrylic acid
MDL	Minimum Detection Limit
MHz	Megahertz
Min	Minute
MIP	Molecularly Imprinted Polymer
mM	Millimolar
MRM	Multiple Reaction Monitoring
MISPE	Molecularly Imprinted Solid Phase Extraction
MS	Mass Spectrometry
NIP	Non Imprinted Polymer
nm	Nanometer
NMR	Nuclear Magnatic Resonance
NSAID	Non steroidal anti-inflammatory drugs
PBS	Phosphate Buffer Solution
PDA	Photodiode Array
PEGDA	Poly(ethyleneglycol)dimethacrylate
PETEA	Pentaerythritol tetraacrylate
PETRA	Pentaerythritol triacrylate
ppb	Parts per billion ($\mu g\ L^{-1}$)
ppm	Parts per million ($mg\ L^{-1}$)
ppt	Parts per trillion ($ng\ L^{-1}$)
Q	Amount of analyte bound to MIP
Q_{max}	Apparent maximum number of binding sites
r	Correlation factor
RAM	Restricted access media
RP	Reversed Phase
RT	Room Temperature
s	Second
SD	Standard deviation
SPE	Solid Phase Extraction
STP	Sewage Treatment Plant
t	Time

t_R	Retention time
TEA	Triethylamine
TFA	Trifluoroacetic acid
TRIM	Trimethylolpropane trimethacrylate
UV-Vis	Ultraviolet and visible
v/v	Volume / Volume
2-VP	2-Vinylpyridine
4-VP	4-Vinylpyridine
w	Weight

VI References

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