Universitat Autònoma de Barcelona

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I dedicate this thesis to my family,

my husband, Mohamad Hafiez Abdullah,

and my beloved son, Muhammad Zhafriel

for their constant support and

unconditional love.

I love you all dearly

Summary

Since a few decades ago, Emerging Persistent Pharmaceutical Pollutants (EPPPs) have been introduced as one type of recalcitrant pollutant sources in water. In this study, synthesis and characterization of molecularly imprinted polymer using selective functional monomer for diclofenac and indomethacin removal in aqueous media via batch mode has been done. Next procedure involved was the characterization of molecularly imprinted solid phase extraction using selective functional monomer for removal and recovery of diclofenac and indomethacin from aqueous media. Then the experimental continued with analytical methods for application of molecularly imprinted polymer using selective functional monomer for diclofenac recovery from water and wastewater.

From the kinetic study, more than 95% of removal was observed for DCF and IDM, with an initial concentration of 5 mg L^{-1} of DCF and IDM within 3 min, agitated at 25 ^{o}C. From the total adsorption study using a cartridge pre-packed with 10 mg of MIP-IDM and MIP-DCF a high adsorption capacity of 600 mg IDM/g MIP and 200 mg DCF/g MIP respectively, were obtained. Scatchard plots were determined to study the homogeneity properties of MIPs finding that MIP-DCF differs to MIP-IDM. Breakthrough curves have been identified during the saturation study using continuous flow mode.

Fourier transform infrared-attenuated total reflectance (FTIR-ATR) has been used in order to study the functional groups in three kinds of different MIP-DCF which were original MIP-DCF, MIP-DCF loaded and MIP- DCF eluting after 10 th times of regeneration. In order to study the morphology, scanning electron microscopy (SEM) was used. Pre-polymerization has been studied using ^{1}H NMR. The shift in the signal observed has been identified with the interactions between amine of AT group with carboxylic acid on DCF. MIP-DCF was chosen for packing into the HPLC column. For the selectivity study, both MIPs were carried out in batch mode. The results show that MIP with AT as the monomer bind to DCF molecules.

For application study, there were three methods has been designed in order to achieved the application objectives for this study. First, the continuous flow mode equipped to UV spectrophotometry detection; second, the optimization of molecularly imprinted solid phase extraction (MISPE) using real water samples; and thirdly, the MIP packed column equipped to high performance liquid chromatography with ultraviolet detection (HPLC-UV) for simultaneous determination. MIP enhance the efficiency in removal and recovery of EPPPs when the MIP has been used as packing media in column.

As conclusion, the developed MIP works as a good sorbent in DCF and IDM removal. The molecularly imprinted technology has shown to be a promising technology for the removal of EPPPs in water. As conclusion, the developed MIP works as a good sorbent in DCF and IDM removal.

Resumen

Desde hace unas décadas, los contaminantes farmacéuticos persistentes emergentes (EPPPs) han sido introducidos como un tipo de fuentes recalcitrantes de contaminantes en el agua. En este estudio, se ha realizado la síntesis y caracterización de polímeros con impresión molecular usando monómero funcional selectivo para la eliminación de diclofenaco e indometacina en medio acuoso a través del modo discontinuo. El siguiente procedimiento implicado fue la caracterización de la extracción en fase sólida molecularmente impresa usando monómero funcional selectivo para la eliminación y recuperación de diclofenaco e indometacina a partir de medios acuosos. A continuación, el experimento continuó con métodos analíticos para la aplicación de polímero impreso molecularmente usando monómero funcional selectivo para la recuperación de diclofenaco de agua y aguas residuales.

En el estudio cinético se observó más del 95% de la eliminación de DCF e IDM dentro de los 3 primeros minutos, con una concentración inicial de 5 mg L^{-1} de DCF e IDM, agitando y a 25ºC. Del estudio de adsorción usando un cartucho empaquetado con 10 mg de MIP-IDM y MIP-DCF, se obtuvo una elevada capacidad de adsorción, concretamente de 600 mg IDM/g MIP y 200 mg DCP/g de MIP. Se utilizó el diagrama de Scatchard para estudiar la homogeneidad de MIP-IDM y MIP-DCF, y los resultados obtenidos mostraron que el proceso de sorción para MIP-DFC es homogéneo, mientras para MIP-IDM es heterogéneo. Durante el estudio de saturación mediante flujo continuo, se identificaron las curvas de ruptura.

Para el estudio de selectividad, ambos MIPs se llevaron a cabo en modo "batch". Se observó que el MIP preparado con AT como monómero se enlaza a moléculas de DCF. El desplazamiento en la señal observada se ha identificado como la interacción entre la amina del grupo AT con el ácido carboxílico del DCF. Se escogió el MIP-DCF para empaquetar en la columna de HPLC.

Para el estudio de los grupos funcionales de tres tipos diferentes de MIP-DCF (MIP-DCF original, MIP-DCF cargado y MIP-DCF eluido después de la 10ª regeneración) se utilizó Fourier Transform Infrared-Attenuated Total Reflectance (FTIR-ATR). El estudio de la morfología se llevó a cabo microscopía electrónica de barrido (SEM). La pre-polimerización se estudió mediante ^{1}H NMR.

Se diseñaron tres disposiciones diferentes para aplicar los MIPs. En primer lugar, el modo de flujo continuo equipado con detección por espectrofotometría UV, en segundo lugar, la optimización de la extracción en fase sólida de impresión molecular (MISPE) utilizando muestras de agua real y en tercer lugar, utilizar la columna de MIP empaquetada en el equipo de cromatografía líquida de alta resolución con detección de ultravioleta (HPLC-UV) para determinación simultánea. La eficiencia en la eliminación y recuperación de EPPPs mejora cuando el MIP se utiliza como medio para empaquetar la columna de HPLC.

En conclusión, el MIP funciona como un buen sorbente en la eliminación de DCF y IDM, y la tecnología de impresión molecular ha demostrado ser prometedora para la eliminación de EPPPs en agua.

Table of Content

List of Figures

List of Tables

ABBREVIATIONS

1.	NSAIDs	Non-steroidal anti-inflammatory drugs
2.	WWTP	Wastewater treatment plant
3.	MIT	Molecularly imprinting technology
4.	ECs	Emerging contaminants
5.	EPs	Emerging Pollutants
6.	EU	European Union
7.	EE2	17-alpha-ethinylestradiol
8.	E2	17-beta-estradiol
9.	EPPPs	Emerging pharmaceutical persistent pollutants
10.	COX	Cyclooxygenase
11.	PG	Prostaglandin
12.	AA	Arachidonic acid
13.	DCF	Diclofenac
14.	K_{ow}	Octanol/water partition coefficient
15.	pK_a	Acid dissociation constant
16.	IDM	Indomethacin
17.	pH	Measure of the hydorgen ion concentration of a solution
18.	DMI	O-desmethylindomethacin
19.	STP	Sewage treatment plant
20.	WWI	Wastewater influent
21.	WWE	Wastewater effluent
22.	$Al_2(SO_4)_3$	Alum
23.	$FeCl_3$	Iron (III) chloride
24.	MBR	Membrane bioreactor
25.	PAC	Powdered activated carbon
26.	CAS	Conventional activated sludge
27.	NDMA	Nitrosodimethylamine
28.	SPE	Solid-phase extraction
29.	LC	Liquid chromatography
30.	GC	Gas chromatography
31.	LC-UV	LC coupled to ultraviolet detection
32.	LC-MS	LC tandem to mass spectrometry
33.	LC-TOF-MS	LC tandem to time of flight – mass spectrometry
34.	HPLC	High performance LC
35.	HPLC-ESI-QTOF-MS	HPLC electrospray ionization - quadrupole - time of flight - mass spectrometry
36.	HPLC-DAD	HPLC – diode array detector
37.	HPLC – ESI – MS/MS	HPLC - electrospray ionization – quadrupole – mass spectrometry/mass spectrometry
38.	GC-MS	GC – mass spectrometry
39.	ISE	Ion-selective electrode
40.	AOP	Advanced oxidation processes
41.	MIP	Molecularly imprinted polymer
42.	MAA	Methacrylic acid
43.	EGDMA	Ethylene glycol dimethacrylate
44.	AIBN	2,2'-Azobisisobutyronitrile
45.	H_2O_2	Hydrogen peroxide
46.	NaOH	Sodium hydroxide

47.	HCl	Hydrochloric acid
48.	LF	Langmuir-Freundlich
49.	FTIR-ATR	Fourier Transform Infrared spectrophotometer equipped with attenuated total reflection
50.	SEM	Scanning electron microscope
51.	NMR	Nuclear Magnetic Resonance
52.	^{1}H NMR	Proton NMR
53.	^{13}C NMR	Carbon 13 NMR
54.	SeRMN	Servei de Resonància Magnètica Nuclear
55.	MISPE	Molecularly Imprinted Solid Polymer Extraction
56.	MIP-IDM	MIP with indomethacin as a template
57.	MIP-DCF	MIP with diclofenac as a template
58.	IBU	Ibuprofen
59.	AM	Acrylamide
60.	2-VP	2 - vynilpyridine
61.	4-VP	4 - vynilpyridine
62.	HEMA	2-hydroxyethyl methacrylate
63.	AT	Allylthiourea
64.	TRIM	Trimethylolpropane trimethacrylate
65.	HOAc	Glacial acetic acid
66.	MeOH	Methanol
67.	TLC	Thin layer chromatography
68.	NIP	Non-imprinted polymer
69.	EDMA	Ethylene dimethacrylate
70.	MIP-IDM-AM	MIP prepared with AM as the monomer using IDM as a template
71.	MIP-IDM-AT	MIP prepared with AT as the monomer using IDM as a template
72,	ε_τ	Dieletric constant
73.	μ	Dipole moment
74.	UV	Ultraviolet
75.	Acetonitrile-d_3	Deuterium (III) acetonitrile
76.	N-H	Amine bonding
77.	N-COR	Amide bonding with R as any alkyl group
78.	TU	1,3-bis-(3,5-bis(trifluoromethyl)phenyl) thiourea
79.	TFA	Trifluoroacetic acid
80.	ppm	Parts per million
81.	PP	Polypropylene
82.	PE	Polyethylene
83.	PTFE	Teflon
84.	SEM	Scanning Electron Microscope
85.	FESEM	Field Emission Scanning Electron Microscope
86.	S.D.	Standard deviation

CHAPTER 1

Introduction

Chapter 1

1.0 INTRODUCTION

Pharmaceuticals have already been shown to be present in the aquatic environment as a class of so-called "emerging" contaminants.[1] The difficulties to separate the cocktail of emerging contaminants have been noticed by many researchers globally. Most of the researchers found a lack of knowledge in the process of recovery beyond the separation which means also the purification process of the target molecules. Thus, this indicates the need of more specific treatments as the use of sorbents to eliminate and recover the emerging contaminants in order to clean water.

In the present chapter, the information on published studies has been reviewed and is shown, focusing on the target molecules diclofenac and indomethacin, kinds of non-steroidal anti-inflammatory drugs (NSAIDs). At the beginning of the chapter, the information on NSAIDs, the challenging in conventional wastewater treatment plant (WWTP) and the detection and removal using various methods, have been briefly explained. At the end of the chapter, the technology called molecularly imprinting technology (MIT) has been introduced as well as the specific objectives of the work.

1.1 EMERGING CONTAMINANTS (ECs) AND EMERGING POLLUTANTS (EPs)

A diverse array of natural and synthetic organic compounds are used by society in vast quantities for a range of purposes including the production and preservation of food, industrial manufacturing processes, as well as for human and animal healthcare.[2] The natural and synthetic organic compounds consumed are discharged and remains in natural water resources which detrimental the water quality worldwide. The circulation of pharmaceuticals which supplied by the manufacturer company have shown in the **Figure 1.1**. The pharmaceuticals have been consumed by human either with prescription or without prescription from the doctors, livestock and aquatic life. In addition, manufacturing company also contribute to this problem in which the company will discharge the pharmaceuticals waste into water. Human,

livestock and aquatic life will discharge the non-metabolized pharmaceuticals via urea and faeces. Most of the pharmaceuticals will become sediment and enter the water supply.

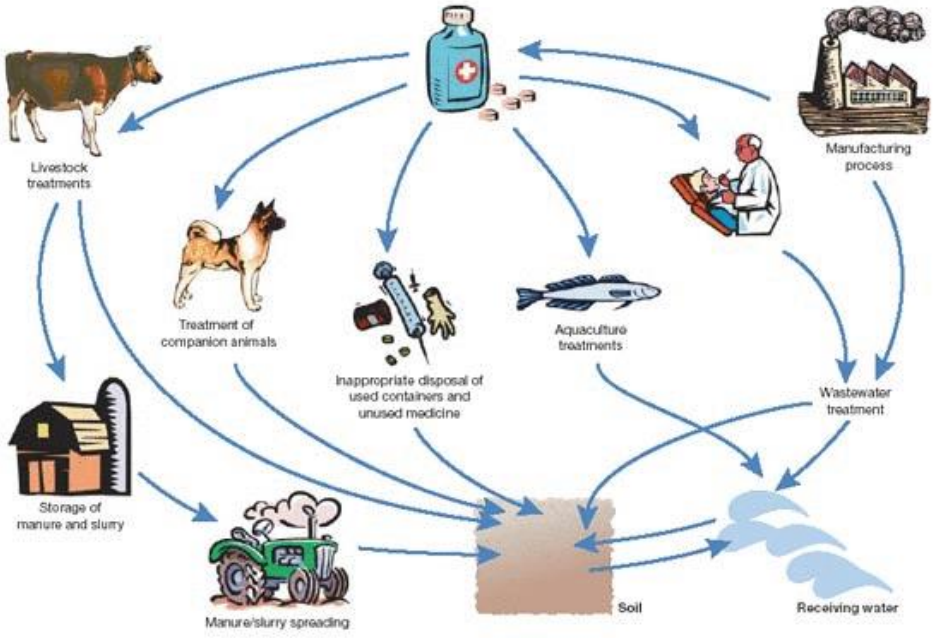

Figure 1.1 The circulation of pharmaceuticals consumption in environment.

Emerging contaminants (ECs) are in essence natural or synthetic substances which have the potential of entering the environment causing adverse ecological or human health effects, being most of them not regulated.[3] ECs have been reported in papers since few decades ago.[4–15] The definition for ECs has varied as well as the types of substances, for instance pharmaceutical compounds which has been listed as the priority compounds to be monitored under ECs. There are many other types of ECs as pesticides (insecticides, fungicides, herbicides) and flame retardants. Directive 2013/39/EU is an amendment to Directives 2000/60/EC and 2008/105/EC which regard with priority substances in the field of water policy.[16]

Pharmaceutical is a group of chemical substances that have medicinal properties and encompass all prescription, non-prescription, and over-the-counter therapeutic drugs, in addition to veterinary drugs.[17] Since a decade ago, pharmaceutical used in human and veterinary medicine, including hormones, have been classified as one type of emerging substances. Three substances with mainly pharmaceutical use have already been selected for inclusion in the first watch list in order to collect sufficient monitoring data for the determination of risk reduction measures: the steroidal estrogens 17-alpha-ethinylestradiol

(EE2), 17-beta-estradiol (E2) and diclofenac.[18] Monitoring data is particularly lacking for many emerging pollutants (EPs), which can be defined as pollutants currently not included in routine monitoring programs at EU level but which could pose a significant risk requiring regulation, depending upon their potential eco-toxicological and toxicological effects and on their levels in the aquatic environment.[17] According to Ladislav, M. and co-workers in the article entitled 'Introduction in Emerging Contaminants in River and Their Environmental Risk', contamination and pollution should not be seen as the same since all pollutants are contaminants, but only those contaminants that can result in adverse biological effects are pollutants.[17]

Two decades ago the adverse biological effect of the non-steroidal anti-inflammatory pharmaceutical diclofenac was observed after the declination number of *Gyps Bengalensis* (vulture) population in Pakistan [19] and then was considered a potential priority pollutant. Previous researchers shown that diclofenac causes renal failure and is lethal to vulture when it feeds on the carcass of a domestic animal that received a normal veterinary dose shortly before death. In Pakistan, diclofenac poisoning was found to be by far the most frequent cause of death.[20] Between 2000 and 2003, it was found a high annual adult and sub-adult mortality (5–86%), resulting in a declining of population (34–95%) associated with renal failure and visceral gout. Oaks and co-workers [19] had observed that diclofenac residues in vulture species were directly correlated with renal failure. Recently, in South Asia the mortality of vulture population has continued increasing and the main cause has been confirmed which came from diclofenac itself.[21] This is due to the illegal veterinary use although the regulations appeared at 2006 to prevent its veterinary use. To the best of our knowledge, diclofenac has been banned by the government of India, Nepal and Pakistan in 2006.[22] The consequences of diclofenac consumption can be also found in wild life especially marine life. Jenny-Maria and co-workers [23] observed diclofenac in the bile of wild fish caught downstream of a WWTPs with the average amount between 6 ng/ml until 95 ng/ml in bream species while 44 ng/mL to 148 ng/mL for roach species.[23] In addition, diclofenac was not only found in wastewater (both underground and surface water), but also was found in tap water and even worse in drinking water.[24] In the previous years, there were many articles reporting on the occurrence of diclofenac in water sources.[5,25,26]

In recent years, diclofenac consumption has been approved in Southern Europe. Nowadays, the vulture populations in Southern Europe has been threatened with the increasing of diclofenac

consumption.[27] In Spain, diclofenac consumption was authorized by the Spanish Drug and Health Products Agency) in March 2013 for the livestock usage. As a result, it has a potential to harm the remaining of the vulture populations in Spain. Thus, the authors urged the implemented consumption of diclofenac in Spain to be banned immediately to avoid undesirable consequences to vulture populations and ecosystem in the country.

According Vieno [28] pharmaceuticals such as diclofenac, naproxen, ibuprofen, ketoprofen, and carbamazepine can be detected in plasma of fish exposed to treated wastewater in aquaria.[23] In addition, Vieno [28] found that diclofenac could be detected in the bile of bream and roach caught from a lake that receives municipal wastewater effluents. Diclofenac concentrations in the lake ranged from 22 to 302 ng/L whereas the concentrations in the bile of bream and roach were up to 95 and 148 μg/L, respectively, roughly 1000 times higher than the aqueous concentrations.

However, there was also certain pharmaceuticals which the level of adverse biological effect was remained unknown until present and display endocrine disruptor properties for instance diclofenac and paracetamol.[29] **Table 1.1** shows the pharmaceuticals frequently found in aquatic environment.

Table 1.1 Pharmaceuticals frequently found in aquatic environment.[18]

Therapeutic class	**Representative compounds**
Analgesic/anti-inflammatory	Ibuprofen, Ketoprofen, Naproxen, Diclofenac, Salicyclic acid (aspirin metabolite), Acetaminophen (paracetamol), Codeine
Antibiotics	Sulfamethoxazole, Ofloxacin, Ciprofloxacin, Norfloxacin, Trimethoprim, Erythromycin, Azithromycin, Clarithromycin
Beta-blockers	Atenolol, Metoprolol, Sotalol, Propanolol
Lipid regulators	Gemfibrozil, Bezafibrat, Clofibric acid (metabolite)
Antidepressants	Diazepam, Citalopram, Paroxetine, Fluoxetine
Antiepileptic	Carbamazepine
Gastric protectors	Ranitidine
Diuretics	Hydroclorotiazida, Furosemide
X-ray contrast media	Iopromida, Diatrizoate, Iopamidol
Antidiabetic	Glibenclamide

'Emerging Pharmaceutical Persistent Pollutants' (EPPPs) is a group of pollutants focused on pharmaceutical compounds with persistence properties, defined as non-easily removed from water and so persistent towards water treatment under conventional procedures. Recently, EPPPs have been detected in trace amounts in water and usually at nanograms per liter until micro grams per liter.[30] EPPPs are one of the major water pollutants problems nowadays.

Pharmaceutical, personal care products, and endocrine disruptors are continuously being released, consciously or unconsciously, into water sources due to poor regulatory frameworks especially in developing countries.[31] Balance of the input and output of pharmaceutical in sewage treatment plants reveals that not all pharmaceuticals are removed during this treatment as they have been designed to be lipophilic and biologically persistent to maintain their therapeutic activity until their specific physiological function on humans or animals has been performed.[32]

1.2 OCCURRENCES OF PHARMACEUTICALS IN NATURAL WATER

The persistent characteristic of pharmaceutical compounds and the by-product toxicity are the main cause of contribution to the high risk effect in human and aquatic life. However, those pharmaceutical are not necessarily the most relevant ones with respect to their environmental risk as previous studies indicates.[33] The occurrence of organic micro-pollutants such as pharmaceutical in water supplies is a key issue in relation to the quality of water.

The synthetic pharmaceutical medicines are important for the needed and patients to heal from the diseases. However, after excretion via urine and feces these benefits turn into possibility of new diseases.

Recently, there is an increment in the number of articles which describe and discuss about the occurrences of EPPPs in natural water.[11,25,34–45] EPPPs are excreted in urine and feces from human and livestock.[46] There are many types of pharmaceutical medicines and related groups that are consumed nowadays such as antibiotics, non-steroidal anti-inflammatory drugs (NSAIDs), anti-epileptics, lipids regulators, beta-blockers, diuretics, contrast media, psycho-stimulants and antidepressants.[47] It is well known that the use of pharmaceutical compounds have been the success for the improvement of life quality in developed countries.

EPPPs are highly likely to be found in water especially in water treatment system in cities which consume clean recycled water from other cities. Hence, the developed analytical methods for removal of EPPPs from water bodies have become an emerging issue for researchers.

The consumers or patients consume the medicines and excrete the non-metabolized compounds through urine and feces and it will pass through the WWTP. Some of the pharmaceutical compounds are not able to be removed during the treatment unless there is an advanced method to remove them. The community consisting of peoples at different ages will use the water every day. The abundance of EPPPs in water resources might risks the community especially old people and children. The pharmaceutical load accumulated in human bodies will give detrimental effect in long term. To date, the toxicity of 'mixture cocktail' pharmaceutical compounds remain unknown and difficult to be separated.

In addition, if the pharmaceutical supplier increases their production, the total load accumulated of EPPPs into wastewater was estimated to be increased. Thus, to control this condition the recovery processes should be developed in wastewater treatment. In this case, the concentration of EPPPs loaded in water bodies can be controlled because recently there were certain pharmaceuticals urged to be banned in order to conserve the water quality since no effective regulations taskforces has been made. The study on develop recovery processes is still on-going. Thus, the present study has been focused on analytical separation techniques in order to develop a recovery methodology.

1.3 NON-STEROIDAL ANTI-INFLAMMATORY DRUGS (NSAIDs)

Non-steroidal anti-inflammatory drugs (NSAIDs) belong to one of the most commonly used therapeutically group of agents for the treatment of pain, fever and inflammation for instances paracetamol, ibuprofen and acetominophen.[48] Paracetamol (a pain reliever but not anti-inflammatory) and over-the-counter non-steroidal anti-inflammatory drugs (NSAIDs), herein referred to collectively as mild analgesics, are among the most sold pharmaceutical drugs worldwide.[29]

The function of the NSAIDs is to block Cyclooxygenase (COX) from the inflammation to be occurred. COX is an enzyme capable of converting arachidonic acid, which is involved in the

formation of cellular membranes.[49] Prostaglandin (PG) is a family of intercellular and intracellular messengers derived from arachidonic acid (AA). Prostaglandins play a key role in the generation of the inflammatory response. Their biosynthesis is significantly increased in inflamed tissue and they contribute to the development of the cardinal signs of acute inflammation.[50]

The initial step in the synthesis of PGs from AA is mediated by cyclooxygenase (COX, also known as prostaglandin-H_2 synthase or prostaglandin endoperoxide synthase), of which two isoforms are recognized, COX1 and COX-2. COX-1 is expressed constitutively in most cell types, and prostanoids derived from COX-1 are thought to be important in gastric and renal homeostasis. COX-2, on the other hand, is the product of an immediate early gene and is rapidly expressed only after exposure of cells to hormones, mitogenic stimuli, and inflammatory mediators, like bacterial lipopolysaccharide. The induction of COX-2, with the resultant production of prostanoids, can contribute to parturition, inflammation, pain, fever, and certain types of cancer.[51]

The two cyclooxygenase isoforms, metabolize arachidonic acid to prostaglandin-H_2, which is subsequently processed by downstream enzymes to the various prostanoids.[51] Under many circumstances the COX-1 enzyme is produced constitutively (gastric mucosa) whereas COX-2 is inducible (sites of inflammation).[49] Therefore, NSAIDs are designed to focus towards COX-2 to block the inflammatory response.

Table 1.2 below shows the classification of NSAIDs according to their COX-1 or COX-2 inhibitory activities. NSAIDs which are classified in Class 1 are the most consumed and so expected to be found in the waste waters, are working towards the COX-2, but can also inhibit COX-1, thus they are not selective.

Ibuprofen and aspirin are classical inhibitors of cyclooxygenases by reducing prostaglandin synthesis. However, aspirin is nonselective compared to ibuprofen.[52] The ability of aspirin to permanently inactivate both COX isoforms indiscriminately explains both its analgesic and anti-inflammatory properties, through COX-2 inhibition, as well as its damaging effects on the gastric mucosa, through COX-1 inhibition.[51]

Table1.2 Classification of NSAIDs according to their COX-1/COX-2 inhibitory activities.[53]

Class	Properties	Examples
Group 1	NSAIDs that inhibit both COX-1 and COX-2 completely with little selectivity	Aspirin, Ibuprofen, Diclofenac, Indomethacin, Naproxen, Piroxicam
Group 2	NSAIDs that inhibit COX-2 with a 5-50 fold selectivity	Celecoxib, Etodolac, Meloxicam, Nimesulide
Group 3	NSAIDs that inhibit COX-2 with a >50 fold selectivity	Rofecoxib, NS-398
Group 4	NSAIDs that are weak inhibitors of both isoforms	5-aminosalicyclic acid, Sodium salicylate, Nabumetone, Sulfasalazine

Praveen and co-workers [53] elaborated the origin of NSAIDs, their mechanism of action at the molecular level such as cyclooxygenase (COX) inhibition, development of selective COX-2 inhibitors, their adverse cardiovascular effects, and some recent developments targeted to the design of effective anti-inflammatory agents with reduced side effects. In their work, the authors found that NSAIDs represent an important class of compounds and developing a safe, effective and economical therapy for treating inflammatory conditions.

Non-steroidal anti-inflammatory drugs (NSAIDs) has been classified as prioritize compounds of pharmaceuticals that are often found as persistent toxic waste and was one of the most widely available drugs in the world.[54] Besides, recent findings indicate that paracetamol and NSAIDs have endocrine disruptive potential during fetal life.[29] In addition, NSAIDs were the most frequently drugs measured in wastewater samples.[5]

1.3.1 Diclofenac and Metabolites

Among the different NSAIDs, the present work is focused on diclofenac (DCF) since it is one of the most consumed and found in natural waters as previously said.[55] The chemical name of diclofenac is (2-(2-(2,6-dichlorophenylamino)phenyl)acetic acid).[28] Diclofenac molecule chemical structure consist of two chloride atoms, an amine group, a carboxylic acid group and two benzene rings as shown in **Figure 1.2**.

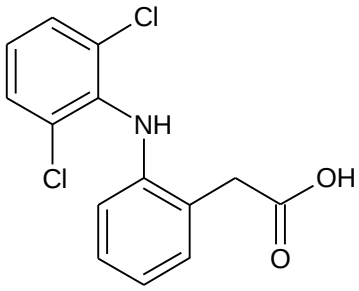

Figure 1.2 Molecular structure of diclofenac.

Drug metabolites can be defined as drug molecules which has been metabolized by the body into a modified form which continues to produce effects in the body. Diclofenac with its metabolites in human body are shown in **Figure 1.3**. Most of the drug is metabolized producing glucuronides and hydroxyl form.

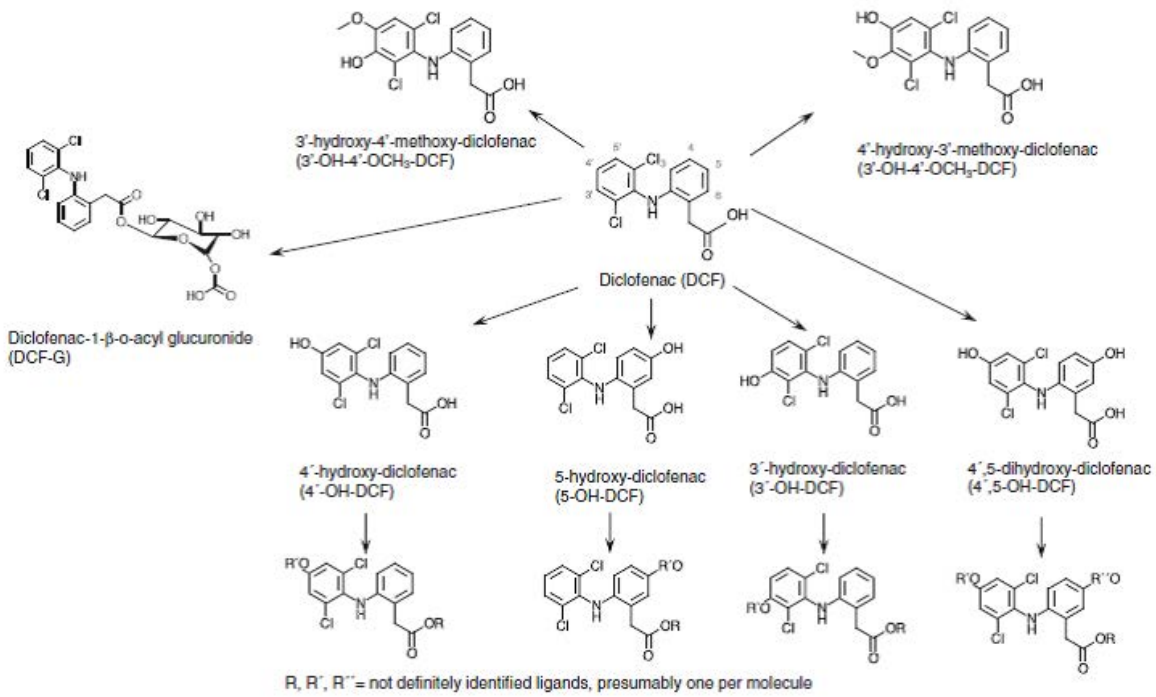

Figure 1.3 Metabolic pathway of DCF in human body.[28] Reprinted with permission.

The most important physical characteristic of diclofenac is log K_{ow} and pK_a. Log K_{ow} is a physical characteristic that describes the hydrophobicity properties expressed as the octanol/water partition coefficient.[56] Diclofenac have log K_{ow} value equals to 4.51.[57] By

referring to the James study ,[56] diclofenac can be classified as an intermediate compounds which can be hydrophobic and hydrophilic simultaneously. However, DCF tends to become hydrophobic when contacting water. Diclofenac pK_a value equals to 4.14.[57] Micro-contaminants having carboxylic acids functionalities with pK_a values much less than 7, such as some nonsteroidal anti-inflammatory drugs, are likely to remain in the solution phase when a sorption process is taking place, and the removal by sorption to settling particles may be limited.[17]

Diclofenac is one of the active pharmaceutical compounds which are most frequently detected in the water-cycle.[58] Muñoz [59] found that diclofenac to be the highest concentration among other NSAIDs in Llobregat river.

Between 2000 until 2010 the estimation of diclofenac consumption in European countries has been found to be different depending on the country being for Germany 953.6 mg $year^{-1}$ $inhabitant^{-1}$, Switzerland 934.1 mg $year^{-1}$ $inhabitant^{-1}$, France 370.1 mg $year^{-1}$ $inhabitant^{-1}$, Sweden 375.9 mg $year^{-1}$ $inhabitant^{-1}$ and Spain 369.9 mg $year^{-1}$ $inhabitant^{-1}$ [60]. The countries with highest consume of diclofenac among European countries were Germany (2613 µg per day/capacity), followed by Spain (2124 µg per day/capacity), Poland (1482 µg per day/capacity) and the lowest consumption was Switzerland (1459 µg per day/capacity).[61]

1.3.2 Indomethacin and Metabolites

The chemical name of indomethacin (IDM) is 1-(4-chlorobenzoyl)-5-methoxy-2-methyl-3-indoleacetic acid and it present as beige powder.[62] The molecular weight of indomethacin is 357.79 g/mol and the melting point is at 155°C. Indomethacin having pKa values ranging from 4.9 to 4.1.[63] The chemical structure of indomethacin is shown in **Figure 1.4**.

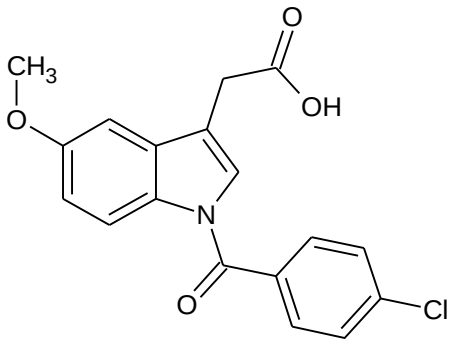

Figure 1.4 Molecule structure of indomethacin.

Indomethacin is a known non-steroidal anti-inflammatory drug (NSAID) broadly used to calm down acute joint and backbone pain and for the treatment of degenerative diseases of the joints and ligaments. Indomethacin is a prime drug used for the treatment of rheumatoid arthritis, gout, and collagen disease. It is a potent inhibitor of cyclooxygenases (COXs), reducing prostaglandin synthesis, relieving pain, and reducing fever in febrile patients.[64] However, recently mild analgesics such as indomethacin have recently been incriminated as potential endocrine disruptors.[65] It has been used since the past few decades for medical purposes as for the treatment of rheumatoid arthritis,[53] Patent Ductus Arteriosus,[66] Bartter syndrome [67] and Gitelman syndrome.[68]

In addition to the mentioned purpose for treatment of different types of diseases, there is an article reporting on the use of nanoparticles with the presence of indomethacin for cosmetic application.[69] The article elaborated the usage of nanoparticles to enhance the indomethacin penetration to skin without increasing the dose of indomethacin uptake or cause gastritis towards patients.

To date, long-term effects of accumulation of indomethacin in the body are still unaddressed. It is believed that the indomethacin concentration found in wastewater was very low. However, long-term effects might occur at much lower concentrations as it can be found frequently and follow different toxicodynamic mechanisms than those extrapolated from short-term studies.[40]

Most of the pharmaceuticals are difficult to be dissolved in water. Thus, the solubility product, K_{sp} of persistent pharmaceutical in water is very low and this includes indomethacin molecules

itself. This introduces the persistent characteristics properties of pharmaceuticals in water. Indomethacin was one kind of the persistent pharmaceuticals in water.

Potentiometric properties of indomethacin have been studied by Zholt and co-workers.[70] Zholt found that the potential of indomethacin molecules was stable in aqueous solution between pH 6 and pH 10. In addition, [70] had chosen pH 8 as the suitable pH media for indomethacin detection in aqueous solution. It is believed that indomethacin might be persistent in natural water as the pH of natural water was approximately between pH 5 and pH 8.

Indomethacin as the parent compound, together with its metabolite O-desmethylindomethacin (DMI) and the conjugates DMI acyl glucuronide and DMI ether glucuronide, have been found in human urine.[71] The chemical structures and diagram is shown in **Figure 1.5.** Indomethacin was mostly observed in wastewater treatment plant compared to its metabolites. Many articles report on finding the parent compound in wastewater rather than its metabolites and conjugated form.[59,72,73] This is because pharmaceutical are often excreted mainly as non-conjugated and conjugated polar metabolites. Conjugates form can, however, be cleaved in sewage treatment plants (STP), resulting in the release of active parent compound as shown for estradiol.[63,74] Fent [63] reported the toxicity of certain pharmaceuticals such as ibuprofen, ketoprofen but not with indomethacin. According to Bussetti [75] study, most of the cases which have been recently reported involved the parent substances (88 chemicals) rather than the transformation products (27 chemicals).

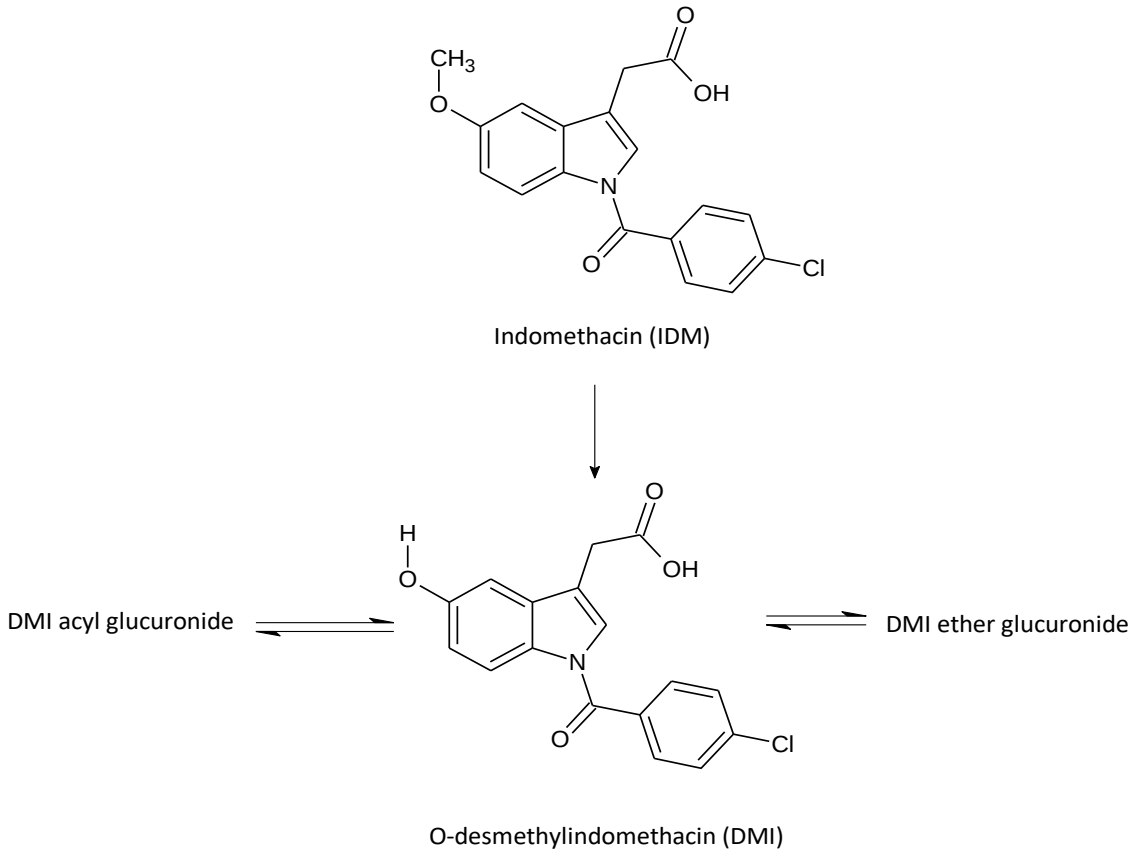

Figure 1.5 The yield of human urine corresponding to the consumption of indomethacin.[71] Data from Vree et al., 1993.

As for all pharmaceuticals, during both human and veterinary usage, a significant proportion of the non-metabolized indomethacin can pass through the body and is thus released into water systems. Indomethacin is typically considered stable in the environment. While acute effects of the exposure of aquatic life to indomethacin or other NSAIDs may not appear immediately, the long-term presence in aquatic systems may lead to chronic toxicity and subtle effects such as endocrine disruption. Therefore, considering the concerns over the potential adverse effects of indomethacin or other NSAIDs on aquatic organisms, it is necessary to monitor the levels of indomethacin and other NSAIDs in water samples frequently.[76] For instance, according to Verlicchi [77] the efficiency removal for indomethacin after passing through the WWTP in Spain hospitals was 37.5% of removal. Hence, this numerical percentage reveals that it was highly persistent in water although it has been passing through the commercial WWTP. Therefore, the new technology needs to be featured to enhance the ability of existing WWTP in removing the EPPPs compounds.

Nowadays, EPPPs become prominent among researchers and this is what most of the environmentalists are concerned about. Many researchers tried to develop methods in order to enhance the IDM dissolution properties and also the number of consumption among community.[69,78–86] As a consequences, the water quality will be affected as the consumption is predicted to be increased.

1.4 RECENT OCCURRENCES OF EPPPs IN SPANISH RIVERS

The pharmaceutical is mainly excreted in urine and feces as such or as metabolites. They are carried to wastewater treatment plants, where they pass through various treatment processes.[87] The continuous increase of the human population has been associated to a tremendous increase of the amount and structural diversity of the chemical compounds released into the environment.[30] Loos [88] had reported on European Union-wide survey of pharmaceutical persistent pollutants in European river waters founding that only 10% of river water samples analyzed could be classified as 'very clean' in terms of chemical pollution. However, in 2013, Loos [89] claimed that the obtained results showed the presence of 80% of the pharmaceutical compounds in European wastewater effluents.

In Spain, few major rivers have become the main focus of water supply treatment for consumption which are Llobregat, Ebro, Guadalquivir and Júcar rivers. For instance, Llobregat River (Catalonia, North Eastern Spain) is characterized by a typical Mediterranean regime and, as such, it suffers from extreme and frequent flow (10–300 m^3/s) fluctuations. Llobregat River suffering from low flows during normal conditions (5 m^3/s) and extraordinary peak events (maximum recorded of 2,500 m^3/s) that periodically reset the system[90]. In addition, the river receives the effluent discharges of more than 55 WWTPs, and at some points especially at drought periods, the effluents may represent almost 100% of the total flow of the river. Besides, in the lower reach there is an important concentration of urban and industrial activities with the corresponding pollution pressures.[91] Llobregat river mass flow loaded per family of pollutants has been described by Köck-Schulmeyer [92] in which pharmaceuticals have been reported as the highest mass flow with more than 4000.000 ng/s compared to estrogens (5000 ng/s), pesticides (50000 ng/s), illicit drugs (25000 ng/s) and alkyl phenols group (220000 ng/s). Thus, the high levels of emerging organic contaminants detected on the river, increases together with the augment of WWTPs and population pressure when moving downstream along the river. In addition, the Llobregat River provides drinking water to the large city of Barcelona.[90]

In Spain, approximately 55% of the consumed top 200 drugs are ingested orally, and approximately 5% of them correspond to NSAIDs.[93] According to Acuña [94] among the seven therapeutic groups of pharmaceutical detected (anti-inflammatories, lipid regulators, diuretics, antihypertensive, psychiatric drugs, β-blockers and antibiotics), concentrations of the NSAIDs group were the highest in all sampling sites, up to three times higher than other groups. The therapeutic groups mentioned previously were among the most frequently found in the aquatic environment.[17] However, it depends on the WWTP because different WWTP will have different kinds of pollutants problem. Therefore, the approaches of treatment would be different.

In 2014, indomethacin was detected in influent with low concentration (0.136 µg/L) whereas the concentration of effluent was unknown and has not been reported previously.[95] For instance, study conducted by Rosario [38] in determination of emerging pollutants in Galicia (North Western Spain) obtained an important finding which diclofenac and indomethacin were not removed at all (0% efficiency removal).

The average amount of diclofenac found in Henares-Jarama-Tajo, Madrid surface water was approximately 0.7 – 156 ng/L whereas in Llobregat River, Spain stated from 0.08 until 18.74 µg/L.[59] In Galicia Spain, diclofenac has been detected as one of the EPPPs which not efficiently being removed from small conventional WWTP using sorption process with the median value for effluent concentration still surpassed 0.1µg/L. Thus, in terms of transportation of matter, diclofenac can be spread through surface water.[38]

Commonly, drugs are designed to be biologically active, and it is possible that unintended effects on non-target organisms and/or receptors occur at lower concentration than the intended therapeutic effects.[40] The effects and toxicity of mixtures of sub-therapeutic levels of complex mixtures of drugs over perhaps 80 years or more are still unknown.[40] Furthermore, it is very complex to understand the mechanism of pharmaceutical in wastewater due to its differences in chemical-physico properties. It is predicted that more advance and persistent pharmaceutical compounds will be produce in order to fulfill the marketing needs. The dissolution of medicine might be increased as the clinical research development were still ongoing. Recently, a wide number of studies on redesign the pharmaceutical drugs to enhance the dissolution properties has been reported.[78,80] It was expected that the number of persistent pharmaceutical will be

increase in water bodies. Thus, it is a very challenging situation in order to remove EPPPs from water supply with a variety of EPPPs that may not sediment easily such as diclofenac at natural ambient. Thus, to control this problem the persistent pharmaceutical pollutants need to be determined and classified according to their percentage degradation under normal ambient degradation in conventional WWTP.

1.5 THE PRIORITIZATION OF EPPPs AND CONVENTIONAL WASTEWATER TREATMENT PLANT

There are several articles reporting on the prioritization of the pharmaceuticals in river water based on the toxicity and group of pharmaceutical[96,97]. Earlier, many of the articles reported the classification according to their types of therapeutic compounds such as ibuprofen is a kind of Non-Steroidal Anti-Inflammatory Drugs (NSAIDs). However, Bueno [5] had classified the pharmaceuticals according to the removal efficiency from high to low level of removal efficiency. For the pharmaceutical compounds that have been classified under primary degree with removal percentage reached 40%, it shows that the persistent property and stability in water is very high and it has high potential to be found in drinking water (diclofenac, indomethacin, triclosan) whereas for tertiary degree with removal efficiency between 70% until 100%, the pharmaceutical such as caffeine, ibuprofen and acetaminophen it is suggested that there is no need of modification of the actual treatment plants since it is very expensive and needs a lot of manpower for maintenance. The secondary degree is between 40% and 70%.

From the point of view, it is suggested that the removal efficiency can be a benchmark in order to prioritize the pharmaceutical that need to be removed. Hence, the baseline of pharmaceutical classification should be made clearly according to the pharmaceutical removal efficiency. However, the removal efficiency might be differed among WWTPs because the removal efficiency will depend on the established treatment in the WWTPs. Different WWTP will obtain different removal efficiency values. According to Muñoz [59] the distribution of pharmaceutical removal efficiency in Llobregat River, Spain the removal efficiency of diclofenac was approximately 60% to 70% (second class) whereas indomethacin was approximately 45% to 50% (third class). In Llobregat River, indomethacin was found at 0.16 µg/L.

It is expected that the amount of pharmaceutical loaded in water will be increased in few decades onwards. Mario and co-workers [17] reviewed that there are three main EPPPs which

has been included in Watch List: steroidal oestrogens, 17α-ethynylestradiol (EE2) and 17β-estradiol (E2), and diclofenac. However, recently the Watch List is still in viewed and progress. The approaches of managing the EPPPs and the consequent benefits for the emerging contaminants reduction will also depend mostly from the politic willingness of the governments of the member states of the EU[17].

In Spain, the frequency detection of diclofenac in wastewater influent (WWI) was 92% whereas in wastewater effluent (WWE) and sludge were 100% respectively.[98] The results reveal the fact that DCF was slightly removed after passing through the WWTP. Then, Aleksandra and co-workers [98] concluded that the elimination for most of the substances was incomplete and improvements of the wastewater treatment and subsequent treatments of the produced sludge are required to prevent the introduction of these micro-pollutants in the Spain water environment. However, the improvement using photodegradation process was feasible since it is very costly and depends only on the electricity supply to degrade the EPPPs in water.

Although, the EPPPs concentration was found in trace amount still it can contribute adverse effect in aquatic life and human being.[99] Recently, there was high number of pharmaceutical compounds detected in water included diclofenac (2200 ng/L) in Llobregat river and the pollutant source was from household waste.[100] Pedrouzo and co-workers [101] found that diclofenac poisoning had become worst with the most frequently detected and highly found in effluents (1032 ng/L). However, diclofenac found in influent was approximately 133 ng/L.[101] From this numerical data it reveals that the pollutant source of diclofenac was continuously contributed until it abundance in WWTP before it can be found in treated wastewater as an effluent. Diclofenac poisoning in water needs attention and urge to be overcome as it now getting extremely worst especially in European countries.

1.5.1 The Challenging in Conventional Wastewater Treatment Plant

In Spain, conventional WWTP commonly consist of primary treatment and secondary treatment. The first treatment involves septic tank, anaerobic filter, alum and chlorine. Septic tank functioned as the reservoir which receive wastewater from households commonly contained with anaerobic filter. Then, the semi-treated water mixed with alum and get sediment. The pollutants tend to sediment and the raw water will be chlorinated as the final stage before passing out as treated water.

In secondary treatment, there is membrane bio-reactor (MBR) function as wastewater filter. Membrane bioreactors hold a promise for the degradation of micro-pollutants, which could be ascribed especially to the high sludge concentration and relative high sludge age at which they operate.[102] However, certain micropollutants that cannot be sediment become sludge during treatment, such as diclofenac.

The recent wastewater treatments involve in a tertiary treatment. Nowadays, there are two main tertiary treatment methods, photodegradation processes and sorption processes. Unlikely, there are two main disadvantages of photodegradation process which make it not practicable in order to remove the pharmaceutical compounds from water bodies. First is the use of high energy and expensive sources in which the ultraviolet rays were used as the main sources for degradation processes. The second factor is the fact that certain pharmaceutical compounds can produce harmful by-products via photodegradation processes such as triclosan, a type of antimicrobial disinfectant. The photodegradation of triclosan produces sub-products (chlorodioxins and chloroanilines) that are highly toxic and persistent in water.[103] Thus, conventional WWTP is unable to remove recalcitrant pharmaceutical compounds although the WWTPs has been equipped with advanced technology such as photodegradation as tertiary treatment. For instance, in hospital effluents, micro-pollutants such as diclofenac and indomethacin shows quite similar trend in removal efficiency.

As can be seen from **Figure 1.6**, the removal efficiency was determining from Verlicchi study [104] which shows the distribution of diclofenac and indomethacin in three different kinds of treatment; primary, secondary and tertiary treatment in WWTPs for hospital effluents.

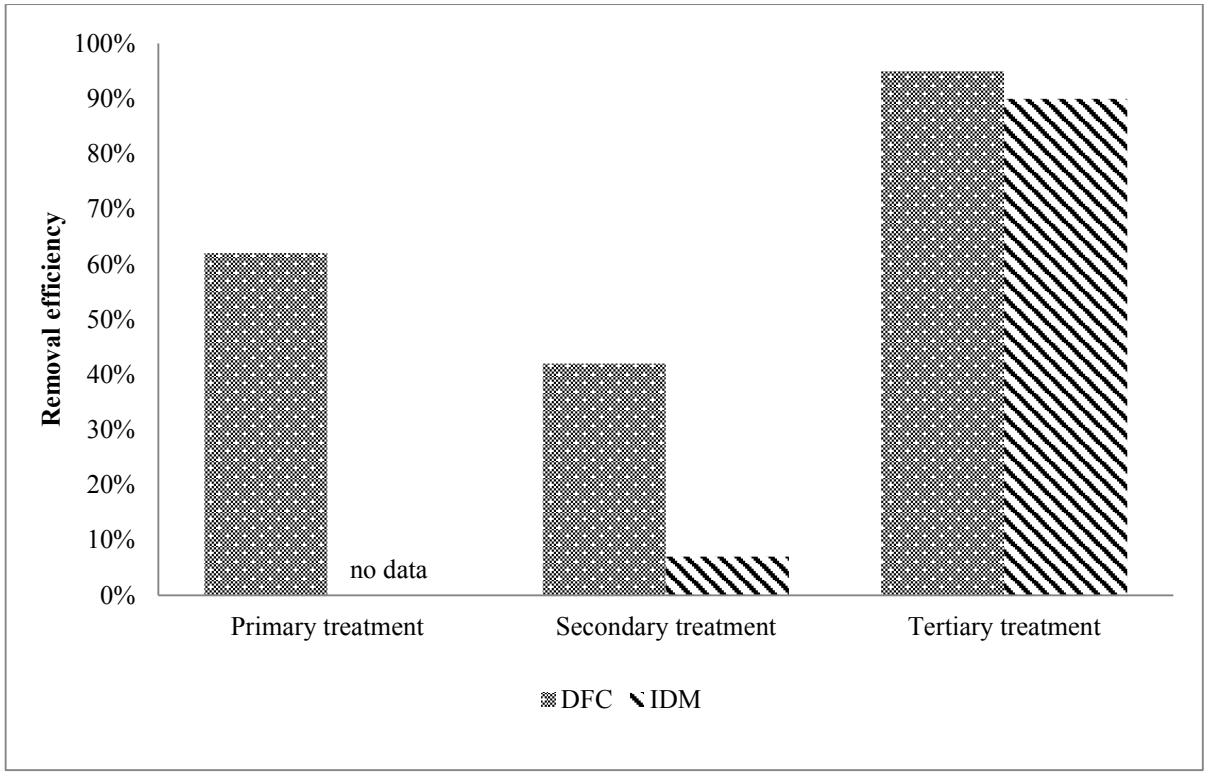

Figure 1.6 Recent trend of average efficiency removal for diclofenac (DCF) and indomethacin (IDM) in hospital effluents according to the primary, secondary and tertiary treatment (Primary treatment: septic tank + anaerobic filter, $Al_2(SO_4)_3$, $Al_2(SO_4)_3$ + flotation, $FeCl_3$, $FeCl_3$ + flotation; Secondary treatment: MBR (DCF:30 days, IDM:30 - 50 days); Tertiary treatment: PAC (8mg/L, 23mg/L & 43 mg/L) in conventional activated sludge (CAS) system. Data from: Verlicchi et al., 2015.[104]

Indomethacin removal methods for instance advanced oxidation process, photocatalyst and powdered activated carbon (PAC) has been reported in many articles previously and also had been summarized by Verlicchi and Luo.[104,105] According to Verlicchi [104] the objective in order to enhance the removal efficiency in secondary treatment using membrane bioreactor (MBR) from 30 until 50 days was unachieved with less than 10% of removal for indomethacin whereas more than 40% for diclofenac. However, for primary treatment there is no data provided for indomethacin and approximately more than 60% of removal for diclofenac. However, in tertiary treatment with powdered activated carbon (PAC) in conventional activated sludge (CAS) system implemented in tertiary treatment using different concentration (8mg/L, 23mg/L and 43mg/L) the efficiency removal was increase and reached approximately 90% of removal.[106] The similar observation also has been agreed by Johnson and co-workers [107] in which about 99% of removal has been achieved by using activated charcoal and nano-filtration.

Although, the removal of indomethacin and diclofenac has been increased in tertiary treatment, there were a few articles reporting on the drawbacks of PAC implementation on WWTPs system.[28,108] PAC can adsorb most of the natural and synthetic organic matters in wastewater which might lead to the extinction of aquatic life. The poor selectivity sorbent for activated carbon has been stated by Meng Dai.[108]

Luo and co-workers [105] reviewed the significance of PAC as the resolution of micro-pollutants in water. However, Johnson [107] stated that the organic discharge from WWTPs helps sustain *detritus* and *periphyton* which have become an important part of diet for fish species such as roach, bream, and stickleback. The decreasing of these organic discharge might cause some of these cyprinid fish populations to come under pressure. In addition, it might be good if there is an ideal level of supplemental organic supply from effluent. If there is too much of organic discharge in water, it may cause oxygen to decrease and asphyxiation whereas if there is too little it would leave the fish species weak and undernourished.

In order to remove trace organics, a variety of tertiary treatment options are available. However, advanced oxidation approaches to treatment are known to cause, at least in some cases, small and highly mobile breakdown products which are a lot less benign then the originals, such as nitrosodimethylamine (NDMA).[109] Moreover, it is likely that most of the advanced technologies such as photocatalyst and advanced oxidation processes would require considerable additional energy requirements, thus potentially increasing the carbon emissions, something that responsible societies should be trying to reduce.[107] Thus, it has to be agreed that the economic, high capacity sorbent and selective would be the best properties should be developed in near future.

1.6 NSAIDs DETERMINATION

As it has been said, non-steroidal anti-inflammatory drugs (NSAIDs) are widely consumed for modern medicinal practices. Approximately 3.000 compounds are approved as constituents in medicinal products.[110] In recent years, several analytical methods have been reported for the determination of EPPPs in aqueous environmental matrices. In this section, the different analytical detection methods for different kinds of EPPPs will be further explained.

1.6.1 Chromatographic Methods

Chromatography is a physical method of separation in which the components to be separated are distributed between two phases, one of which is stationary (stationary phase) while the other (the mobile phase) moves in a definite direction.[111] The most common procedures for determination of EPPPs in aquatic environmental matrices consist in pre-concentration by solid-phase extraction (SPE) followed by separation and determination using liquid (LC) or gas chromatography (GC).[112]

1.6.1.1 Liquid Chromatography

Liquid chromatography (LC) can be defined as a separation technique in which the mobile phase is a liquid and it can be carried out either in a column or on a plane.[111] Liquid chromatography generally utilizing very small particles as a stationary phase and a relatively high inlet pressure is often implemented for column chromatography. There are many articles reporting the determination of pharmaceutical compounds using hyphenated LC coupled to ultraviolet detection (LC-UV),[113,114,115] to tandem mass spectrometry (LC-MS), [36,105,112,117] to time of flight – mass spectrometry (LC-TOF-MS),[4,118,119] to electrospray ionization - quadrupole - time of flight - mass spectrometry (HPLC-ESI-QTOF-MS),[89,120] or using high performance liquid chromatography – diode array detector (HPLC – DAD),[54,121,122] high performance liquid chromatography – electrospray ionization – quadrupole – mass spectrometry/mass spectrometry (HPLC – ESI – MS/MS).[87] Many researchers commonly use liquid chromatography method for EPPPs determination. However, liquid chromatography needs a pre-preparation such as solid phase extraction in prior to analyze the real samples.

1.6.1.2 Gas Chromatography

Gas chromatography (GC) has been defined as a separation technique in which the mobile phase is a gas and it is always carried out in a column.[111] Commonly, there are many articles using gas chromatography mass spectrometry (GC-MS). [123,124] However, the main drawback of gas chromatography for EPPPs analysis is that this technique is limited to volatile and thermally stabile compounds.[125] Because most of EPPPs are polar substances, they need to be derivative before injection into the gas chromatography.[112] Gas chromatography also can be categorized as an expensive instrument.

1.6.2 Spectrophotometry

Spectrophotometry, a relatively inexpensive and easy handling technique with good precision and accuracy of analysis, offers the practical and economic advantages over the other techniques but it lacks the required selectivity and sensitivity for the determination of the low level of the analyte in complex matrices.[126] Recently, there are some articles reporting on simultaneous detection of methocarbamol and ibuprofen or diclofenac potassium in mixtures using spectrophotometry showing a good performance.[127]

1.6.3 Potentiometric Method

Potentiometric method with ion-selective electrodes (ISE) can provide valuable and straight measures of indomethacin in complex mixtures, as it makes possible the direct determination of ions in solution with high selectivity. Most ion-selective electrodes are low-cost, their use and maintenance are very simple, and assay procedures involving such electrodes are generally simple and fast.[128]

1.7 NSAIDs REMOVAL METHODS

Non-steroidal anti-inflammatories drugs (NSAIDs) is the therapeutic group most commonly detected in water.[5] Thus, new efficient removal methods are highly needed in order to remove them from water. Rivera and co-workers [8] had summarized that there are two big processes which have a potential to be implemented in real WWTP degradation processes (advanced oxidation processes) and sorption processes for example carbon. On the contrary, both methods have drawback which need to be reviewed.

1.7.1 Degradation Processes

Degradation processes have been studied more than decades ago. Degradation processes consist of advanced oxidation processes (AOP) and biodegradation. Advanced oxidation processes have been widely used in water and wastewater treatment for the removal of organic and inorganic contaminants as well as to improve biodegradability of industrial wastewater.[129] There are four types of technologies approaches via advanced oxidation processes:

- based on ultraviolet radiation,
- based on ozone,
- based on gamma radiation
- electro-oxidation without and with active chlorine generation.

The further elaboration will be focusing on the popular treatments such as AOP based on ultraviolet radiation. Nowadays, there are an increasing numbers of articles reporting on photodegradation of EPPPs removal from water[130–139].

According to a previous study, advanced oxidation processes show high percentage of EPPPs removal in water and a photodegradation process has reached a pilot scale study [140]. For instance, advanced oxidation processes are potential treatment processes that can improve the effectiveness of diclofenac removal in municipal wastewater treatment plants.[141] However, the process of degradation may produce more toxic by-product [38,142,143].

There are thousands of pharmaceuticals that are still unknown in terms of toxicity after passing through the advanced oxidation processes. However, degradation processes are not focused only on the advanced oxidation process methods. Any method that underwent a 'degradation' process may contribute to the production of more toxic by-products. As an example, research conducted by Becker and co-workers [144] observed that although antibiotics had been degraded by enzymatic treatment with *fungal laccase* another problem had arisen since degradation does not mean elimination of the toxicity of the EPPPs. Hence, the challenge of the degradation methods is the elimination of toxicity of the EPPPs and by-products from water. Generally, advanced oxidation processes are costly and the implementation in WWTPs will be limited by the competitive removal of interfering matters and producing toxic by-products.

Together with the possibility of release more toxic constituents compared to parent compounds, advanced oxidation processes also need a high energy supply. Furthermore, advanced oxidation processes are not applicable in large scale, neither in real situation in surface water treatment plants because of the condition of the field which are often non ideal for photodegradation, hence removal along a river, where residence times are typically much shorter than lakes.[61]

1.7.2 Sorption Processes

Sorption processes consisted of adsorption and/or absorption processes. The term sorption is defined as the full range of processes whereby matter is partitioned between gas, aqueous and/or solid phases. In geochemical systems, this includes adsorption of matter at the surfaces of solid particles (minerals and organic matter) or at the air–water interface, and absorption into the solids during surface precipitation or solid phase diffusion.[145]

Adsorption process is considered as a preferable method for the removal of pharmaceutical because it does not produce undesirable by-products, is user-friendly and cheap. There are many articles reporting on EPPPs sorption processes using different kind of adsorbents such as powder activated carbon [106,139,146–150], molecularly imprinted polymers (MIP) [108,126,151–157], biomass [158,159], ion exchange resins and zeolites [146]. Molecularly Imprinted Polymers will be elaborated further in the next section.

1.8 MOLECULARLY IMPRINTING TECHNOLOGY (MIT)

Molecularly imprinting technology (MIT) has become a promising scientific research in wastewater treatment especially for EPPPs recovery. Molecularly imprinted polymer (MIP) concept was proposed by Polyakov in 1931.[160] This concept has been expanded and developed according to different application purposes. The unique of MIT, it does not diminish the molecular properties of target compounds compared to other method and it give benefits from the economic point of view as it can be re-collected and reused. Audrey and co-workers [161] summarized that current wastewater treatment technologies have limitations for removing emerging contaminant and there was a gap of potential for new technologies being both effective and economically feasible. MIP does not require high-technology to be fabricated. It is inexpensive, has recovery properties and has potential to remove the pharmaceutical pollutants in high percentage of removal depending on the active sites of the molecularly imprinted polymer.

Molecular imprinting is an efficient method to introduce specific molecular recognition sites into a polymeric matrix.[162] Molecularly imprinting technology has been used as relevant method in removing EPPPs and high percent of recovery usually has been reported. Since molecularly imprinting technology is working with some kind of polymers, the efficiency

removal and recovery will depend on the ratio and types of each reagent in fabricating the polymer.

The main advantages of MIPs are their high affinity and selectivity for the target molecule (template). MIPs also have higher physical strength, robustness, resistance to elevated pressure and temperature and inertness against various chemicals (organic solvents, acids, bases, and metal ions) compared to biological media such as proteins and nucleic acids. Furthermore, their production costs are low and their lifetimes can be as long as several years at room temperature.[163,164]

There are three types of approaches in molecular imprinting:

1. Covalent imprinting: the polymerizable derivatives are co-polymerized with a cross-linking monomer pioneered by Günter Wulff and co-workers.[165]
2. Non-covalent: was introduced by Mosbach and co-workers [166], and is based on the formation of relatively weak non-covalent interactions (hydrogen bonding or ionic interactions) between template molecule and selected monomers before polymerization. The non-covalent approach is the most widely used for the preparation of MIPs thanks to its versatility.[167]
3. Semi-covalent: aims to unite the advantages of the covalent and non-covalent approach.[165]

In order to synthetize the MIPs, Cormack, and co-workers [168] had reviewed in details the MIPs synthesis procedures. Template, functional monomer, cross-linker, initiator and the porogen solvent are the obligatory chemicals in developing the MIPs. The template is the central importance in all molecular imprinting processes and it directs the organization of the functional groups pendent to the functional monomers. The binding interaction between template and functional monomer will produce the mold-shaped in which the target molecule can be trapped into the imprinted binding sites and can be released once the elution process takes place.

Functional monomer usually exceeds the template in ratio 4:1 for non-covalent bonding.[163] In imprinted polymers synthesis, the cross-linker also fulfill important functions. The cross-linker is important in controlling the morphology of the polymer matrix, serves to stabilize the imprinted binding sites and imparts mechanical stability to the polymer matrix in order to retain

its molecular recognition capability.[164] According to published studies, the most successful non-covalent imprinting systems are based on methacrylic acid (MAA) which is cross-linked with ethylene glycol dimethacrylate (EGDMA).[165] Initiator is needed for the initiation of free radical polymerizations in the presence of template.[168] The common initiator used in many articles is 2,2'-Azobisisobutyronitrile (AIBN).[122,162,169] Porogen solvent plays an important role in creating the pores in macroporous polymers.[168] The porogen solvent depends on the dissolution of template and functional monomer. The common porogen solvent which has been reported in many articles are chloroform,[76,155,165,170] toluene [121,122,152] and acetonitrile [171].

The most common polymerization method used is bulk polymerization. Firstly, the template is well-dissolved with functional monomer using porogen solvent to produce complex molecule. Then, cross-linker and initiator are added into the solution and thermal polymerization process takes placed at standard temperature 60°C.[163] Next stage the template is removed and the template cavities remain empty for the separation process. **Figure 1.7** shows the process of polymerization starting from mixing until the MIP is ready for analysis purpose.

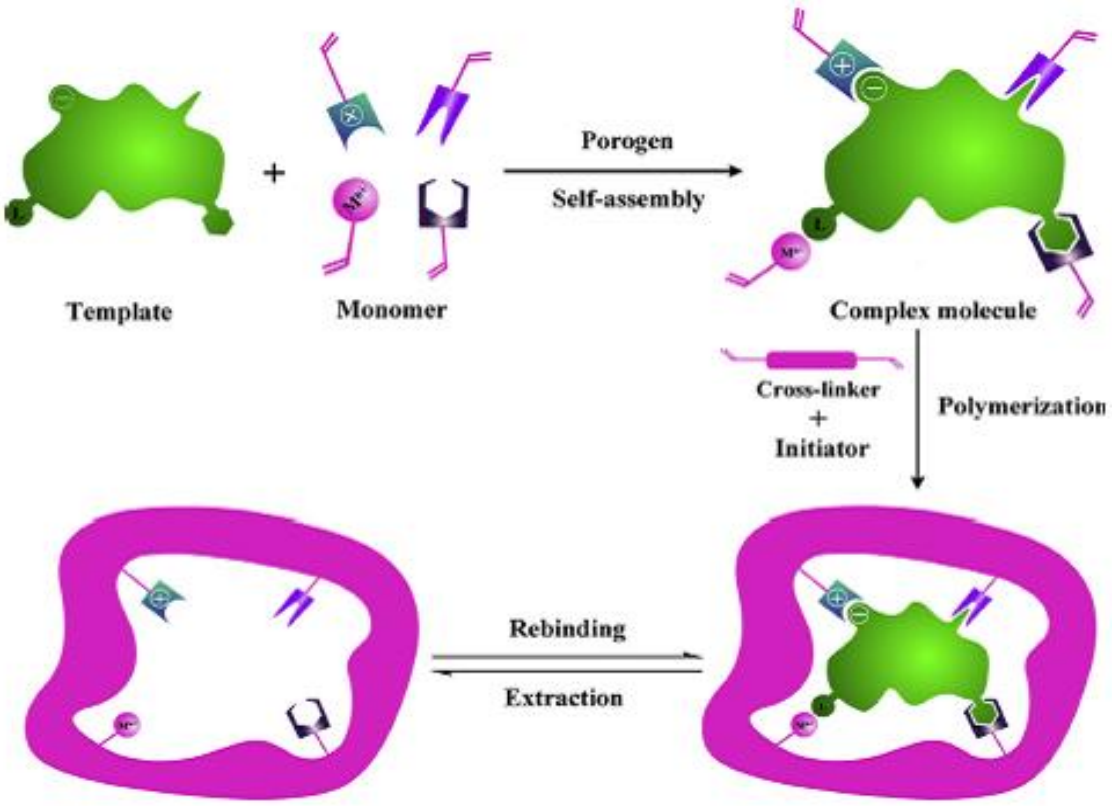

Figure 1.7 Scheme for molecular imprinting.[172] Reprinted with permission.

In order to produce successful MIPs, the degradation properties of target compound must be well-known because the polymerization is carried out at 60^{o}C so it must be stable at certain temperature, otherwise the molecular printing of the template would not be successful since the template would be degraded. Diclofenac degradation has been studied by Dungarani and co-workers [173] in which the stress degradation studies shows that diclofenac underwent degradation in acidic and alkaline conditions, whereas, it was relatively stable when exposed to dry heat (70^{o}C), oxidation (30% H_2O_2), and in photolytic conditions. In addition, with the presents of alkaline media (0.1 N NaOH, 1 ml), in the range of 30 minutes to 90 minutes the degradation occurred in range 59.47% to 78.49% whereas diclofenac in the acidic media (1 N HCl, 1 ml) within the same range of time was totally degraded.[173]

1.8.1 Typical Parameters Analyzed for MIPs

1.8.1.1 Binding Sites Between MIP and Analytes

The study of the adsorption isotherm is the common way to study sorption-desorption process of adsorbents. There are two types of sorbent binding sites which are homogenous and heterogeneous model. The differentiate between homogenous and heterogeneous sorbent as shown in **Figure 1.8**.

In homogeneous models, the important adsorption isotherms are Langmuir, Freundlich and Langmuir-Freundlich. Langmuir adsorption isotherm, originally developed to describe gas–solid-phase adsorption onto activated carbon, has traditionally been used to quantify and in its formulation, this empirical model assumes monolayer adsorption (the adsorbed layer is one molecule in thickness).[174] The Freundlich isotherm is originally empirical in nature, but was later interpreted as adsorption to heterogeneous surfaces or surfaces supporting sites of varied affinities and has been used widely to fit experimental data.[135] However, MIPs has been classified as heterogeneous adsorbents (depends on certain case study) and it is expected that some of the MIPs synthesis would not fitted with Langmuir isotherm model (homogenous adsorbents). Umpleby [175] had suggested that the Langmuir-Freundlich (LF) isotherm is able to model MIPs of both homogeneous and heterogeneous distributions at both high and low concentrations.

Figure 1.8 Schematic of homogenous and heterogeneous sorbents.

For heterogeneous adsorption characterization, Scatchard plot has been used in many kinds of MIPs. Commonly, most MIPs do not fit the homogenous model.[176] Based on Bi-Langmuir formula, the graph producing high and low affinity binding sites which referred to two lines in graph represents heterogeneous affinities whereas one straight line in graph represents homogenous affinities.

In order to study the actual process occurring in MIPs, it is important the knowledge of the binding sites for pre-sorption and post-sorption/desorption. Hence, Fourier Transform Infrared spectrophotometer equipped with attenuated total reflection (FTIR-ATR) will be used to study this parameter **(Figure 1.9)**. One important parameter to be studied is the determination of active functional groups for pre and post sorption/desorption process which is used to investigate the interaction between the target molecule and the active functional sites before after sorption process. Generally, in order to detect the functional groups in compound of polymer, Fourier Transform Infrared (FTIR) will be used.

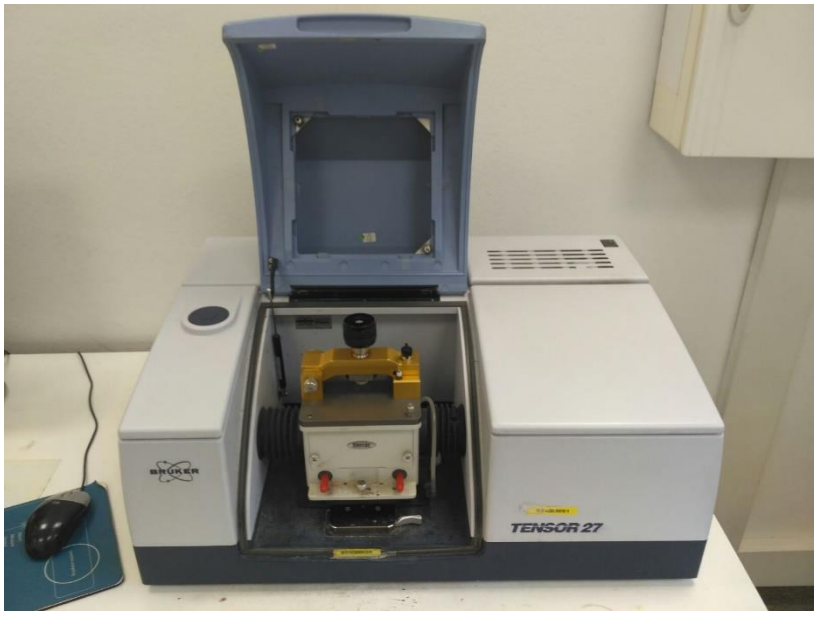

Figure 1.9 Fourier Transform Infrared spectrometer equipped with attenuated total reflection (FTIR-ATR). Picture from Servei d'Anàlisi Química, Universitat Autònoma de Barcelona.

1.8.1.2 Surface Morphology Properties

The scanning electron microscope (SEM) is commonly used to examine the structure and surface morphology of the MIPs[177] to characterize the porosity of sorbent and pores homogeneity **Figure 1.10**.

Figure 1.10 Scanning Electron Microscope (SEM). Equipment used from Servei de Microscopia, Universitat Autònoma de Barcelona.

1.8.1.3 Pre-polymerization Study

The pre-polymerization study was conducted in order to understand structure-property relationships in polymers for improvement and optimization behaviour.[178] In environmental applications, Nuclear Magnetic Resonance (NMR) was well known in characterize the interaction between compounds at molecular level. In addition, Nuclear Magnetic Resonance application was necessary to be used in order to characterize the reaction between monomers and templates in terms of developing the cross-linked-polymer and the active functional sites. Generally, in the production of MIP there are two types of NMR widely used; proton Nuclear Magnetic Resonance (^{1}H NMR) and Carbon 13 Nuclear Magnetic Resonance (^{13}C NMR). There is different power supply provided depending on the analysis purpose such as 250 Hertz, 350 Hertz, 360 Hertz, 400 Hertz **(Figure 1.11)**, 500 Hertz and 600 Hertz.

Figure 1.11 ^{1}H NMR 400 MHz. Picture from Servei de Resonància Magnètica Nuclear (SeRMN), Universitat Autònoma de Barcelona.

1.9 GENERAL OBJECTIVE

Considering MIP as a powerful sorbent with high affinity and selective for using as stationary phase, there are many previous articles showing that different polymers have different ability in order to separate a so-called 'mixture cocktail'. Mixture cocktail can be referred as the mixture of many kinds of drugs in water. The main objective of the present work is the development of MIPs able to separate pharmaceutical compounds to be used as packing material for an HPLC column.

1.9.1 Specific Objectives

There are three specific objectives to be achieved in this study:

1. Synthesis and Characterization of New Molecularly Imprinted Polymer in Batch Mode using diclofenac or indomethacin individually as the template.

    i. To synthetize successive new molecularly imprinted polymer using indomethacin and diclofenac individually as a template with 1-allylthiourea as the monomer via bulk polymerization.

    ii. To characterize the MIPs properties such as: binding properties, the influence of media, the influence of different pH solutions and the selectivity properties in binary mixture via batch mode for MIP with indomethacin as a template and MIP with diclofenac as a template.

    iii. To study the pre-polymerization of complex formed between functional monomer and templates.

2. Pre-polymerization and adsorption isotherm study using Molecularly Imprinted Solid Polymer Extraction (MISPE).

    i. To investigate the homogeneity and sorption isotherm of MIP with indomethacin as a template (MIP-IDM) and MIP with diclofenac as a template

(MIP-DCF) using successive total sorption study via pre-packed cartridge analysis.

ii. To characterize the functional groups of MIP with diclofenac as a template (MIP-DCF); MIP with diclofenac as a template (MIP-DCF) loaded with diclofenac; and MIP with diclofenac as a template (MIP-DCF) after 10^{th} regeneration using Fourier Transform Infrared.

iii. To characterize the surface morphology of MIP with indomethacin as a template (MIP-IDM) and MIP with diclofenac as a template (MIP-DCF).

3. To develop an analytical methodology for application of Molecularly Imprinted Polymer for diclofenac recovery.

i. To develop online detection flow analysis using ultraviolet spectrophotometry for three components in mixture (DCF, IDM and IBU).

ii. To analyse the recovery in nmol of diclofenac until 10^{th} of cycles in natural water using MIP with diclofenac as a template (MIP-DCF) and analysis by High Performance Liquid Chromatography equipped with Ultraviolet detector.

iii. To use the MIP (with diclofenac as a template, MIP-DCF) as stationary phase for packing a column to use in High Performance Liquid Chromatography equipped with Ultraviolet detector to separate DCF and IBU in a mixture.

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CHAPTER 2

Synthesis and Characterization of Molecularly Imprinted Polymer Using Selective Functional Monomer for Diclofenac and Indomethacin Removal in Aqueous Media via Batch Mode

Chapter 2

Synthesis and Characterization of Molecularly Imprinted Polymer Using Selective Functional Monomer for Diclofenac and Indomethacin Removal in Aqueous Media via Batch Mode

2.1 INTRODUCTION

2.1.1 Molecularly imprinted polymer

Molecularly-imprinted polymers (MIPs) are synthetic materials with artificially generated recognition sites being able to rebind a target molecule specifically in preference to other closely-related compounds.[1] More recently, molecularly imprinted polymers (MIPs) are attracting widespread attention due to their prominent selectivity properties. MIPs are synthetic polymers possessing specific cavities designed for target molecules.[2] Synthesis of MIPs will undergo polymerization reaction. Polymerization reaction is known as a very complex process, which could be affected by many factors, such as type and concentration of the monomer, cross-linker and initiator, temperature and time of polymerization, the presence or absence of magnetic field, and volume of the polymerization mixture.[3] Recently, the most common method used to obtain MIPs is the free radical polymerization. Generally,The synthesis of MIPs procedure is performed under mild reaction conditions (e.g., temperature lower than 80 °C and atmospheric pressure) in bulk or in solution, and it is tolerant for a wide range of functional groups and template structures.[4] Generally, MIPs have been prepared as monoliths by traditional bulk polymerization.[2] Bulk polymerization is the most popular and general method to prepare MIPs due to its attractive properties, such as rapidity and simplicity in preparation, with no requirement for sophisticated or expensive instrumentation, and purity in the produced MIPs.[3] In preparing MIPs via bulk polymerization, this procedure needs template, monomer, initiator, cross-linker and porogen solvent.[5] The process started by mixing the template and monomer in the porogen solvent. After that, the crosslinker and initiator has been added followed by degassing procedure. Then, polymerization occurred for a few hours. After that, the prepared polymer was crushed and located into the sohxlet extraction in order to remove the template. The process of bulk polymerization synthesis as shown in **Figure 2.1**.

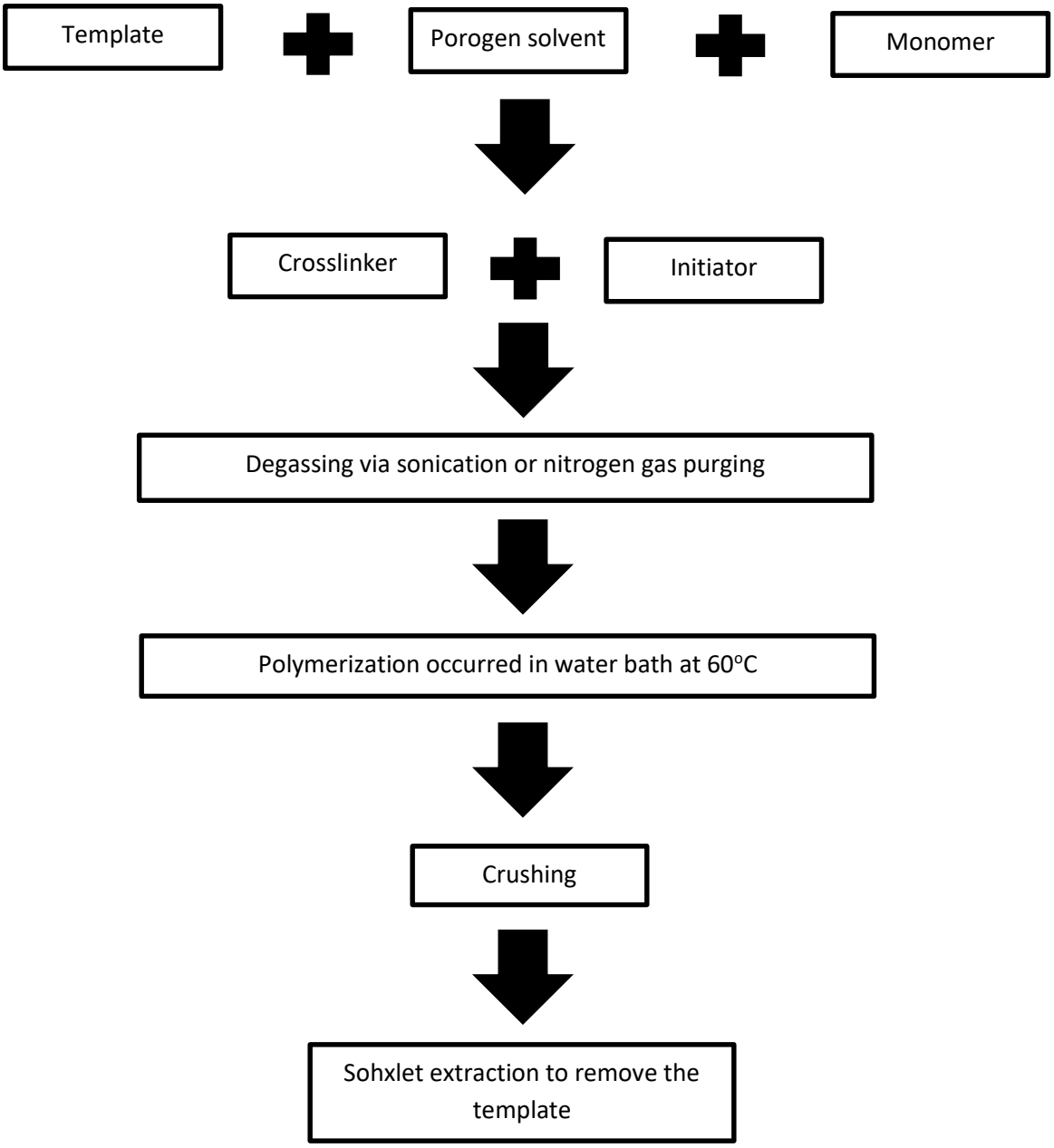


Figure 2.1 Fundamental process for bulk polymerization synthesis.

2.1.1.1 MIPs with IDM as the template

In the present work, bulk polymerization has been implemented in order to synthetised new MIPs. Previously, Yang, and co-workers [6] had synthetised MIPs using two different methods which are bulk and suspension method. Yang and co-workers [6] had discovered that bulk polymerization shows higher efficiency removal in terms of percentage removal compared to

suspension polymerization. Thus, in this study bulk polymerization has been choosed as the main procedure in synthesis of MIPs with IDM as the template.

2.1.1.2 MIPs with DCF as the template

Preparation of MIPs with DCF as the template has been studied by many researchers.[7,8] Recently, the precipitation polymerization method has been implemented due to its highly selective properties and total sorption amount. However, likely it depends on the types of monomer and the active functional sites on the MIPs.[9] According to Sun and co-workers [10] the researchers had developed MIPs using bulk polymerization and observed approximately 70 nmol of DCF sorbed as the uptake capacity per 10 mg of polymer. Recently, the preparation MIPs via molecularly imprinted polymer sorbent in hollow fiber combined with fiber optic-linear array spectrophotometry which has been developed by Amiri and co-workers.[8]

2.1.2 Monomer

For MIPs preparation using IDM as the template, Yang, and co-workers had compared the synthetised MIPs with different monomers acrylamide (AM) and methacrylic acid (MAA) via bulk polymerization and observed that AM was more efficient in removing the IDM from water.[11] However, the following compounds are the common functional monomers employed in non-covalent molecular imprinting for a variety of templates used **(Figure 2.2)**.

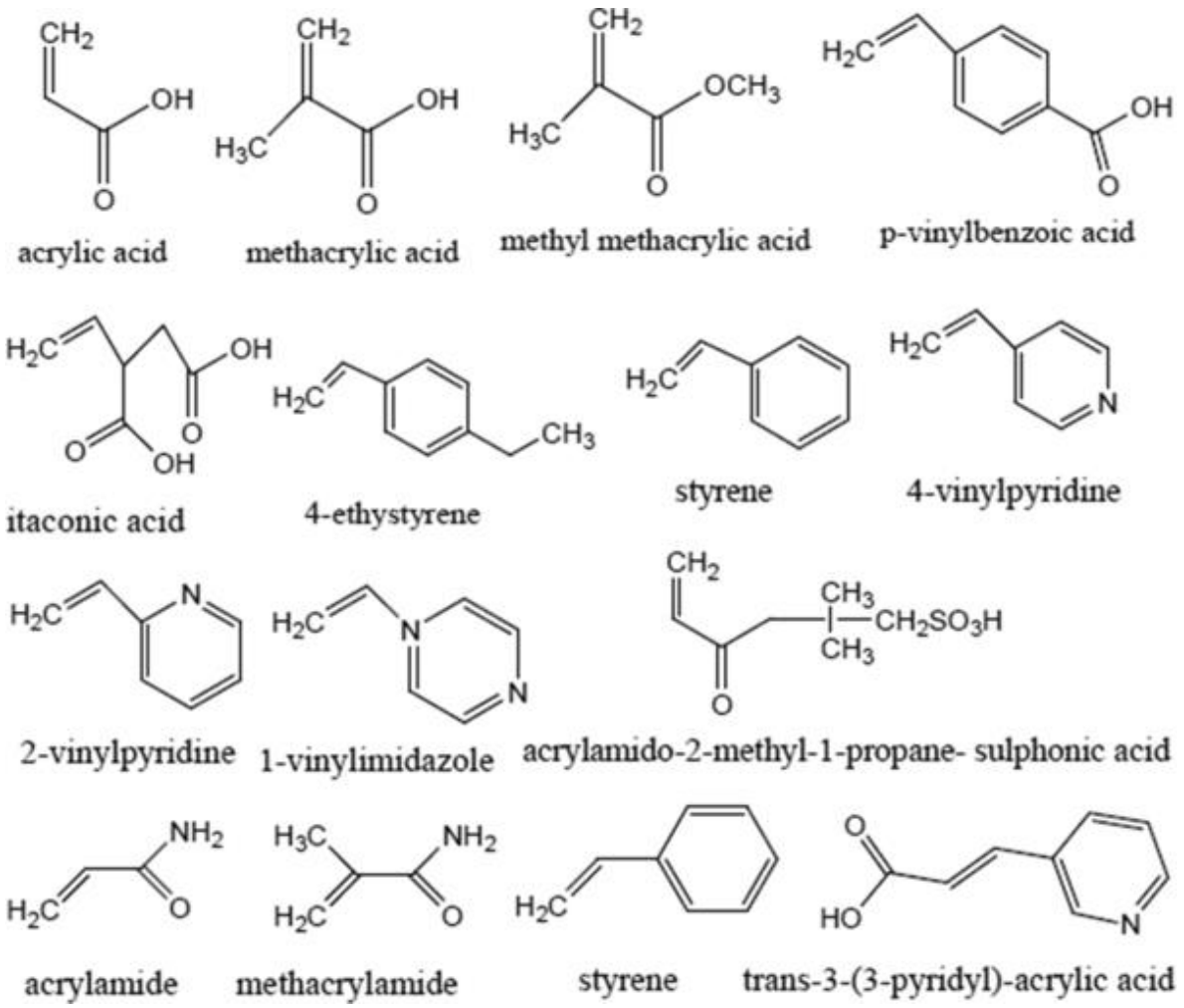


Figure 2.2 Common functional monomers employed in non-covalent molecular imprinting.[12] Reprinted with permission.

For MIPs preparation using DCF as the template, commonly used monomers for molecular imprinting include methacrylic acid (MAA), acrylic acid, 2- or 4-vinylpyridine (2- or 4-VP), acrylamide (AM), trifluoromethacrylic acid and 2-hydroxyethyl methacrylate (HEMA).[3] Recently, there were many articles reported on 2-VP and 4-VP due to its good functional sites that well-interacted with DCF.[13,14] In this study, the monomer that has been used was allylthiourea (AT). This monomer has thiol group works as the functional monomer and was highly soluble in acetonitrile, the polar organic solvent. To the best of our knowledge, to date, there is still none article reported on AT as the monomer in fabrication of MIPs for DCF and IDM removal.

2.1.3 Initiator

Many chemical initiators with different chemical properties can be used as the radical source in free radical polymerisation. Normally they are used at low levels compared to the monomer, e.g. 1 wt.%, or 1 mol.% with respect to the total number of moles of polymerisable double bonds.[15] The azo initiator or so called Azobisisobutyronitrile (AlBN) **(Figure 2.3)** can initiate free radical polymerizations and has been used in many articles involving MIPs preparation using DCF and IDM as template.[11,13,14] AlBN also known as 2,2′-Azobis(2-methylpropionitrile) and 2-(azo(1-cyano-1-methylethyl))-2-methylpropane nitrile. AlBN can be conveniently decomposed by photolysis (UV) or thermolysis to give stabilised, carbon-centred radicals capable of initiating the growth of a number of vinyl monomers.[15]

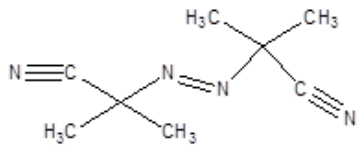


Figure 2.3 Molecule structure of AlBN.

2.1.4 Cross-linker

The cross-linker is important to control the morphology of the polymer matrix, serves to stabilize the imprinted binding sites and imparts mechanical stability to the polymer matrix in order to retain its molecular recognition capability.[16] Some authors found that cross-linker has a major impact on the physical characteristics of the final polymers and much less effect on the specific interactions between the template and functional monomers. Ethylene glycol dimethacrylate (EGDMA) and trimethylolpropane trimethacrylate (TRIM) are the most commonly employed.[4] However, there were many articles reporting the use of EGDMA as the cross-linker for DCF and IDM removal.[5,7,10,13] Other potential cross-linkers are shown in **Figure 2.4**.

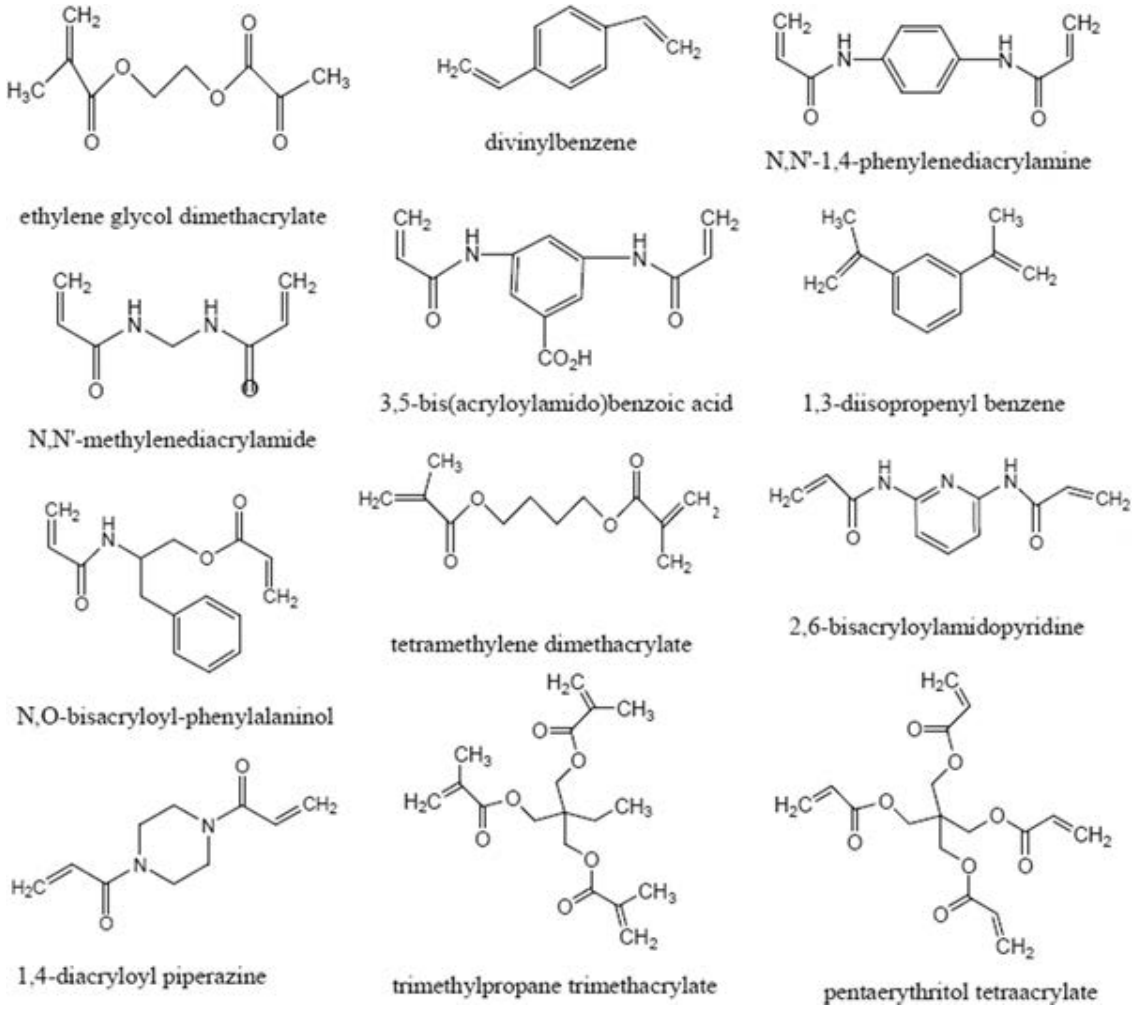


Figure 2.4 Potential cross-linkers used in MIPs.[12] Reprinted with permission.

2.1.5 Porogen solvent

The solvent serves to bring all the components in the polymerisation, i.e. template, functional monomer(s), cross-linker and initiator into one phase.[15] Nature and volume of the solvent play also an important role in the molecular imprinting process. Solvent is responsible for creating the pores in macroporous polymers hence it is commonly called 'porogen'.The most common solvents used for MIPs synthesis are toluene, chloroform, dichlorometane or acetonitrile.[4]

In this study, the solvent which has been used was acetonitrile since AT as the monomer and IDM also DCF as the template are well-soluble in acetonitrile. The volume also play a main role in order to get high removal efficiency of target molecule, IDM and DCF.

2.1.6 Template removal method

At present, soxhlet extraction is the most popular method to remove template from crosslinked polymer matrices.[3] Ellwanger and co-workers [17] suggested that the soxhlet extraction could be improved somewhat by extraction in glacial acetic acid (HOAc) for 24 h. In the present work, soxhlet extraction with HOAc and methanol (MeOH) combination was implemented. Yang and co-workers [11] had implemented successive washing steps with methanol/acetic acid (9:1, v/v) in a soxhlet apparatus. In this study, the same procedure has been used in order to removed the template from MIPs.

2.1.7 Interaction of functional sites for non-covalent polymerization

Understanding the basis for optimization of non-covalent methods for molecular imprinting is important for two reasons: the methodology is far easier than covalent methods, and it produces higher affinity binding sites, versus covalent methods.[18]

A pre-polymerization study has been developed in the present study to investigate the interaction between functional sites of monomer and active functional group of analyte. High affinity and low affinity of MIPs depend on the active functional sites bonded to the active functional group on analyte in pre-polymer complexes during the polymerization phase. As shown in **Figure 2.5**, the less number of interactions between template and monomer, the lower affinity sites are produced. In addition, according to Spivak [18] the increased number of binding interactions in the polymer binding site may account for greater fidelity of the site, and thus impart greater affinity and selectivity to the site. This would suggest that the number of functional groups in the polymer binding site is not determined directly by the solution phase pre-polymer complexes; rather, it is determined during polymerization. Moreover, Chen [3] observed that heterogeneous binding sites often exist in MIPs formed by non-covalent interactions because the pre-polymerization step is a non-well defined process, which results in the formation of complexes with different ratios of template to monomer, and then leads to different binding sites.

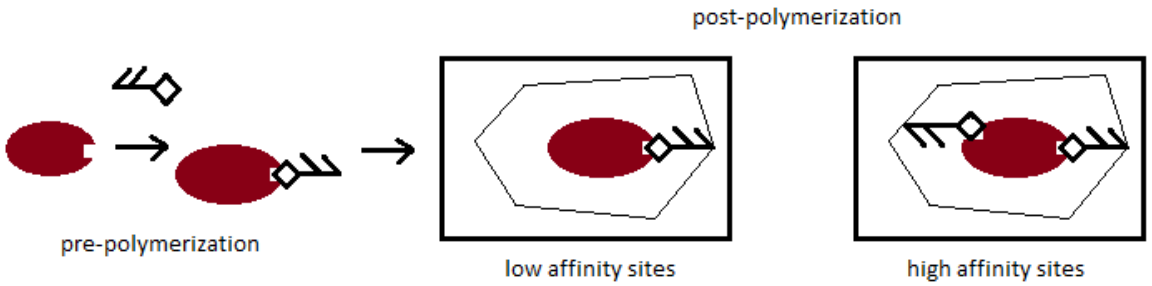


Figure 2.5 The pre-polymerization and post-polymerization schematic diagram.

2.2 OBJECTIVES

The main objectives of this study are:

i. To synthetize successive new molecularly imprinted polymer using indomethacin and diclofenac individually as a template with 1-allylthiourea as the monomer via bulk polymerization.

ii. To characterize the MIPs properties such as: binding properties, the influence of media, the influence of different pH solutions and the selectivity properties in binary mixture via batch mode for MIP with indomethacin as a template and MIP with diclofenac as a template.

iii. To study the pre-polymerization of complex formed between functional monomer and templates.

2.3 EXPERIMENTAL

2.3.1 Reagents and equipments

Reagents:

- Indomethacin (IDM), allylthiourea (AT), acrylamide (AM) and ethylene glycol dimethacrylate (EGDMA) were from Sigma Aldrich with 98-99% of purity. TLC grade.
- 2,2-azobisisobutyronitrile (AIBN) was from Acros Organic with 98% of purity.

- Diclofenac-Na and ibuprofen was from Cayman Chemical with purity higher than 99%.
- Acetic acid (TLC grade) and hydrochloric acid were from J. T.Baker with 96% and 37-38% of purity respectively.
- Sodium hydroxide from Panreac with purity of 98%.
- Chloroform and acetonitrile (HPLC grade) were from VWR Chemicals with 99.5%-99% of purity.
- Acetonitrile-d_3 from Sigma Aldrich, Spain with 99.9% purity and analytical grade.

Equipments/Instruments:

- Water bath from E. Gabarro A-G. **(Figure 2.6)**
- Soxhlet extraction apparatus set from VWR company. **(Figure 2.7)**
- Centrifuge from Orto Alresa model Digicen. **(Figure 2.8)**
- Mortar grinder from Retsch. **(Figure 2.9)**
- UV double-beam spectrophotometer from UNICAM, model UV-2 200.
- 400 MHz Nuclear Magnetic Resonance (^{1}H NMR) from Merlin, Germany. The instrument was self-serviced at Servei de Ressonància Magnètica Nuclear (SeRMN), Universitat Autònoma de Barcelona, Spain.

Figure 2.6 Water bath.

Figure 2.7 Soxhlet extraction apparatus.

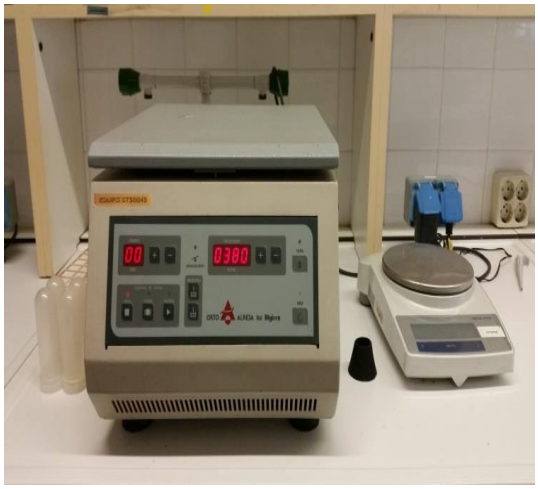


Figure 2.8 Centrifuge.

Figure 2.9 Automated mortar grinder.

2.3.2 Synthesis of new Molecularly Imprinted Polymers (MIPs)

The synthesis has been prepared by using IDM and DCF as template with 1-allylthiourea as the monomer via bulk polymerization individually. In the present work, λ_{max} has been chosen for each compound from UV spectra calibration with IDM: 260 nm, DCF: 280 nm, IBU: 220 nm. From the literature, similar values are reported in the few articles found, IDM: 254 nm [19], DCF: 276 nm [20] and IBU: 220 nm.[20]

2.3.2.1 Synthesis of MIP using IDM as template

A new procedure for preparing molecularly imprinted polymer was used based on reference (17): Indomethacin (IDM, 1.0 mmol), 1-allylthiourea (AT, 4.0 mmol), ethylene glycol dimethacrylate (EGDMA, 20 mmol), and 2,2-azobisisobutyronitrile as an initiator (AIBN, 0.12 mmol) were dissolved in 4.0 mL of acetonitrile.[21] The solution was sonicated and sparged with N_2 for 5min.[10] Then, the sealed solution was polymerized at 60ºC in a water bath for 24 h. Successive washing steps with methanol/acetic acid (9:1, v/v) were performed in a Soxhlet apparatus.[6,22,23]. The final washing step used 25 mL of methanol for three cycles and later was centrifuged at 5300 rpm for 3 min to remove acetic acid residual. The supernatant of methanol was analysed using UV spectrophotometer in order to confirm that there was no more template eluting from the polymer particles. The polymer was dried at 60 °C under vacuum overnight and stored at room temperature.[6] Next, the polymer was ground using automated mortar for 5 min and sieved to yield a particle size between 28 µm and 100 µm. The process was repeated until the size of particles was in the selected range. The polymer particles then were dried at

room temperature overnight. The non-imprinted polymer (NIP) with absence of template was synthetised using the similar procedure as mentioned above but without the addition of template.

2.3.2.2 Comparative study using different monomers

The comparison between the monomers allylthiourea (AT) and acrylamide (AM) has been studied. For the comparative studies, the MIP-IDM was synthesized according to Yang article [11] and was then compared with MIP-IDM synthetised using AT as the functional monomer. The summary of the reagents used for the MIPs preparation is shown in **Table 2.1.**

Table 2.1 Reagents for MIP-IDM preparation using different monomers.[11,21]

Polymer	Template	Monomer		Initiator	Cross-linker		Solvent volume
		AT	AM	AIBN	EGDMA	EDMA	
MIP-IDM-AM	1 mmol	-	4 mmol	0.24 mmol	-	20mmol	8 mL (Chloroform)
MIP-IDM-AT	1 mmol	4 mmol	-	0.12 mmol	20 mmol	-	4 mL (Acetonitrile)

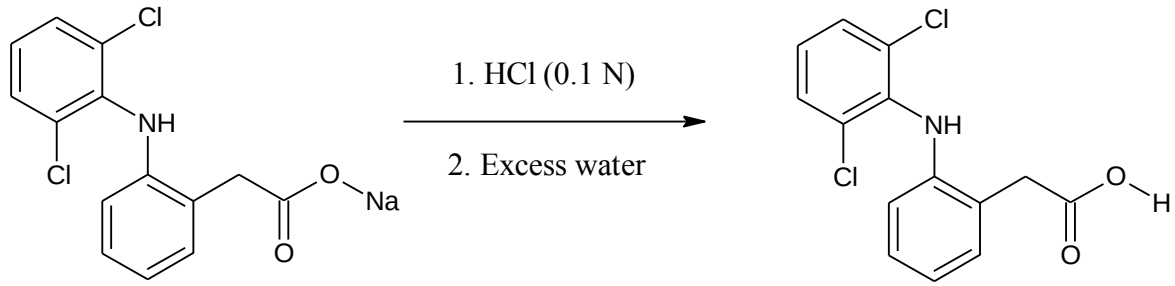
2.3.2.3 Comparative study in porogen solvent volume for synthesis with IDM as template

The synthesis procedure was carried out similarly to the described in section **2.3.2.1** but usingdouble volume of porogen solvent (acetonitrile), so instead of 4mL it was used 8 mL in order to see the effect.

2.3.2.4 Synthesis of new MIP using DCF as template

In order to synthetize the MIP-DCF, firstly the synthesis of DCF acid from DCF salt was required. The following procedure is the synthesis of DCF acid from DCF sodium salt. DCF sodium was dissolved in water at a concentration of 7 mg/mL. When the DCF sodium was

completely dissolved, it was acidified with an equal molar amount of hydrochloric acid (HCl). This solution was stirred using a magnetic stir bar and plate for 10 min. Since the DCF acid was not dissolved in water, it was immediately precipitated out of solution.[24] The free acid of DCF was a suspension in water. **Figure 2.10** shows the chemical reaction. The mixture was filtered using 0.45 µm filter paper and a vacuum apparatus. The filtrate was washed with dilute HCl (0.001 N) and excess amounts of water to remove any sodium chloride and unreacted DCF sodium. The powder was allowed to dry under a hood, collected and stored in a clear glass vial and prepared for next purpose.[25] For the synthetise of MIP-DCF purpose, the whole procedure followed in **2.3.2.1** for the synthesis of MIP-IDM was repeated but replacing the template.



DCF sodium DCF acid

Figure 2.10 Chemical reaction scheme for the transformation of DCF acid to DCF salt.

2.3.3 Binding Properties Study

2.3.3.1 Kinetic study

10 mg of MIP-DCF and NIP were individually weighted and placed in 5 mL tubes. 2 mL of 5 mg/L DCF solution were poured into the tubes[6] and were covered using aluminium foil. After the solutions were agitated for the required time up to 120 min, the solutions were filtered using the syringe filter (mesh size: 0.22 µm). The solutions were then analysed by UV spectrophotometer. The absorbance value according to the selected wavelength (λmax) was determined. The procedure was performed in triplicate. The similar method as above wasrepeated for MIP-IDM and NIP using IDM solution. The percentage of removal (% removal) was calculated using the **Equation 2.1** with $[Analyte]_i$ is the initial concentration of analyte and $[Analyte]_f$ is the final concentration of analyte. The concentration was obtained

from calibration curve of absorbance versus concentration. The calculation was used for all the experiments relates to percentage of removal.

$$\%\, removal = \frac{[Analyte]_i - [Analyte]_f}{[Analyte]_i} \times 100\% \qquad \text{..... Equation 2.1}$$

2.3.3.2 Total adsorption study

10 mg of MIP-DCF and NIP were individually weighted in 5 mL tubes. Different concentration solutions of 2 mL DCF were poured into 5 ml tubes. DCF solutions were prepared between 1 mg/L until 25 mg/L due to its limit of solubility in 5% of ACN:water at ambient temperature. The solutions were agitated for 1 h and covered using aluminium foil. Then, the solution was filtered using the syringe filter (0.22 μm). Finally, solutions were analysed by UV spectrophotometer. The similar method as above was repeated for MIP-IDM and NIP using IDM solution.

2.3.3.3 Statistical analysis for total adsorption study between MIPs using different template

The F-test was used to assess the significance of difference in standard deviation of total sorption between MIP-IDM and MIP-DCF in order to study the reproducibility of MIP synthetised using different template. Results with $p < 0.05$ were considered to be statistically significant.

2.3.4 Effect of solution media

Medium plays a main role in order to isolate the target molecule at high removal efficiency by the MIP. Acetonitrile, or hydrogen cyanide, has been chosen in this study because it is miscible in water and it can dissolve the target molecule (IDM and/or DCF). Furthermore, acetonitrile combined with water has been used as mobile phase in liquid chromatography system since a long time ago, which can be interesting for further applications of the MIP. Acetonitrile has less dieletric constant, ε_τ compared to water at 20ºC. The polarity of the adsorbates can be assessed in various ways, including dipole moment, μ and relative dielectric constant, ε_τ with

the properties of water (μ: 1.85 D, ε_τ: 80.1) and acetonitrile (μ: 3.84 D, ε_τ: 38.8) show that both molecules are highly polar.[26] Dielectric constant is ratio of the electric field strength in vacuum to that in a given medium in which recently called relative permittivity.[27] Different concentrations of 2 mL DCF solutions were prepared in different media such as 100% of ethanol, acetonitrile and chloroform. The range of the DCF concentration was between 1 mg/L and until 1000 mg/L. The solutions were placed, as in previous experiments, in 5mL tubes containing 10 mg of MIP-IDM or NIP. The solutions were covered using aluminium foil and agitated for 1 h. Then, the solutions were filtered using the syringe filter (0.22 μm). The solutions then were analysed by UV spectrophotometer.

2.3.5 Effect of pH

10 mg of MIP-DCF was individually weighted in 5 mL tubes. 2 mL of 15 ppm DCF solutions were prepared in different pH range from 3 to 12. The solutions were added to the tube and agitated for 1 h then were filtered using the syringe filter (mesh size: 0.22μm). The solutions were analysed by UV spectrophotometer.

2.3.6 Selectivity study via batch mode

2 mL of each 5 mg/L mixture solutions consisting of target molecule and interference (DCF, IBU and/or IDM) were added to 10 mg of MIP-DCF or MIP-IDM in 5 mL falcon tubes. The solutions were agitated for 1 h and covered using aluminium foil then filtered using the syringe filter (mesh size: 0.22μm). All analysis were prepared in triplicates. The solutions then were analysed by UV spectrophotometer.

2.3.7 Pre-polymerization study via ^{1}H NMR

A non-covalent molecular imprinting approach was followed to prepare the MIP and NIP. Molecular recognition of the template molecule by imprinted polymers is based on the intermolecular interaction between the template molecule and functional groups in the polymer. Thus, to study the interaction between the template molecule and the monomer in pre-polymerization complexes it is important to predict the recognition mechanism of the imprinted polymer.

In the present work, the interaction between template and monomer in pre-polymerization complexes was studied by using ^{1}H NMR spectroscopy which is now being widely used within the field of molecular imprinting.[13] To do so, a series of samples were prepared with a fixed concentration of template (DCF), 0.05 mol/L, and varying concentration of monomer (AT), 0.10 mol/L (Mixture 1), 0.30 mol/L (Mixture 2) and 0.50 mol/L (Mixture 3) in acetonitrile-d_3. ^{1}H NMR spectra were acquired at room temperature.[10]

2.4 RESULTS AND DISCUSSION

2.4.1 Influence of porogen solvent volume

Porogen solvent used in this study was acetonitrile which was similar to a previous article reported.[21] When the volume of solvent increase to double, the removal efficiency was poor **(Figure 2.11)**. Thus, the combination of each reagents for synthetizing MIPs is important in order to enhance the removal efficiency of target molecule. In this case, the less volume consumed produce much better MIPs in terms of sorption capacity. In addition to that, precipitation method has become a trendy and shows very good performance in removal efficiency for instance the study investigated by Dai and co-workers in which they observed that the removal efficiency was much higher with the capacity reached until approximately 300 mg DCF per g MIP. The MIPs was synthetised via precipitation method which commonly needs a lot of porogen solvent volume more than 4 mL and usually up to 60 mL. Bulk polymerization is an environmental-friendly process due to the less porogen solvent used to prepare the MIP. The less volume used and the no striring needed during polymerization, make the bulk polymerization better rather than precipitation polymerization in terms of enhancing the sorption capacity, due to the well-templated during polymerization process.

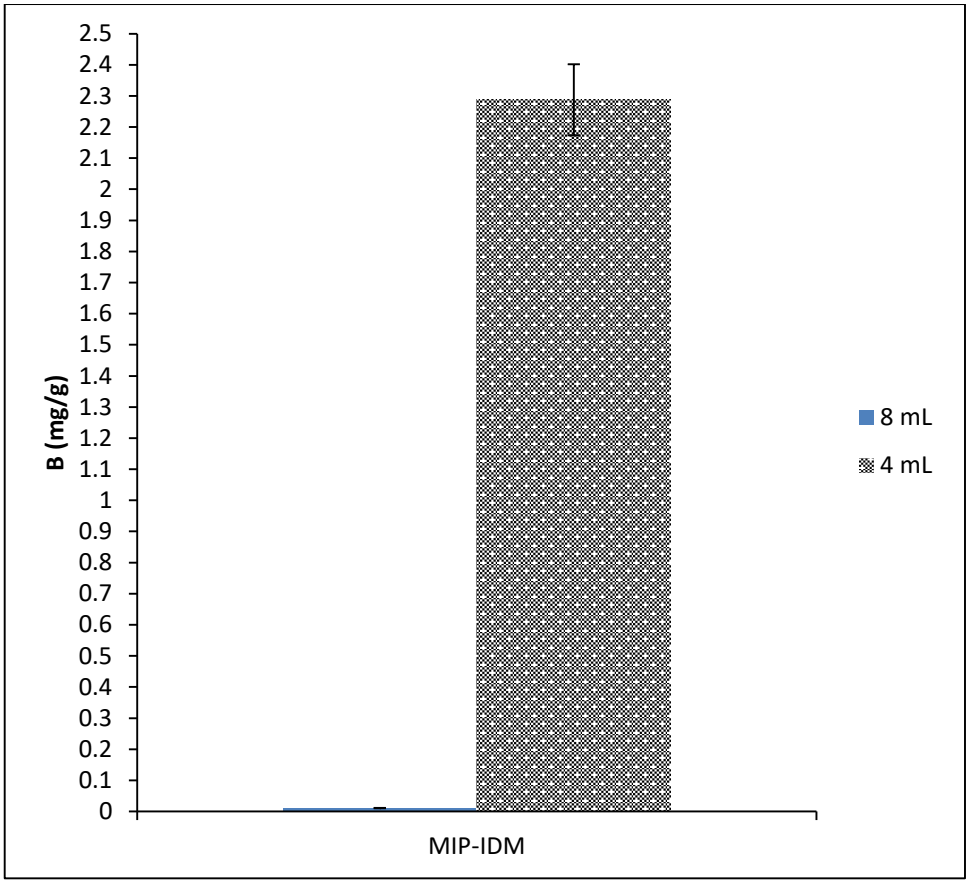


Figure 2.11 The total sorption by MIP-IDM in different porogen solvent volume during MIP-IDM synthesis using AT as a monomer.

Renkecz and co-workers [28] has successful prepared highly crosslinked polymer microparticles by precipitation polymerization using high monomer loadings (≥25% v/v) which generally would lead to bulk monoliths. A typical formulation for polymerization was used for microspheres polymer in which the functional monomer, (0.147 mmol), crosslinking monomer, (0.733 mmol) and initiator, AIBN (0.011 mmol, 1.8 mg, 1.3 mol% of the monomers) were dissolved in 750 µL of co-solvent/PO mixture (50/50 v/v%). However, the recipe for polymerization was in small ratio in order to control the particle size which they found in range ±1 µm.

Other article has been reported on using high volume of porogen solvent. Dai and co-workers [13,14] has synthetised the MIP via precipitation polymerization with DCF as the template and used 60 mL of porogen solvent (toluene).[13] In the present study, the bulk polymerization uses only 4 mL of porogen solvent (acetonitrile) for synthesis. Result obtained from the present

work shows that the synthesis method is better compared to Dai study [13,14] since the volume of porogen solvent used is much lower than Dai study.

2.4.2 Kinetic and sorption capacity studies in batch mode

For the IDM removal by MIP-IDM and MIP-DCF, the kinetic experiment shows important differents between MIP-IDM with NIP and MIP-DCF with NIP **(Figure 2.12 & Figure 2.13)**. According to the result obtained, more than 90% removal by MIP-IDM and MIP-DCF respectively whereas approximately less than 30% removal by NIPs. The binding is mainly due to the fact that the formed sites in the cross-linked polymers for MIP-IDM and MIP-DCF has been well-templated. In addition, it is probably because of the high affinity of cavities and memory properties. All the experiments have been done in a mixture solution of acetonitrile and water (5% in volume ratio).

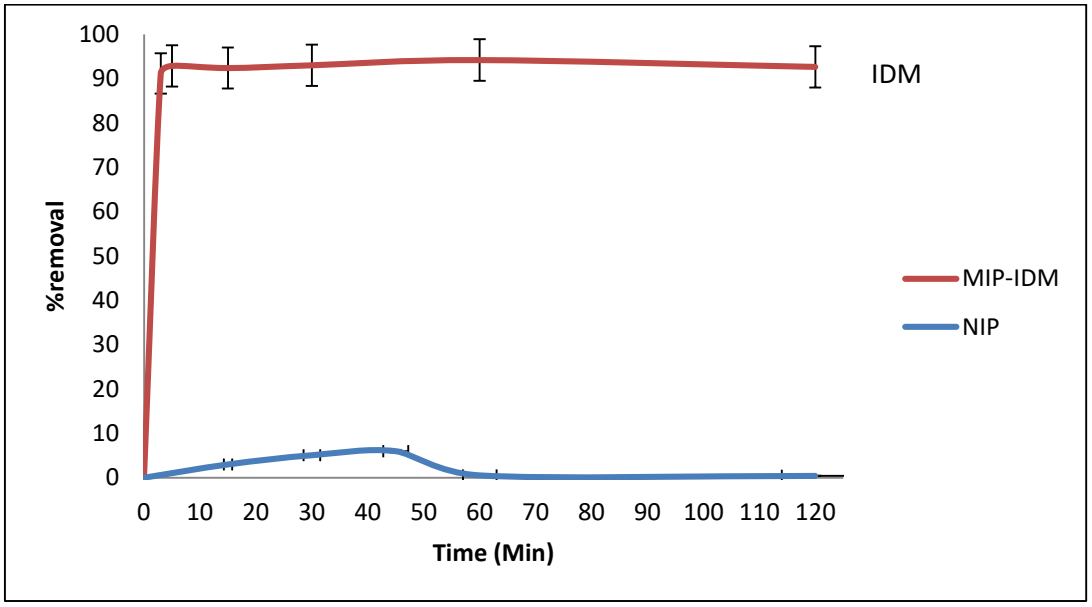


Figure 2.12 Sorption kinetic curve of MIP-IDM and NIP with initial IDM concentration of 5 µg/mL via batch mode. 10 mg sorbent (NIP and MIP-IDM), volume solution: 2 mL, room temperature.

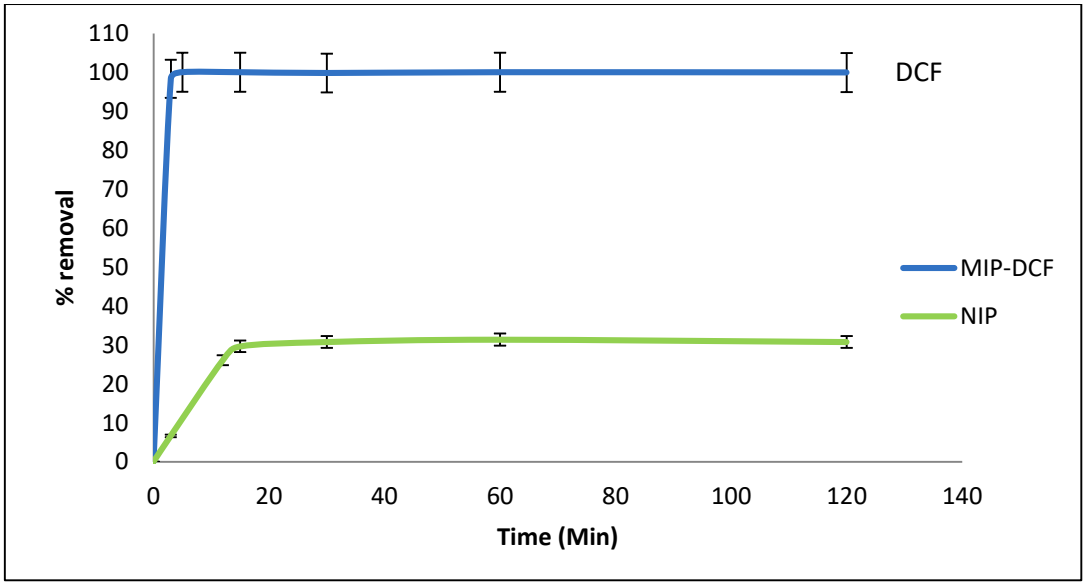


Figure 2.13 Sorption kinetic curve of MIP-DCF and NIP with initial DCF concentration of 5 µg/mL via batch mode. 10 mg sorbent (NIP, and MIP-DCF), volume solution: 2 mL, room temperature, extraction media: acetonitrile:water (5% v/v).

It is well accepted that two-parameter adsorption kinetic equations (Lagergren first-order and second-order) are useful tools to describe the adsorption properties of a sorbent.[29] The Lagergren pseudo first-order and second-order equations can be linearly expressed as **Equations (2.2)** and **(2.3)**, respectively where Q_e and Q_t are the adsorption capacities (mg/g) of MIP at equilibrium and at time t, respectively; k_1 and k_2 are the first order and second order rate constants respectively. In **Figure 2.14**, the graph of Q (mg/g) versus time (min) from **Equation 2.1** has been plotted and both MIPs were obeyed the Lagergren pseudo first order model.

$$Ln\,(Q_e - Q_t) = LnQ_e - k_1 t \quad \text{..... Equation 2.2}$$

$$\frac{t}{Q_t} = \frac{1}{k_2 Q_e^2} + \frac{t}{Q_e} \quad \text{.....Equation 2.3}$$

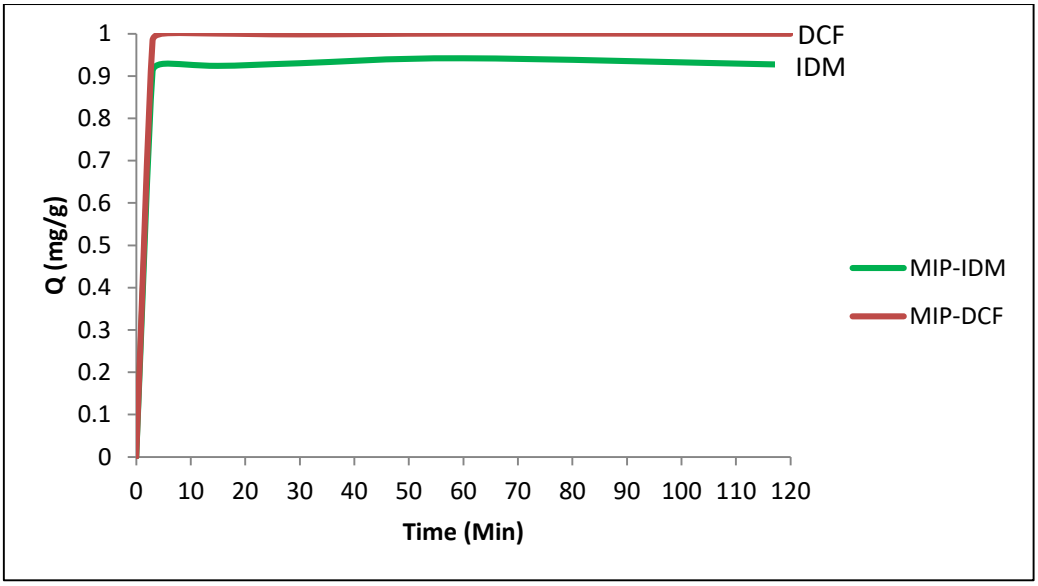


Figure 2.14 Legergren pseudo first order model plot for IDM or DCF sorption, in individual solutions, with initial concentration of 5 µg/mL via batch mode and using 10 mg of sorbent (MIP-IDM or MIP-DCF respectively).

In order to characterize the total sorption or saturation profile of MIPs, the analysis of the sorbed amount for different initial concentrations of IDM or DCF via batch mode have been done. However, the solubility of target molecule in water is limited. Thus, in this study the use of a mixture of acetonitrile and water (5%v/v) has been used to dissolve the pharmaceutical compounds.

The concentration of IDM was limited up to 25 µg/mL **(Figure 2.15)**. For concentrations higher than 25 µg/mL the IDM does not dissolve in the acetonitrile:water due to its hydrophobic behaviour and ithas tendency to agglomerates and precipitate so the absorbance reading decreases.

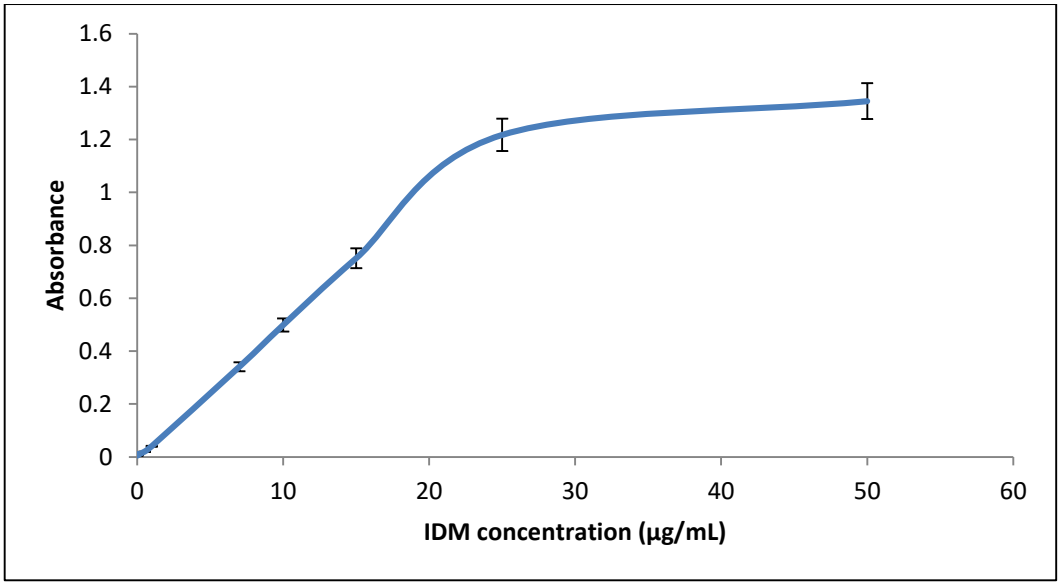


Figure 2.15 The limitation of IDM solubility in acetonitrile/water (5% v/v) reached maximum concentration at 25 µg/mL of IDM.

One significant research has been developed by Sun and co-workers (Sun et al., 2008) [10]. They develop a MIP founding an adsorption capacity of 19.1 µmol/g which is equal to 5.6564 mg/g by using bulk polymerization method. This method is a common method in preparation of MIP. However, it was time-consuming and the purity might be disputed since the MIP need to be crushed and sieved in order to have the particles size in certain range. However, as long as the bulk polymerization can produce high affinity polymers and selective, it still can be considered as a good approach.

In **Figure 2.16**, it is shown the efficiency removal by MIP-IDM within the range of 0.5 µg/mL to 25 µg/mL. As it is seen, the Q increases uniformly as the initial concentration also increases. The regression linear line for the total sorption is obtained being $y = 0.1919x + 0.0355$ with R^2 equals to 0.9993 and $y = 0.1956x - 0.0795$ with R^2 equals to 0.9999 for DCF and IDM respectively, being y the Q and x the concentration of pharmaceutical. The removal by MIP is mainly due to the specific interaction created by the template and not by the polymer itself. However, the removal by NIP with capacity loaded almost zero because NIP does not have imprinted properties and NIP itself could not remove the analyte from aqueous media. The removal of IDM using NIP show significant differences compared to MIP with approximately zero sorption of capacity. The same trend has been observed for the DCF removal by MIP-DCF as shown in **Figure 2.17**.

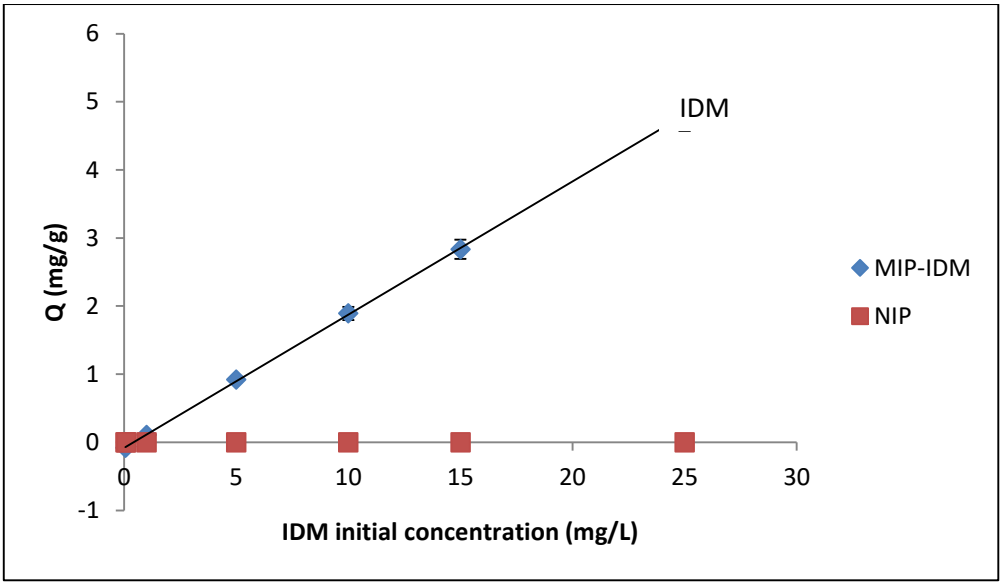


Figure 2.16 Sorption capacities (mg/g) for IDM removal within 60 min agitation for both MIP-IDM and NIP respectively. Solution volume: 2mL, Extraction media: acetonitrile/water (5%), room temperature

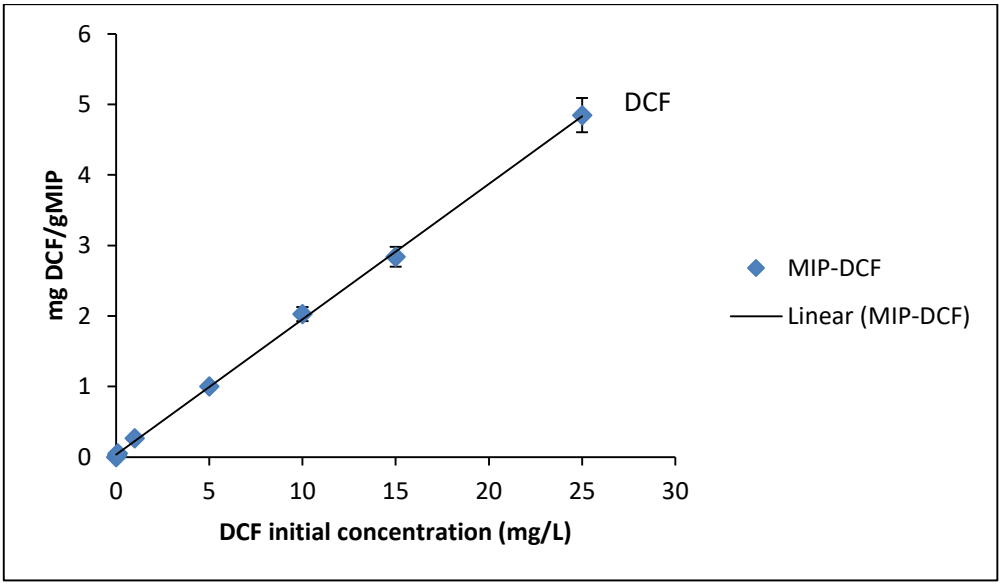


Figure 2.17 Sorption capacities (mg/g) for DCF removal by MIP-DCF within 60 min agitation. Solution volume: 2mL, Extraction media: acetonitrile/water (5%), room temperature.

Since the total sorption of target molecule using batch mode did not reach the plateau, other analysis method was suggested via molecularly imprinted solid phase extraction (MISPE). The total sorption value for MIP-IDM and MIP-DCF and the adsorption isotherm model via MISPE will be explained in **Chapter 3**.

2.4.3 Comparative study between different monomers and extraction time

For IDM removal, Yang [6] used acrylamide (AM), as previously said, as the functional monomer in preparation of MIP via bulk polymerization. AM has been used widely in many synthesis of MIP for a variety purposes.[30] However, according to the results found in our study, AM showed good sorbent properties but not because it can produce well-templated cross-linked polymer. The MIP and NIP with AM as the monomer have the ability to interact with the target molecule but the interaction between functional sites of AM was less efficient compared to AT in removing the IDM. Thus, MIP-AM showed approximately similar amount of IDM sorbed **(Figure 2.18)**. However, the one synthetised with AT as the monomer showed big differences between MIP-IDM (2.3 mg/g) and NIP (0.0 mg/g). This significant differences show that the cross-linked polymer was well-imprinted and the interaction occurred during the extraction process was not because of the polymer itself, otherwise it would also have been extracted by the NIP, but because of the molecular imprinting on the MIP.

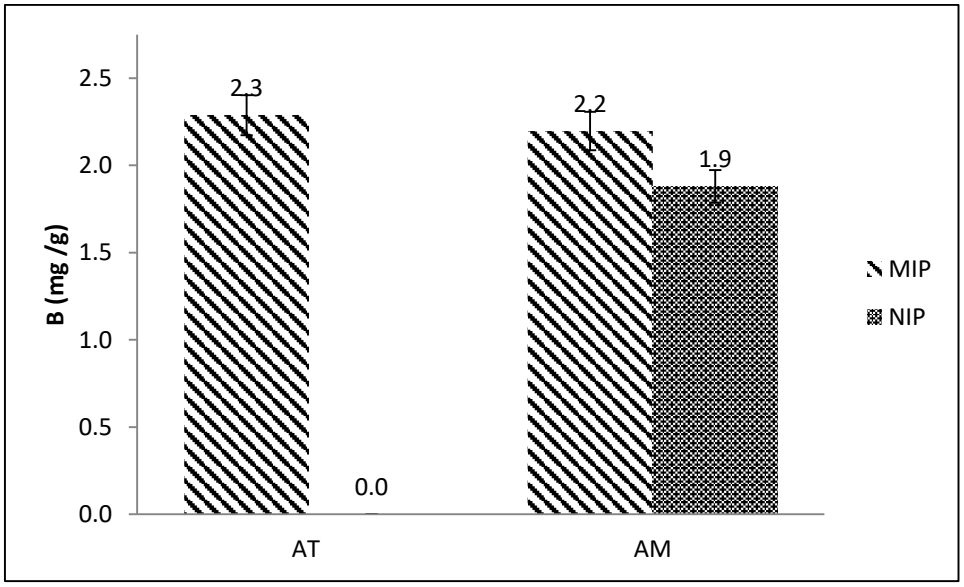


Figure 2.18 Comparison of sorption capacities (mg/g) for IDM removal with initial IDM concentration 5 µg/ml within 60 min agitation for both MIP-IDM and NIP respectively using different monomers which were AT and AM; Solution volume: 5mL, Extraction medium = acetonitrile/water (5% v/v).

The extraction time in the present work was 1 h (agitated) whereas in the Yang study [11] the extraction process was incubated for 10 h. So, the developed work shows a significant

improvement in terms of extraction time and simplified work by using new MIP with AT as the monomer.

The monomer which has been used in this study was N-allylthiourea **(Figure 2.19)**. N-allyltiourea molecular active functional site (thiourea) has the ability to attract the template via hydrogen bonding. The thiourea is miscible in water since it contains an active functional group –thiol in the structure. However, to the best of our knowledge, only one article has been found so far using allylthiourea as the monomer in synthesis of MIPs using DCF and IDM as the template.[21]

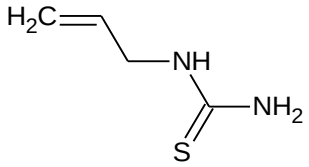


Figure 2.19 1-allylthiourea

Methacrylic acid (MAA) **(Figure 2.20**) has been used as a 'universal' functional monomer due to its unique characteristics, being capable to act as a hydrogen-bond donor and acceptor, and showing good suitability for ionic interactions.[3] There is an article reporting on the use of MAA as functional monomer.[29] However, interestingly, this is contrary to a study conducted by Yang, and co-workers who used acrylamide (AM) **(Figure 2.21)** and MAA as the functional monomer in order to differentiate the IDM efficiency removal between two types of monomers. AM also has been used in many kinds of purpose especially in preparation of MIPs. Yang, and co-workers indicated that AM was much better compared to the universal functional monomer, MAA. Thus, it shows that the 'good' monomer would likely depends on the objectives of research and synthesizing method.

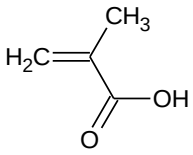


Figure 2.20 Methacrylic acid

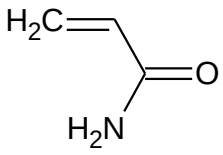


Figure 2.21 Acrylamide

2.4.4 Influence of medium solvent for the pharmaceutical dissolution for the removal process using MIP with AT as the functional monomer

2.4.4.1 Acetonitrile/water mixture (5% v/v)

Blank analysis has been carried out in the first place. It was performed using the same procedure with absent of target molecule. For the blank analysis **(Figure 2.22)** in acetonitrile/water (5% v/v), no peak was found at all at 260 nm for IDM. Besides, it can be assumed that there was no IDM still attached to the cross-linked MIPs which would be realeased from the MIP to the solution if a combination of acetonitrile and water was used during removal study. The medium of acetonitrile andwater shows a good combination for further experiments. Acetonitrile was used for further study because it has a less dieletric constant value which means that acetonitrile does not interfere in bonding interaction during removal process.

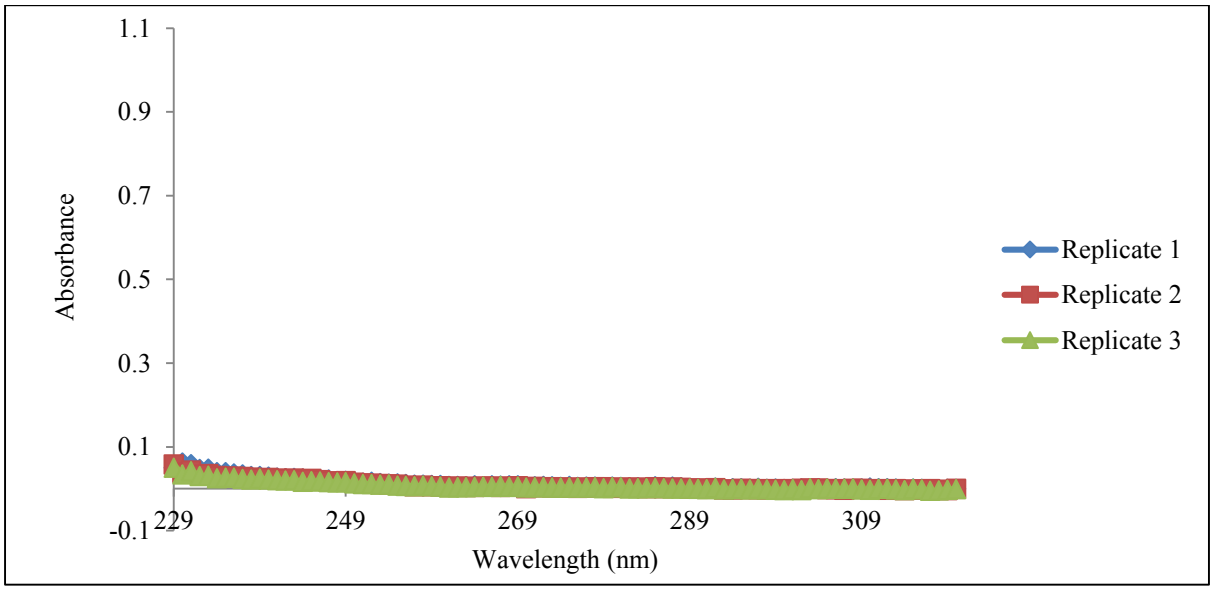


Figure 2.22 Spectrum of blank analysis after agitated with MIP-IDM for 1 h in extraction medium of acetonitrile/water (5%v/v).

2.4.4.2 Acetonitrile (100%)

The usage of acetonitrile as the medium has been reported in previous studies.[10,6] According to Yang and co-workers, the experiments were carried out in acetonitrile as the extraction medium with low amount of IDM sorbed into MIP-IDM-AM (0.175 mg IDM/g MIP in 1 mL

of solution) whereas in the present work the MIP-IDM-AM synthetised by using the formulation shown in **Table 2.1** the IDM sorbed was 0.44 mg IDM/g MIP in 1 mL of solution. The results shows that synthetised MIP in present study was 4 times more than Yang study. In addition, MIP particles have tendency to return into solution when acetonitrile was used. Hence, the present work shows an efficient method in terms of solvent media used in removing IDM from aqueous media.

Yang and co-workers use acetonitrile (100% v/v) as the media for the removal of IDM via MIPs due to the insolubility of IDM in water. The MIP synthetised in the present study is about 2 times more efficient than the Yang study. However, by using the MIP-IDM synthetised using AT as the monomer, the IDM sorbed was 0.46 mg IDM/g MIP in 1 mL of solution.

In this study, significant result was observed when using acetonitrile as the medium. As can be seen in **Figure 2.23**, spectra shows different absorbance value for different types of solution. The blank absorbance was much higher compared to 5 mg/L IDM solution. It was due to the MIP was dissolute and return to the solution. The porogen solvent used for synthetised MIP-IDM was acetonitrile. Furthermore, the absorbance of after sorption without centrifuge shows much higher compared to blank and 5 mg/L IDM solution. It is because after centrifuge, the absorbance reading much less than after sorption without centrifuge.When the solution was centrifuged, the MIP particles tends to sediment and when the MIPs were still in the solution the absorbance reading gave higher value due to the conjugated cross-linked between particles and the target molecules. All the solutions were filtered before using spectrophotometer.

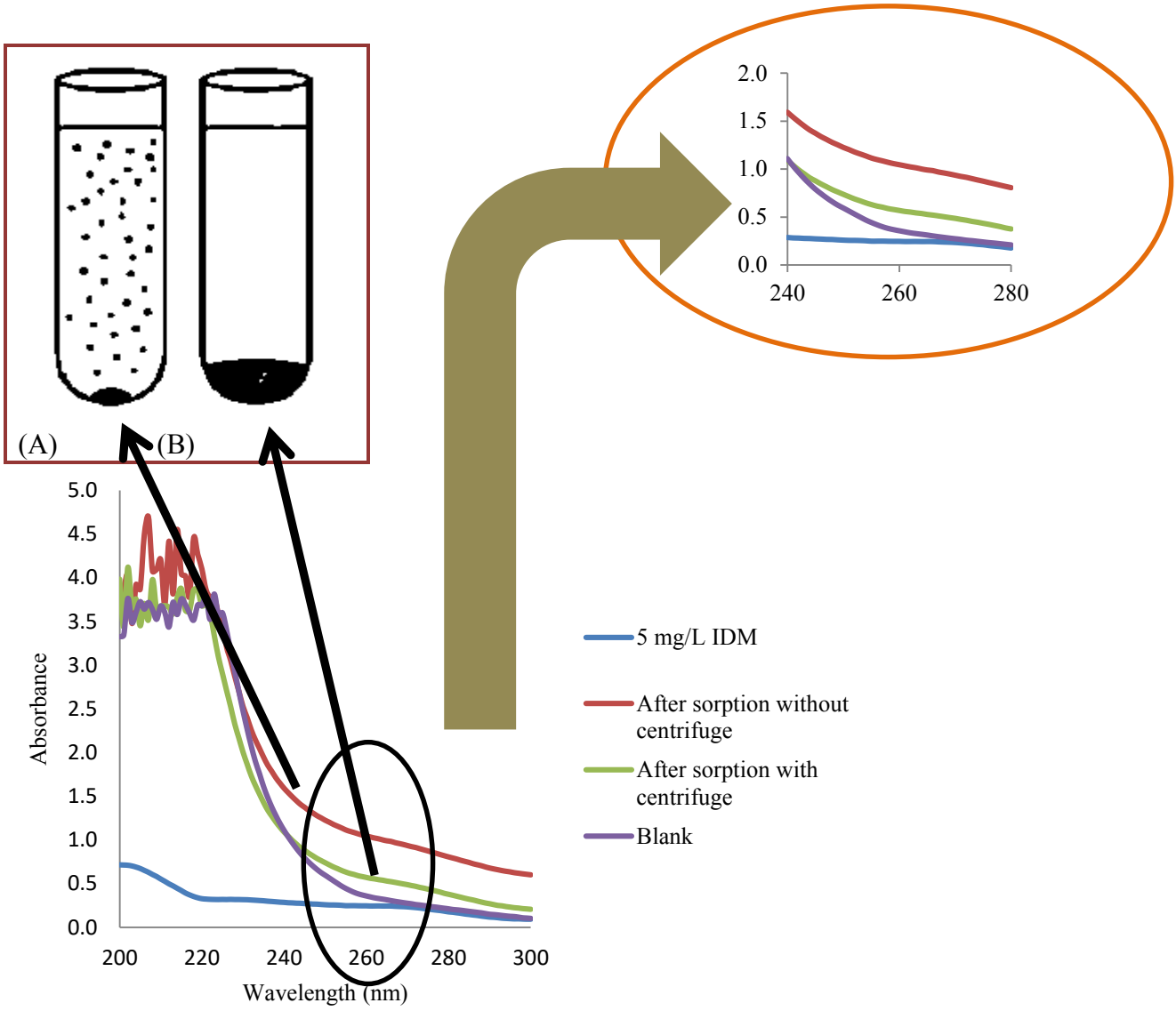


Figure 2.23 Spectra of different solutions for pre-removal by MIP-IDM in extraction medium of ACN (100%) with initial IDM concentration of 5 µg/mL, post-removal without centrifuged, post-removal with centrifuged and blank solution after agitation with MIP-IDM, solution volume: 2 ml, λ_{max} = 260 nm.

2.4.4.3 Other organic solvents

The sorption of IDM by MIP-IDM in organic solvents such as chloroform has been reported previously[6]. The organic solvent as has been used due to the immiscibility of IDM and DCF in aqueous medium. However, in this study, different types of organic solvent such as chloroform and ethanol have been used in order to characterize the amount of IDM sorbed to the MIP-IDM (mg IDM per g MIP-IDM). As can be seen from the **Figure 2.24** below, it can be concluded

that different medium give different amount of IDM sorbed to the MIP-IDM being chloroform 50 mg/g, whereas ethanol 30 mg/g. Chloroform is a non-polar organic solvent whereas ethanol is a polar organic solvent. In terms of sorption sorbed value, Dai and co-workers observed that the DCF sorbed to MIP was 300 mg/g when the extraction medium used was methanol/water (1/1), a combination of organic solvent in aqueous medium. It was estimated that chloroform or ethanol in 100% per volume was not suitable to be used in this study due to the expected lower total sorption capacity compared to chloroform as the medium. In particular, the imprinted polymers proved to be applicable in aqueous buffers for specific molecular recognition under the optimal binding conditions.[31] Thus, further experimental in the present work has been carried out with a combination of organic solvent in aqueous medium.

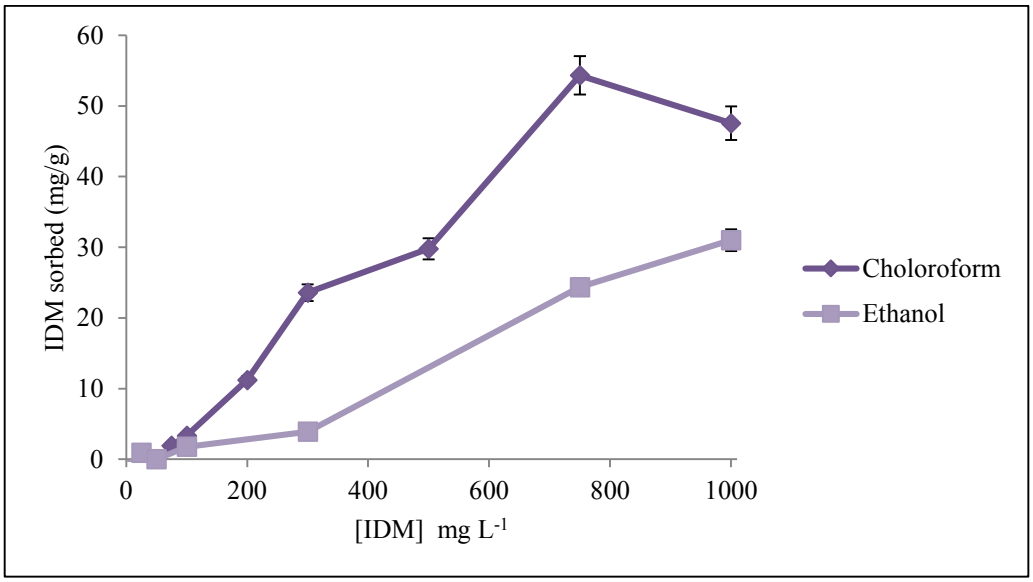


Figure 2.24 Sorption of IDM in different medium (1) chloroform and (2) ethanol, 100% v/v, solution volume: 2 mL via batch mode, 10mg of MIP-IDM.

2.4.5 Influence of pH

pH commonly affects the process of removal if the target molecule contain acidic or/and basic functional sites. According to the study carried out by Asiabi [1] MIPs fabricated tends to isolate the target molecule under neutral pH. However, it was dependent on the target molecule and the functional sites in the cross-linked polymer (MIPs). In the present study, the pH range from 3 to 8 was studied. From the obtained results, the efficiency removal was approximately 90% for both MIP-IDM and MIP-DCF **(Figure 2.25)** because the target molecules were considered stable at low pH (pH 3 – pH 7) and both were acidic pharmaceuticals. Dai and co-workers

found a similar patern comparing with the MIP-IDM synthetised in the present study wherein the removal efficiency works well.[13] The efficiency removal value was calculated using **Equation 2.1**.

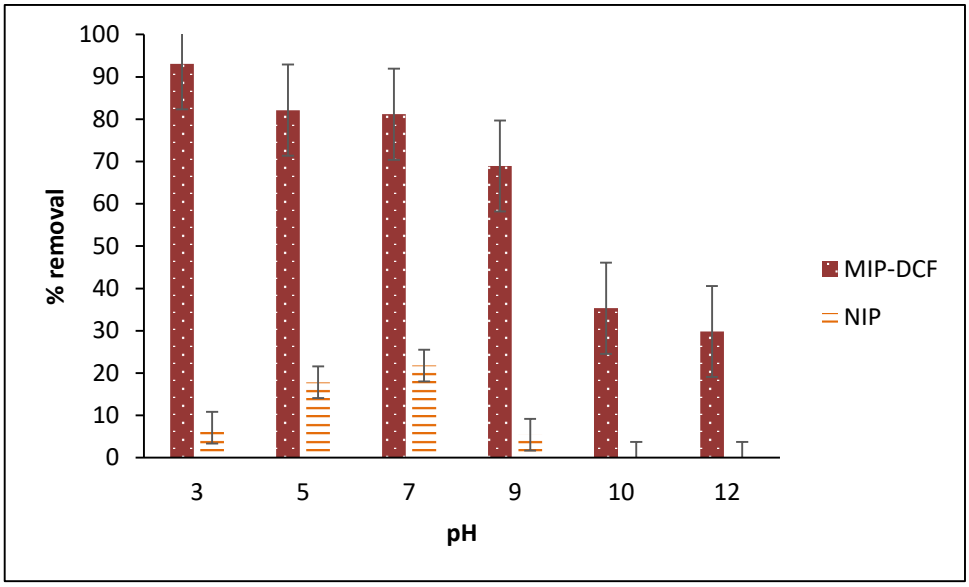


Figure 2.25 % removal of DCF in different pH solutions (pH 3 – pH 10) using MIP-DCF and NIP, initial concentration: 15 µg/mL of DCF, extraction medium: acetonitrile/water (5% v/v) and agitated for 1 h.

The sorption capacity was approximately 3.0 ± 0.4 mg DCF/ g MIP within neutral pH range (5-9) in 15 µg/mL of DCF solution as initial concentration. With good correlation in regression linear line as mentioned in section **2.4.1**, it was estimated that at 5 µg/mL the total sorption capacity of DCF would be 1.0 ± 0.1 mg DCF/ g MIP. DCF is a very soluble in neutral-alkaline medium (50 g /L) and is an acidic pharmaceutical (pKa = 4.15) that becomes almost insoluble below pH 4, so below this pH value, diclofenac precipitates.[32] Thus, at pH 3 the DCF removal shown in **Figure 2.26** (2.5 mg DCF/g MIP) can be caused by the precipitation of DCF and not by using MIP as the sorbent.

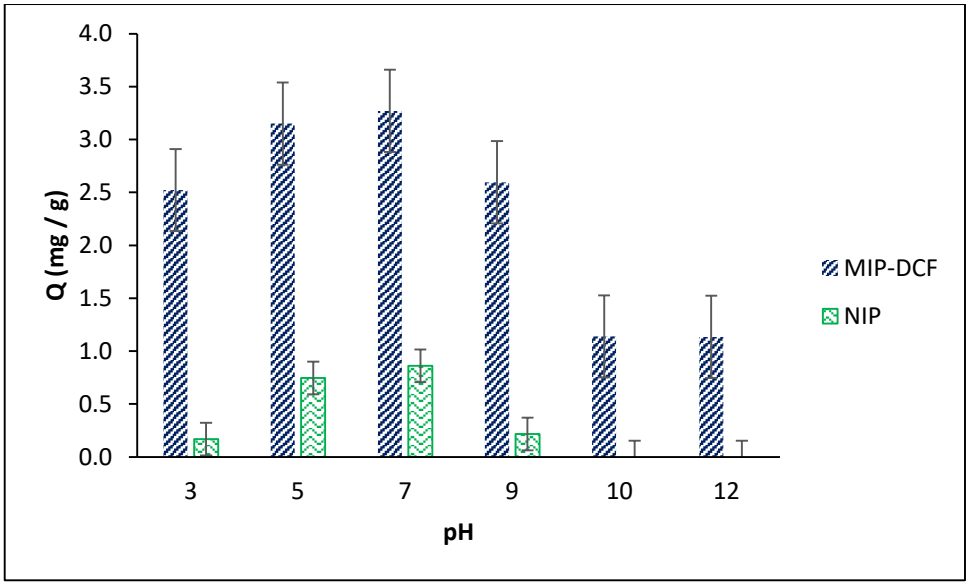


Figure 2.26 DCF sorbed in different pH solutions (pH 3 – pH 10) using MIP-DCF and NIP, initial concentration: 15 µg/mL of DCF, extraction medium: acetonitrile/water (5% v/v) and agitated for 1 h.

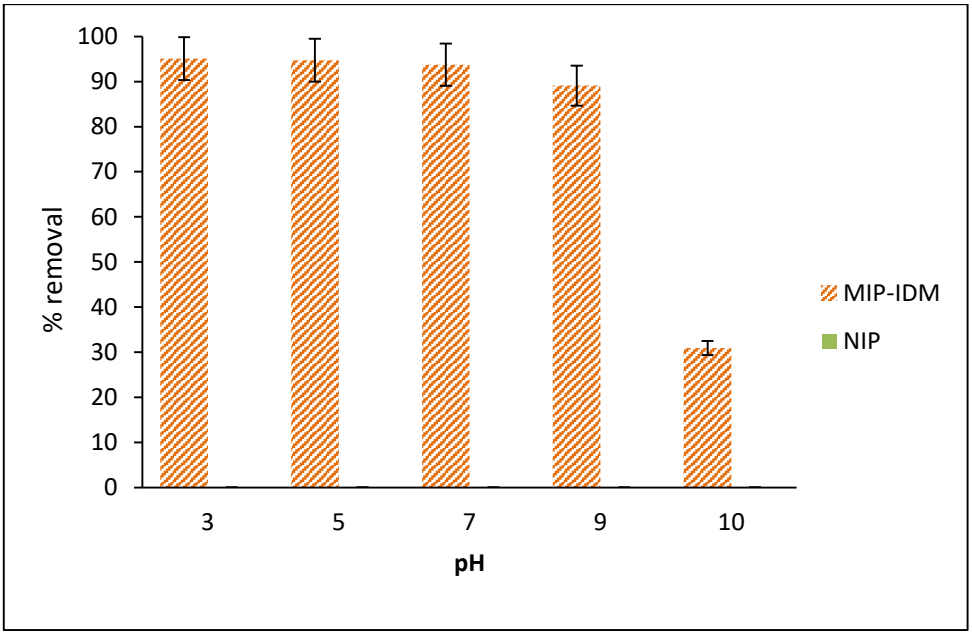


Figure 2.27 % removal of IDM in different pH solutions (pH 3 – pH 10) for the IDM removal using MIP-IDM and NIP, initial concentration: 15 µg/mL of IDM, extraction medium: acetonitrile/water (5% v/v) and agitated for 1 h.

For IDM, as shown in **Figure 2.28**, the sorption capacity was approximately 2.78 ± 0.09 mg IDM/ g MIP within neutral pH range (5-9) in 15 µg/mL IDM as initial concentration. With good correlation in regression linear line as mentioned in **2.4.1** section, it was estimated that at

5 µg/mL IDM (initial concentration) the total sorption of IDM would be 0.46 ± 0.06 mg IDM/ g MIP.

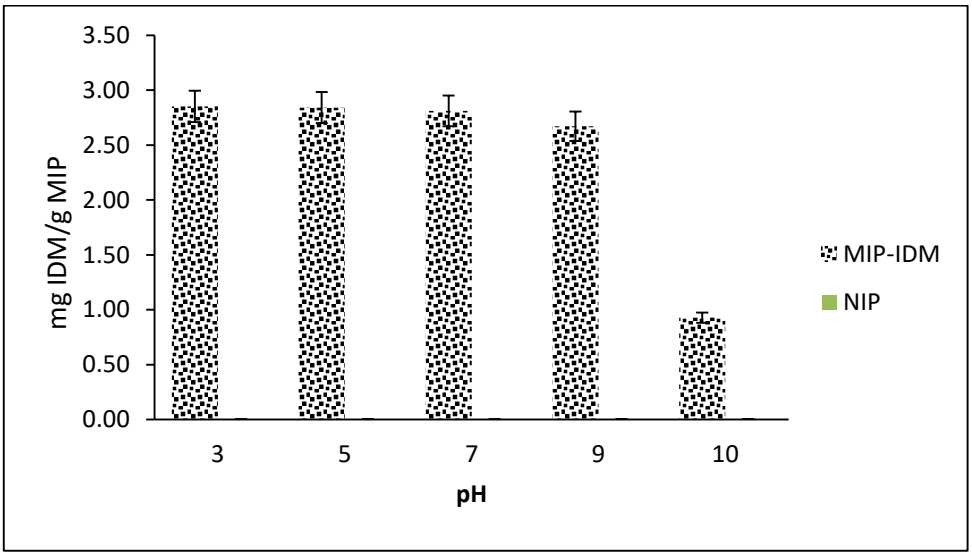


Figure 2.28 Sorbed of IDM in different pH solutions (pH 3 – pH 10) for the IDM removal using MIP-IDM and NIP, initial concentration: 15 µg/mL of IDM, extraction medium: acetonitrile/water (5% v/v) & agitated for 1 h.

2.4.6 Selectivity study via simultaneous detection

Detection via spectrophotometer is a simple and direct detection analysis. Hence, in this study, spectrophotometer has been choosen as the instrument for the IDM, DCF and/or IBU detection in two components in a mixture.

To perform the study, simultaneous detection for two components in a mixture consisting of IDM, DCF and/or IBU using MIP-DCF and MIP-IDM was carried out. For MIP-IDM, the interference would be DCF and IBU whereas for MIP-DCF, the interference would be IDM and IBU. In this study, the solutions were tested and being analysed in the presence of interference in order to study the selectiveness properties. Different mixtures consist of two pharmaceuticals in aqueous medium (5% of ACN/ water) with initial concentration of 5 µg/mL for each one.

As can be seen from the **Figure 2.29-Figure 2.31**, zero order absorption spectra shows severe overlapping between each compound. In this study, the two components in a mixture consist of target molecules and one interference will be discussed.

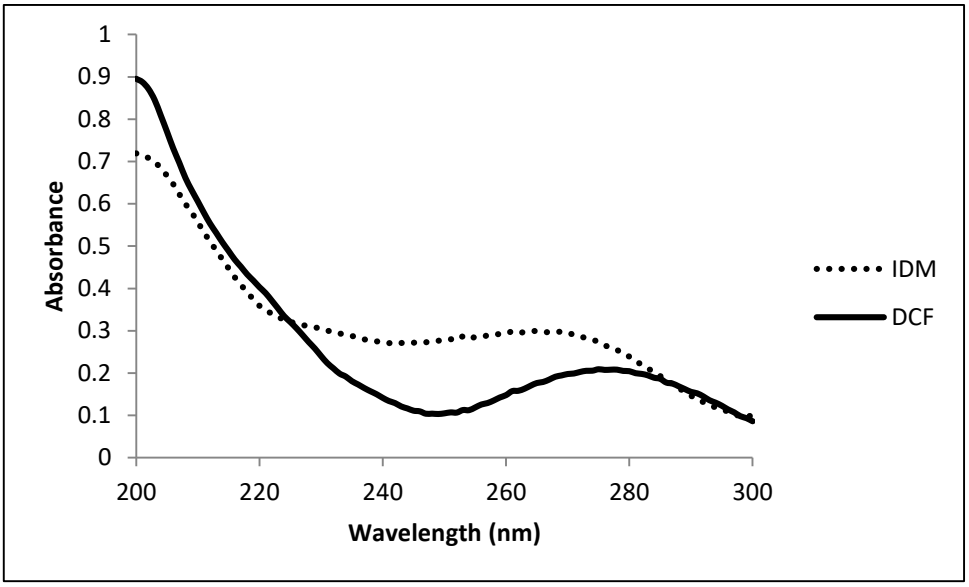


Figure 2.29 Zero-order absorption spectra of IDM and DCF in mixture 1 with concentration at 5 µg/mL individually prepared using 5% (v/v) ACN/water as blank.

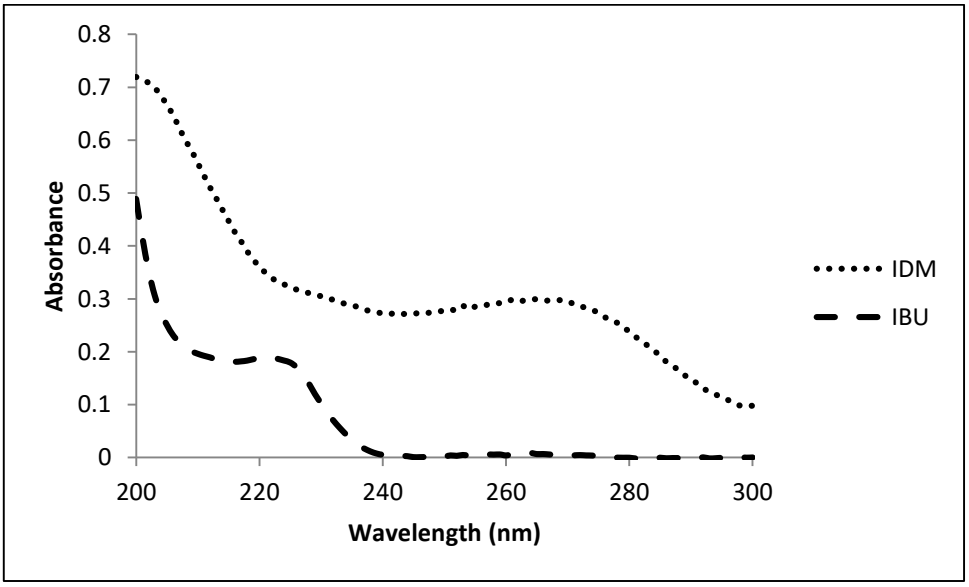


Figure 2.30 Zero-order absorption spectra of IDM and IBU in mixture 2 with concentration at 5 µg/mL individually prepared using 5% (v/v) ACN/water as blank.

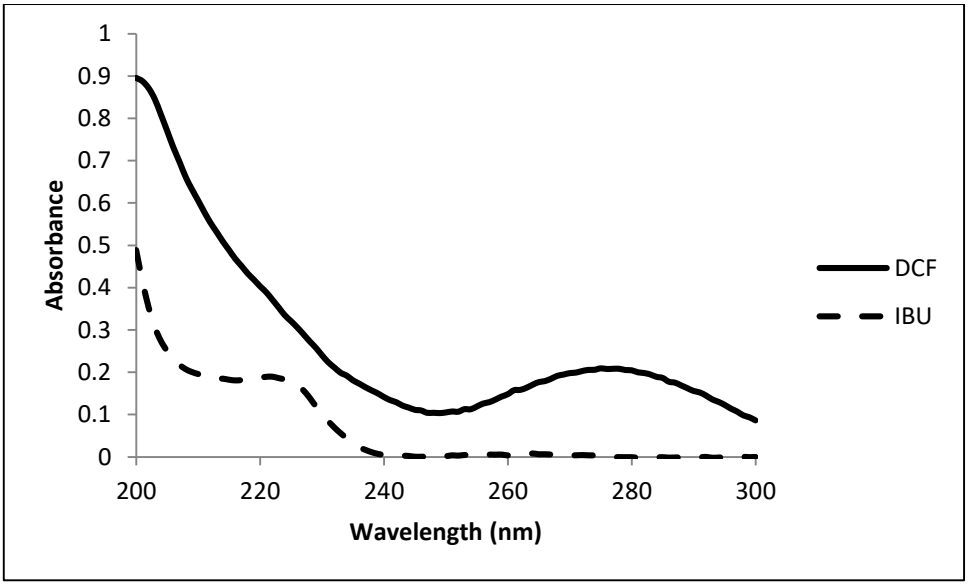


Figure 2.31 Zero-order absorption spectra of DCF and IBU in mixture 3 with concentration at 5 µg/mL individually prepared using 5% (v/v) ACN/water as blank.

Based on the Beer's Law,

$A = \alpha b C$, ……..…**Equation 2.4**

with

α = absorptivity vector,
b = length of the path cuvette wall = 1 cm
C = concentration at certain wavelength

Consider a mixture of two compounds, X and Y:

$A_m = \alpha_x C_x + \alpha_y C_y$……..……..**Equation 2.5**

A_m = Absorbance for mixture
α = slope
C = concentration certain wavelength

Table 2.2 shows the linear equations for each different mixtures at different maximum wavelength (λ_{max}) for IDM (260 nm), DCF (280 nm) and IBU (220nm). The slope were

determined from the calibration curve for each target molecule at different maximum wavelength, λ_{max}. Next, the calculation on amount sorbed in terms of μmol for each types of MIPs has been performed.

Table 2.2 Determination of IDM, DCF and/or IBU in the two components mixtures.

Template	Mixture	Equations	
MIP-IDM	Mixture 1 (IDM + DCF)	0.0144x + 0.0123y = 0.0290	At 260 nm
		0.0178x + 0.0088y = 0.0560	At 280 nm
	Mixture 2 (IDM + IBU)	0.0221x + 0.0093z = 0.2295	At 260 nm
		0.0178x + 0.0003z = 0.0597	At 220 nm
MIP-DCF	Mixture 1 (IDM + DCF)	0.0144x + 0.0123y = 0.0320	At 260 nm
		0.0178x + 0.0088y = 0.0560	At 280 nm
	Mixture 3 (DCF + IBU)	0.0123y + 0.0001z = 0.0300	At 260 nm
		0.0246y + 0.0093z = 0.2600	At 220 nm

*x: IDM, y: DCF and z: IBU amount sorbed in μmol

The selective study has been carried out between MIP-IDM and MIP-DCF in different mixtures:

- Mixture 1: IDM + DCF
- Mixture 2: DCF + IBU
- Mixture 3: IDM + IBU

In mixture 1 with MIP-IDM, the result indicated that MIP-IDM slightly favored to isolate DCF molecule rather than IDM molecule **(Figure 2.32)**. However, the removal by MIP-DCF in mixture 1 shows that the removal slightly favored to isolate DCF molecules **(Figure 2.33)**. It was believed that the functional sites on the target molecule will influence the molecule interaction traped into the unoccupied template. The other reason for this it is because the size of template moulded was sufficient for DCF molecule size to be trapped into the unoccupied templates of MIPs fabricated. This is due to the size of target molecule and functional sites on MIPs that have an ability to interact via hydrogen bonding with the N-H functional group in

DCF instead of N-COR functional group that attached on IDM molecule. Refer to the schematic reaction in **2.4.7**.

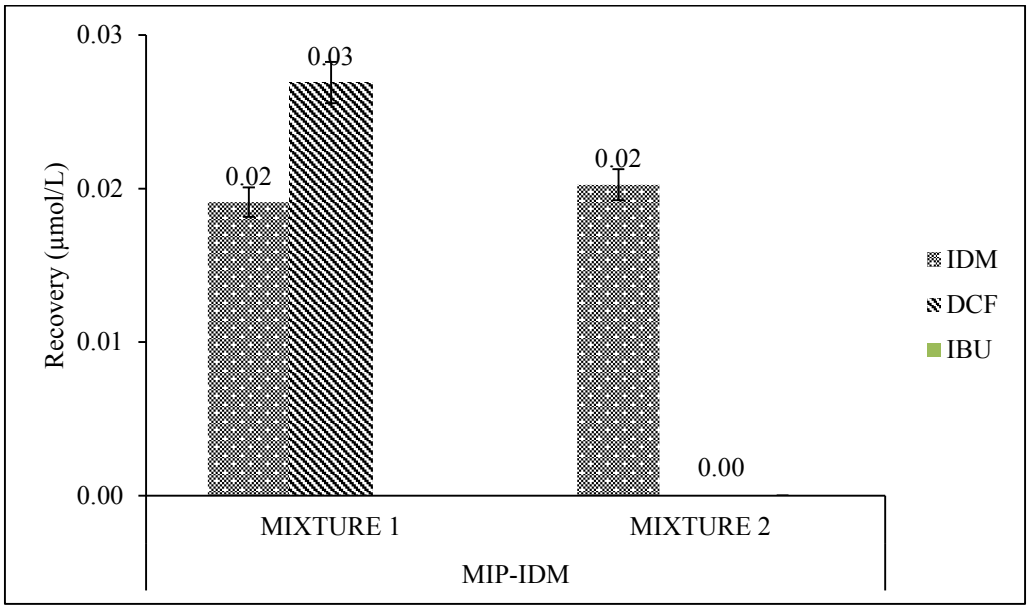


Figure 2.32 Sorbed (µmol) uptake by MIP-IDM in different mixtures of solution with the initial concentration of 5 mg/L via batch mode, Mixture 1: (IDM ± 0.0001 µmol) + (DCF ± 0.0002 µmol); Mixture 2: (IDM ± 0.0001 µmol) + (IBU ± 0.0007 µmol).

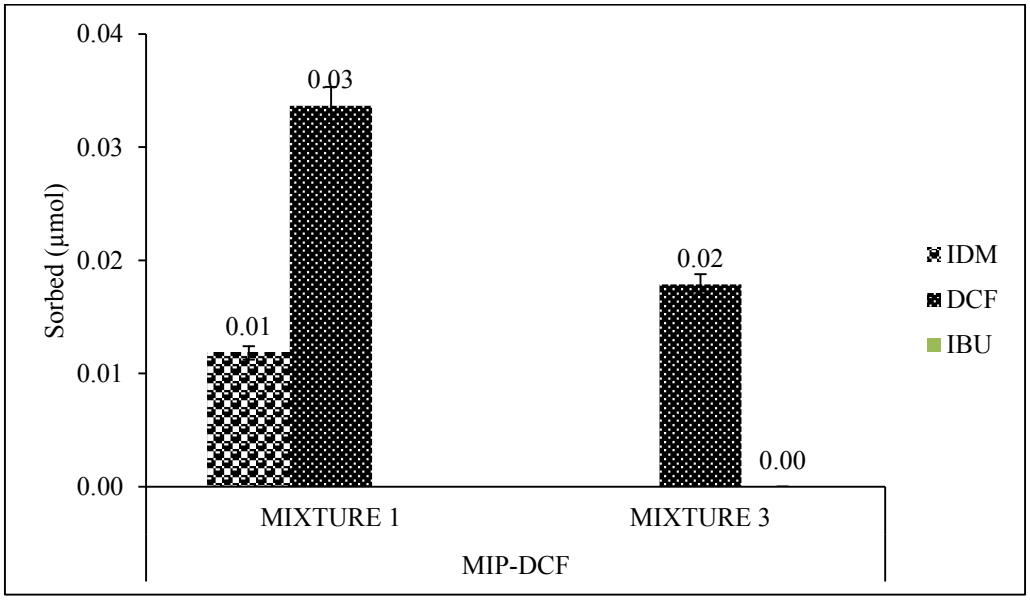


Figure 2.33 Sorbed (µmol) uptake by MIP-DCF in different mixtures of solution with the initial concentration of 5 mg/L via batch mode, Mixture 1: (IDM ± 0.0006 µmol) + (DCF ± 0.0007 µmol); Mixture 3: (DCF ± 0.0004 µmol) + (IBU ± 0.0012 µmol).

According to published studies, a few selectivity studies was conducted in individual solutions containing the interference only and not in a mixture containing both, analyte and interference. One example is the study by Amiri and co-workers where the DCF solution was prepared individually and called it as interference study.[8] This was practically unselective procedure towards the analyte according to the definition of selectivity. Selectivity can be defined as the ability of differentiating and quantifying the analyte in the presence of other components from its matrix.[33] Hence, in our study the two components in a mixture were used with the presence of analyte and interference.

Competitive functional sites works as imprinted fingerprint in which it will become selective for N-H functional sites instead of other functional groups **(Figure 2.34)**. Hence, this can be another benefits of the DCF isolation for selective properties since DCF have the N-H functional group in the molecule. The priority of selective properties will be N-H > N-COR. Further elaboration on the schematic reaction in section **2.4.7**.

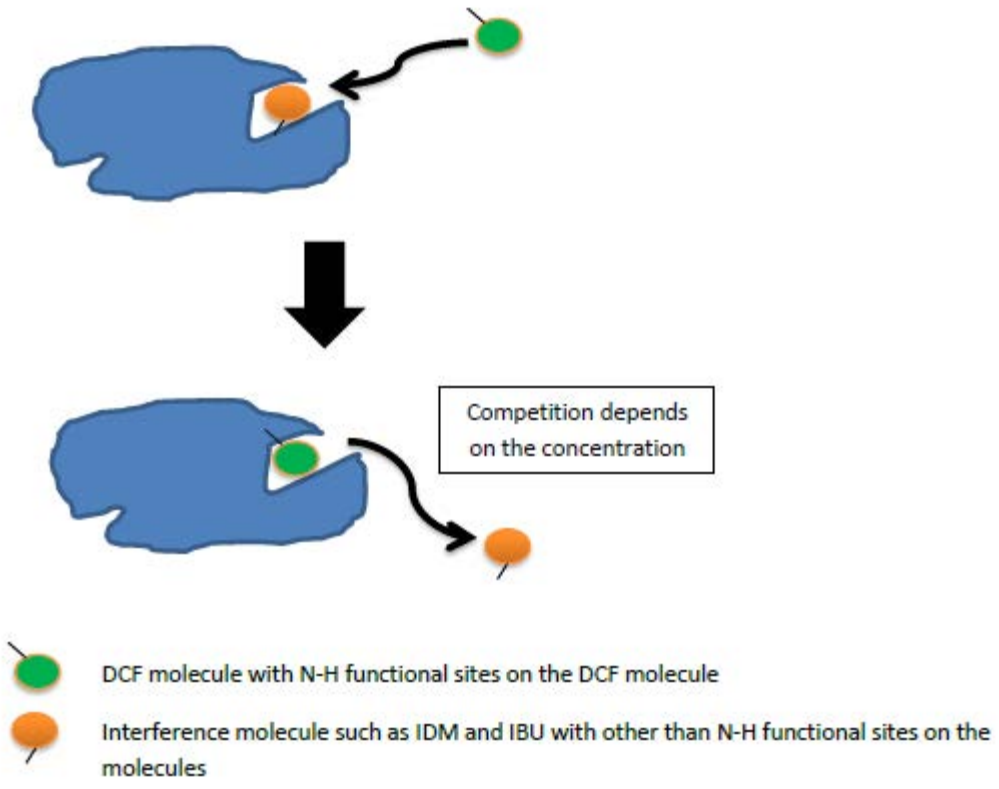


Figure 2.34 The selective and competition of functional sites, AT towards N-H functional group on target molecule.

Steric hindrance in the center of target molecules in IDM also might be the caused of this matter. However, for a mixture containing IBU, there was almost zero sorption by MIP-IDM and MIP-DCF respectively. The molecule structure of DCF, IDM and IBU as shown in **Figure 2.35.** As can be seen, there is a carboxylic acid functional group able to interact with thiol groups. . The theory about the interaction between the carboxylic acid group with thiol groups has been elaborated in detail by Xiao Pei, and co-workers.[34] However, at the center of the compounds there are another active functional groups likely to interact via hydrogen bonding with functional sites on MIPs. Nevertheless, there is no functional group at the center of IBU. Hence, the sorption of IBU by MIP-IDM and MIP-DCF was almost zero..

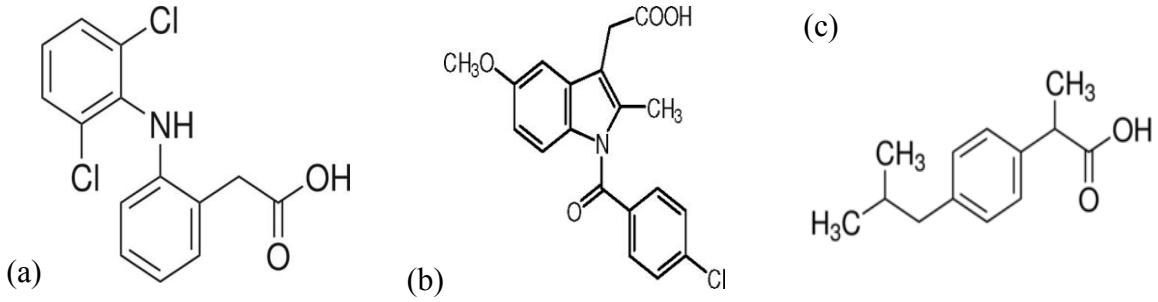
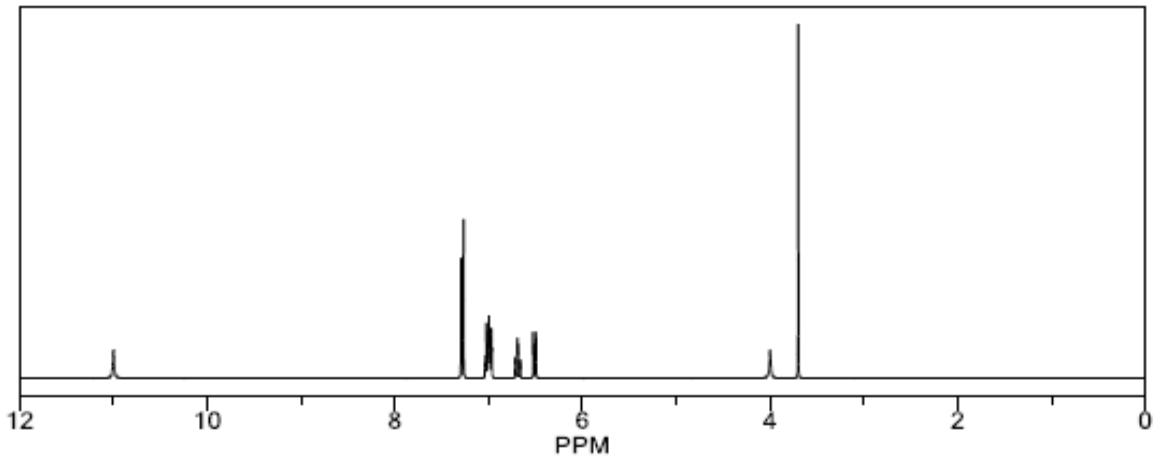


Figure 2.35 Molecule structure of DCF (a), IDM (b) and IBU (c).

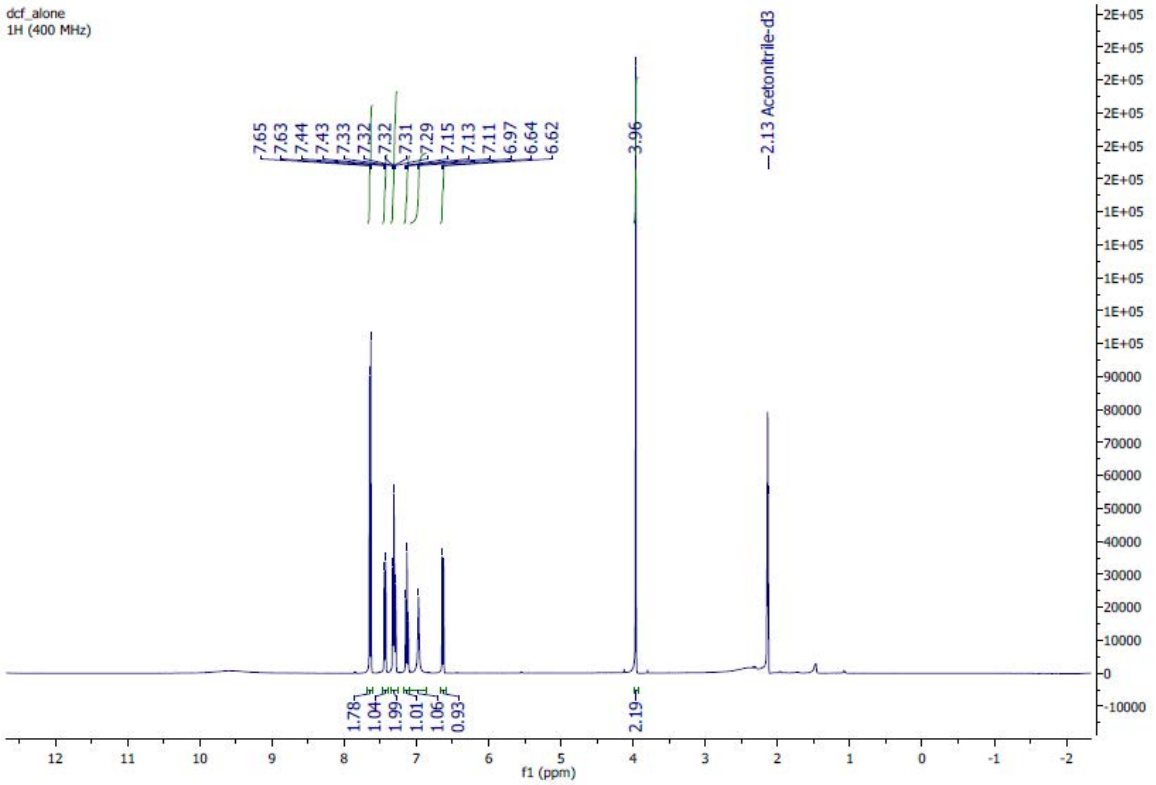
2.4.7 Pre-polymerization study using ^{1}H NMR spectroscopy

In **Figure 2.36**, it is shown the simulation spectra obtained and the experimental result for both, the DCF and the AT. In the experimental result of pure AT, the observed peaks were broad because amino moieties of thiourea groups give intra and intermolecular hydrogen bonds that show an enlargement of signals. However, for the experimental of pure DCF in acetonitrile-d_3, the spectrum was approximately similar to the simulation spectrum.

(a) Simulation of pure DCF.



(b) Experimental spectra of pure DCF.



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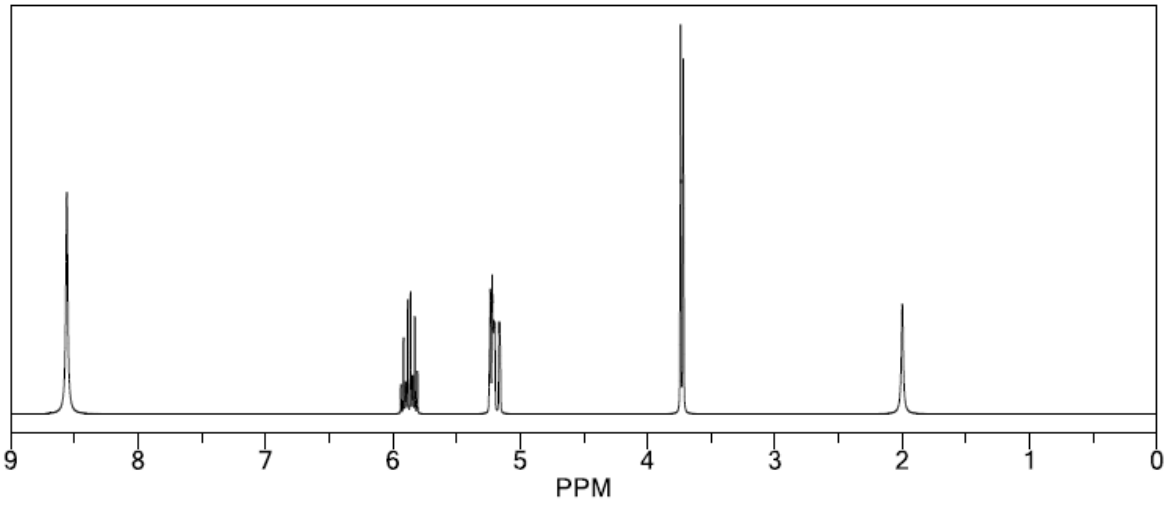
(c) Simulation of pure AT.



(d) Experimental of pure AT.

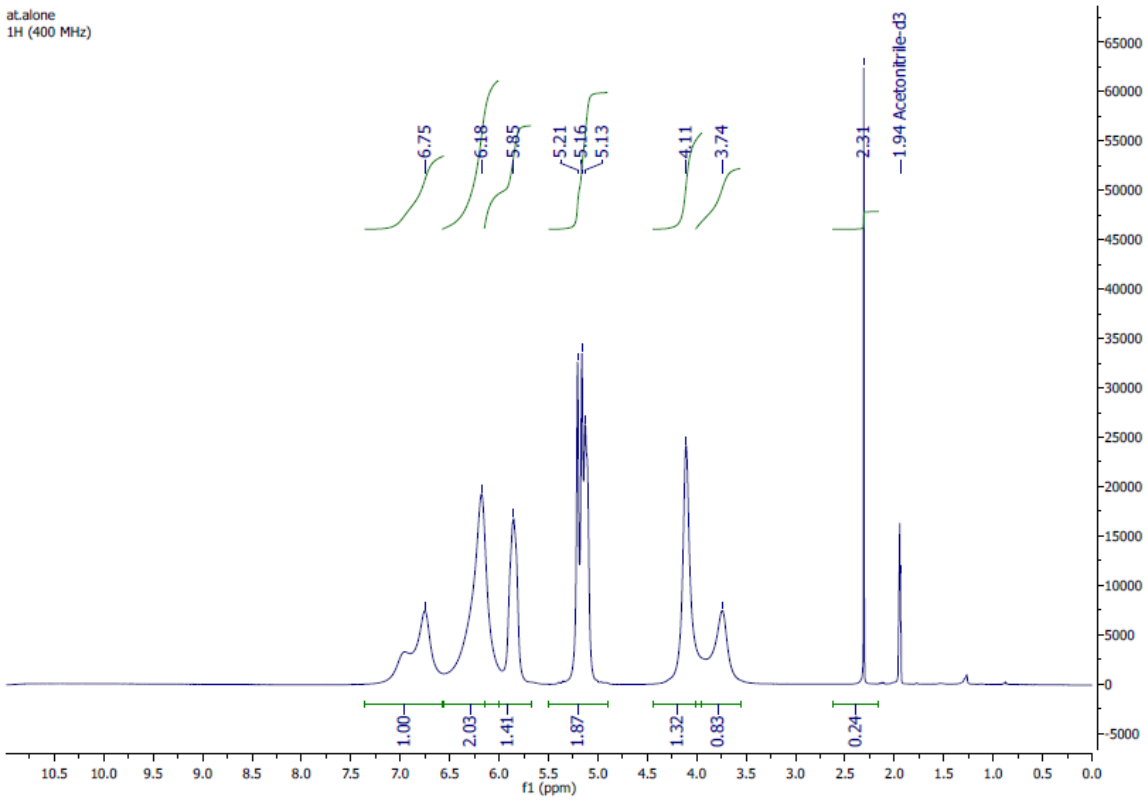


Figure 2.36 ^{1}H NMR spectra of (a) simulation of pure DCF (b) experimental of pure DCF (c) simulation of pure AT and (d) experimental of pure AT in 1 mL of acetonitrile-d_3 at 400MHz.

For pre-polymerization study, proton NMR has been used to study the interaction between monomer (AT) and template (DCF). The results show more than one active functional sites so it can be estimated that the MIPs will have high affinity towards the analyte in sorption process.

From the results obtained, generally the reaction involved for pre-polymerization was estimated as forward process because the results obtained in mixtures (Mixture 1, Mixture 2 and Mixture 3) shows that the peak in pure DCF or pure AT were decreased and certain significant peaks could not be found in the mixtures spectra. In addition, by applying the Le Chatelier principle, the pre-polymerization occurred with the product favor to be yielded. In this case, the product is the complexes formed.

In order to produce high number of active imprinted sites on the imprinting polymer, it is well known that the ratio between monomer and template is crucial.[10,13,35] According to the previous study conducted by Sun [10] the ratio between monomer and template was found to be optimum at 4 mmol:1 mmol Applying Le Chatelier's principle to the complexes formed prior to polymerization, increasing the binding affinity of the complexes in the pre-polymerization mixture would predict an increase in the pre-polymer complexes. Correspondingly, there is an increase the number of final binding sites in the imprinted polymer, resulting in an increased binding or selectivity factor per gram of polymer.[18] The structure of the complex formed between DCF and MIP is suggested in **Figure 2.37**.

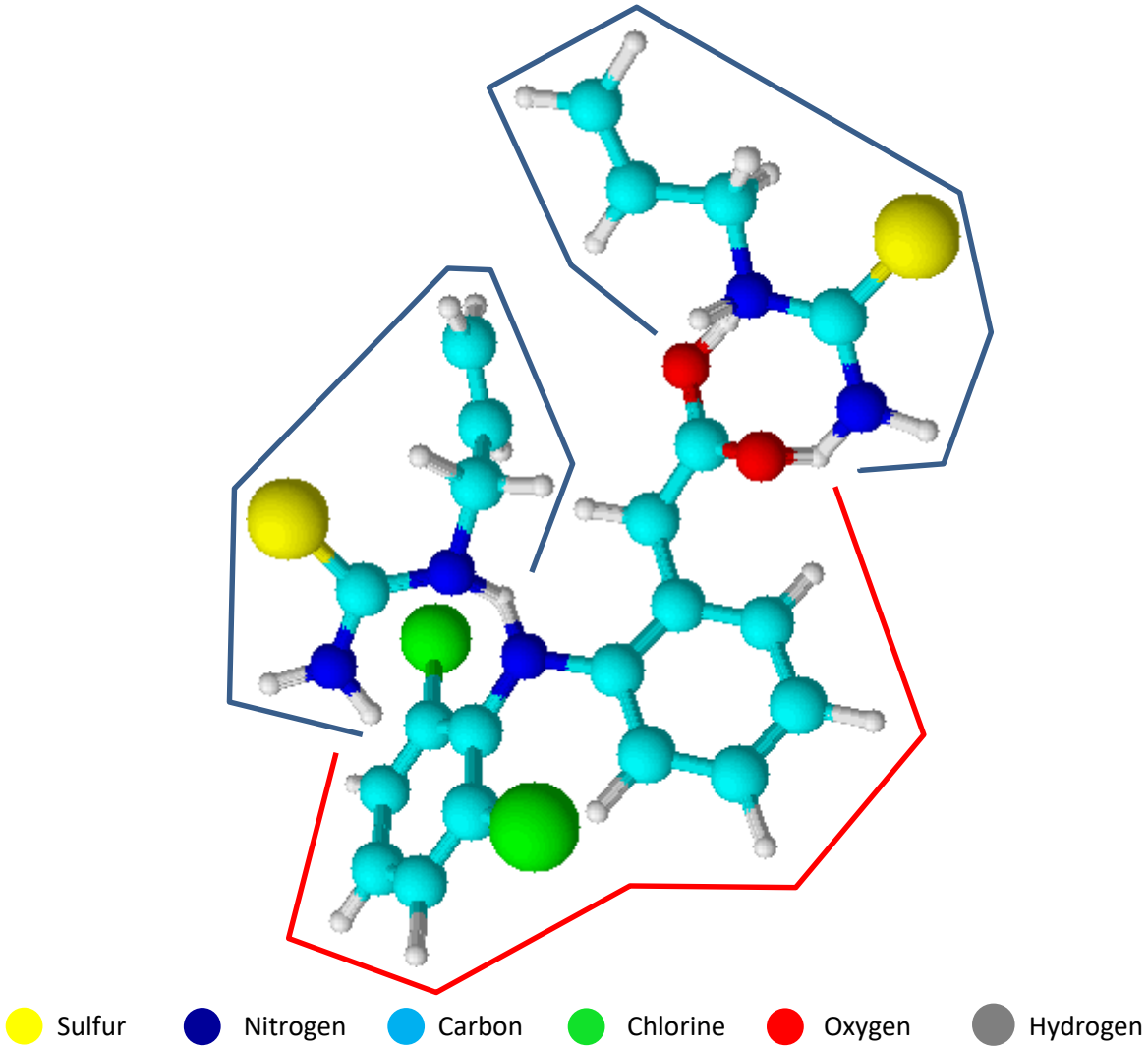


Figure 2.37 Complexes structure formed between DCF (—) and AT (—) with two active functional sites.

From the ^{1}H NMR spectroscopy results, a chemical shift was observed from 6.1 ppm in mixture 1 (low AT concentration) to 6.4 ppm in mixture 3 (high AT concentration) corresponding to the amino moieties of thiourea group of AT molecule bonded to the carboxylic acid at DCF molecule via hydrogen bonding **(Figure 2.38)**.

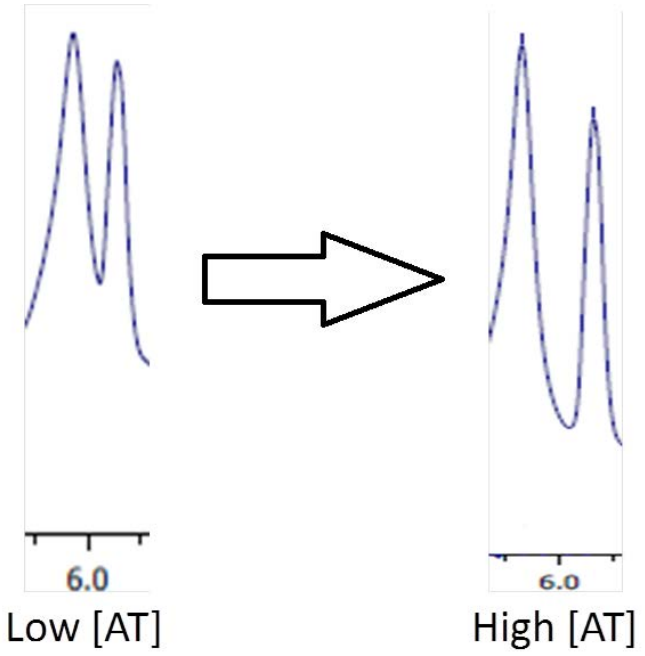


Figure 2.38 Significant peak shows chemical shift of carboxylic acid react with amino acid group on AT from 6.10 ppm (at low AT concentration) to 6.40 ppm (at high AT concentration) of ^{1}H NMR spectra at acetonitrile-d_3 solvent and at 400MHz.

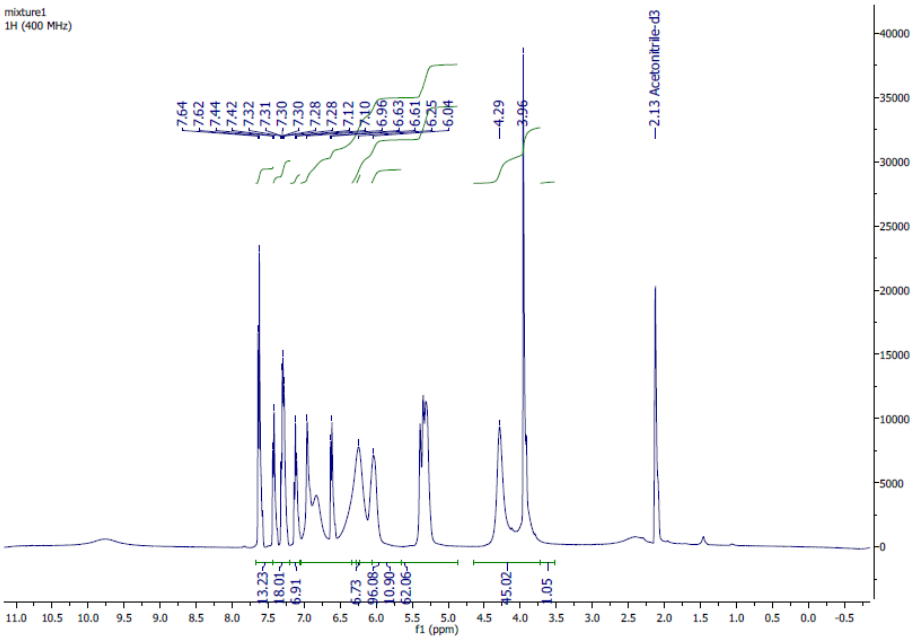
Similar finding was reported by Zhang [34] for the reaction between thiourea and carboxylic acid in open ring compounds. In their work, when the amount of 1,3-bis-(3,5-bis(trifluoromethyl)phenyl) thiourea (TU) (functional monomer) was increased at fixed trifluoroacetic acid (TFA) (template) concentration, the downfield chemical shifted was occurred from 7.83 ppm to 7.94 ppm.[34] The authors mentioned that TU work as anion receptor coordinated with carboxylate by double hydrogen bonding interactions, and was expected to stabilize the anion and lower the pKa of the oxy-acid, thus allowing increase of the electrophilicity of the activated cationic substrate.[34]

However, the results obtained shows that the downfield chemical shifted occurred in the present work was contradicted to the results reported by Sun [10] in which the author claimed an upfield chemical shift, instead of downfield, which occurred from 11.20 ppm to 10.85 ppm.

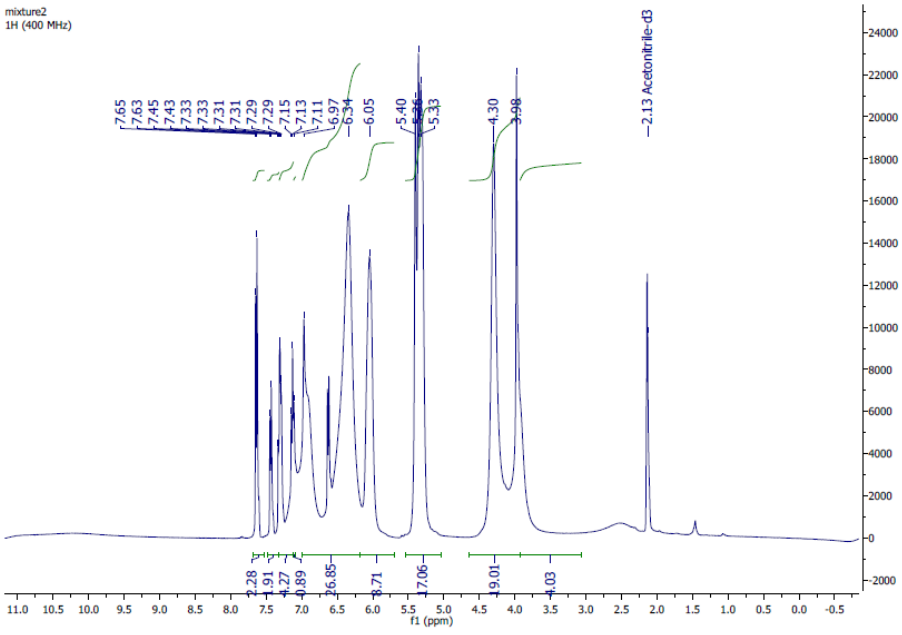
Another downfield chemical shifted observed in our work occurred from 6.60 ppm to 6.90 ppm due to hydrogen bonding between amino moieties of thiourea group of DCF and nitrogen atom at AT. Similar finding was reported by Sun [10] in which downfield chemical shifted was

occurred from 7.70 ppm to 7.82 ppm. All the sepctra for the different mixtures prepared are shown in **Figure 2.39**.

(a) Mixture 1



(b) Mixture 2



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(c) Mixture 3

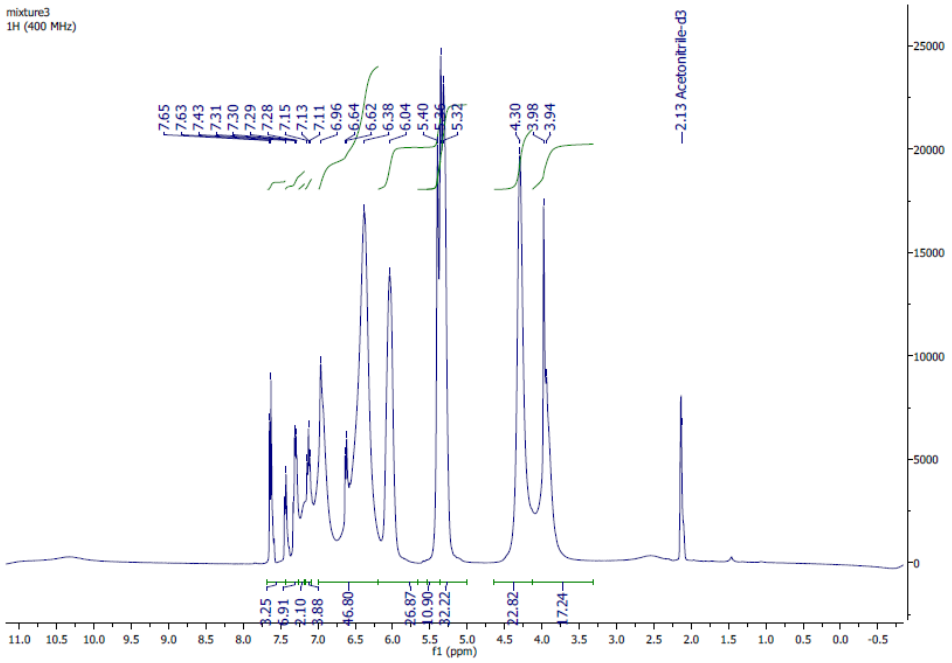
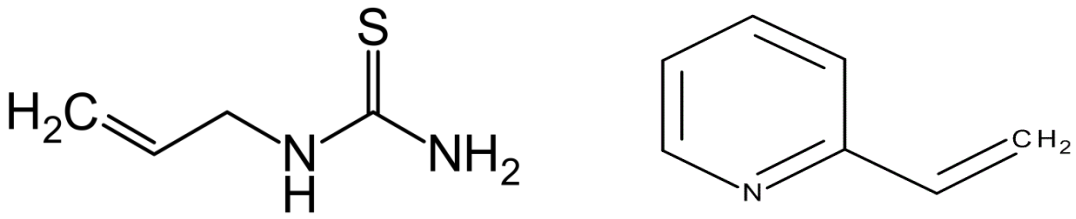


Figure 2.39 [1]H NMR spectra of mixtures consist of (a) 0.1 mol/L AT and 0.05 mol/L DCF (b) 0.3 mol/L AT and 0.05 mol/L DCF (c) 0.5 mol/L AT and 0.05 mol/L DCF in 1 mL of acetonitrile-d_3 at 400MHz.

However, Sun [10] used 2-vynilpyridine (2-VP) as the functional monomer for synthesizing the polymers **(Figure 2.40)**. The clear differences between the two monomers is the only one active functional site on 2-VP molecule whereas there are two active functional sites on AT molecule. Nitrogen atom in 2-VP molecule (pyridine functional site) is less easily protonated compared to the one on the aliphatic amino moieties of thiourea group. Hence, the reaction between amino acid group with carboxylic acid group is favoured with aliphatic amino acid compared to pyridine functional sites. To the best of our knowledge, deshielded occurred during downshield chemical shifted whereas shielded occurred during upfield chemical shifted.[36] Thus the pyridine active functional sites on 2-VP molecule was shielded due to the high steric hindrance and less protonated compared to aliphatic amino acids. Besides, the observation in present work shows that deshielded was occurred and the aliphatic amino acids were easily protonated. Till to date, study on comparison effects of aliphatic amino acids and aromatic amino acids with carboxylic acid is still unknown.



AT 2-VP

Figure 2.40 Molecule structure of monomers, thiourea (AT) and 2-vynilpiridyne (2-VP).

In this study, the mechanism reaction of MIP-DCF during polymerization has been proposed based on the results observed **(Figure 2.41)**. In the proposed mechanism, there are two active functional sites on one AT molecule. Thus, it can be estimate that AT as the functional monomer gives higher affinity sites for DCF as the template compared to 2-VP.

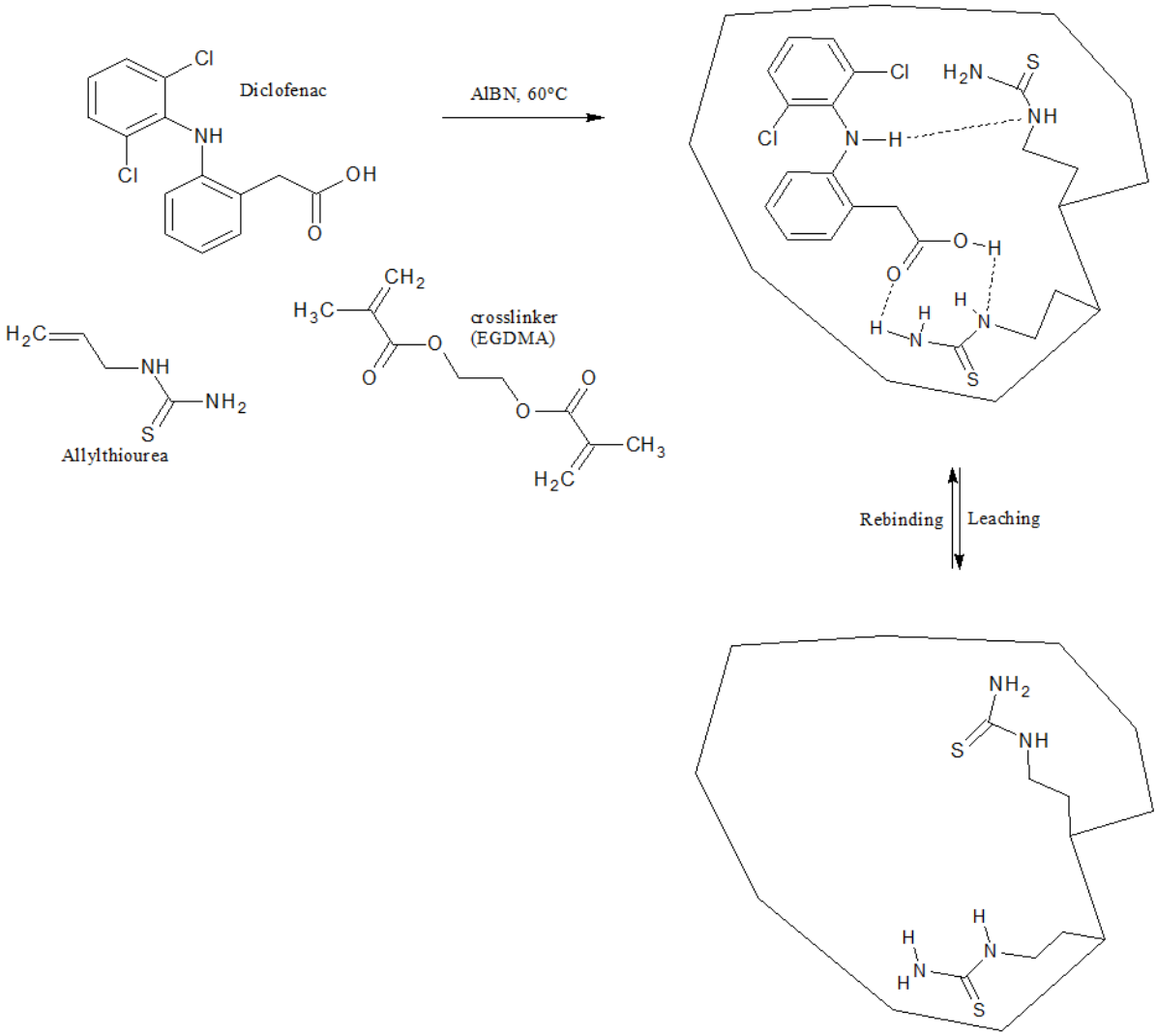


Figure 2.41 Scheme of proposed complexation mechanism of DCF and AT as the functional monomer in MIP-DCF.

2.5 CONCLUSION

The new molecularly imprinted polymer (MIP) with DCF and IDM as the template using allylthiourea (AT) as the monomer via bulk polymerization was successfully synthetised.

- One of the significant findings to emerge from this study is that there was more than 90% of efficiency removal within 3 min for both MIP-DCF and MIP-IDM compared to NIP. The present work is much faster and less laborious due to high affinity and well-templated cavities was formed.

- According to the Lagergren pseudo first-order, both MIP-IDM and MIP-DCF were reached at 0.9 mg/g and 1.0 mg/g of IDM and DCF respectively. Medium used during removal processes has been tested in the present study using acetonitrile (100%), ethanol (100%) and chloroform (100%). However, the aqueous consist of acetonitrile-water (5% in volume ratio) is the best medium for further study. pH of the solution during removal processes also has been tested and the optimum pH value in order to achieved high sorption capacity is at neutral pH (pH 7).

- The selectivity study shows that the synthetised MIP was possessed towards DCF instead of IDM due to the N-H functional group located at the center of DCF instead of IDM compound which favor react to the active functional sites of the MIPIn addition, the size of DCF molecules was smaller than IDM molecules. Hence, DCF was favor to be trapped into the cavities.

- The experimental results obtained in pre-polymerization study was consistent to the suggested scheme reaction of MIP synthesis.

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CHAPTER 3

Characterization of Molecularly Imprinted Solid Phase Extraction using Selective Functional Monomer for Removal and Recovery of Diclofenac and Indomethacin from Aqueous Media

Chapter 3

Characterization of Molecularly Imprinted Solid Phase Extraction using Selective Functional Monomer for Removal and Recovery of Diclofenac and Indomethacin from Aqueous Media

3.1 INTRODUCTION

As decribed in section **2.1**, Molecularly Imprinted Polymers or MIPs are synthetic materials prepared in the presence of a template that serves as a mold for the formation of a template-complementary binding site.[1] During the preparation of MIPs, three-dimension structure cavities are generated after polymerization and after template extraction the cavities formed are complementary in size, shape, and chemical functionality to that of templates.[2] MIPs are obtained by polymerising functional and cross-linking monomers around a template molecule, leading to a highly cross-linked three-dimensional network polymer.[3] MIPs are stable, robust, resistant to a wide range of pH, solvents and temperature, relatively cheap and user-friendly.[4] There are two approaches in preparing the MIPs, non-covalent and covalent interaction.[5] Currently, non-covalent imprinting has become the most popular and general synthesis strategy for MIT.[6]

3.1.1 Molecularly Imprinted Solid Phase Extraction (MISPE)

Molecular imprinting technology is a quite simple and easy method to provide versatile, efficiently and economically receptors for improvement of selectivity in the determination of molecules.[7] Solid phase extraction (SPE) is a very active area in the field of separation science and more than 50 companies currently make products for SPE. However, in the last decade the molecular imprinting technology has been implemented in solid phase extraction, so-called Molecularly Imprinted Solid-Phase Extraction (MISPE), is by far the most advanced technical application of MIPs.[8] The use of MIPs as selective materials allows performing a customized sample treatment step prior to the final determination. The unique of MISPE method is that the MIPs can be washed and re-use instead of using batch mode analysis. The continuous flow mode shows a promising method of analysis instead of batch mode which commonly cannot represent the real application of analysis. This is of special interest when the sample is complex

and the presence of interferences could prevent final quantification by typical chromatographic techniques coupled to common detectors. Due to the inherent selectivity provided by MIPs, past years have seen a growing interest in application and it has been extensively reviewed.[9] MISPE has been commonly used in separation analysis. **Figure 3.1** shows a scheme of a MISPE cartridge. The sample reservoir is made from polypropylene or glass whereas the filter elements are usually made from polypropylene (PP), polyethylene (PE) or teflon (PTFE), and during the analysis it must be ensured that the compounds of interest are not sorbed onto the filter. Commonly, MIPs will be weighted and put in between the filter elements or frits in order to isolate the MIPs by allowing the solution to flow out from the cartridge.

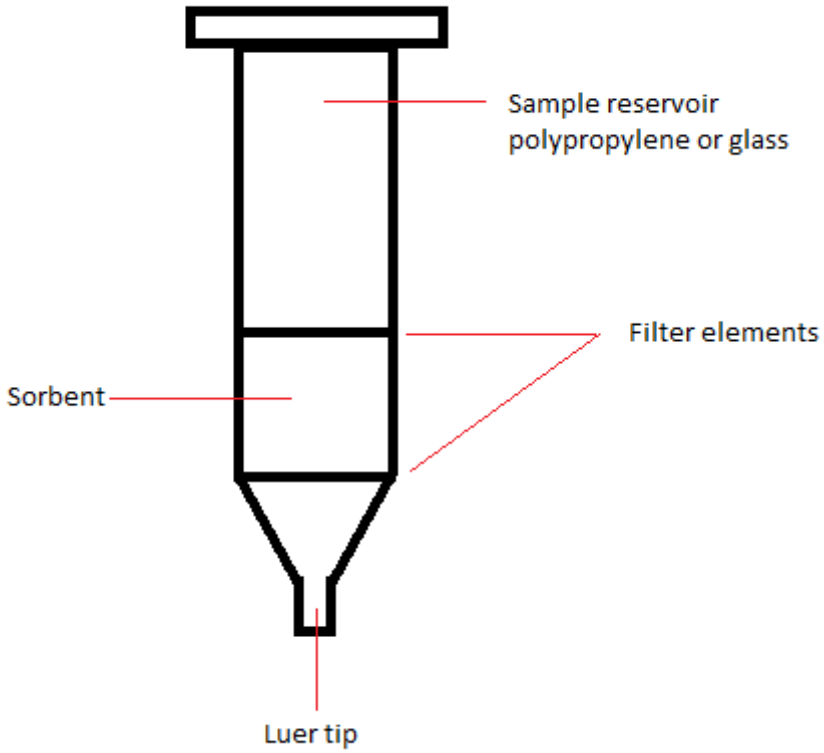


Figure 3.1 Basic cartridge for molecularly imprinted solid phase extraction (MISPE).

The basics of the MISPE operation is as follows. Firstly, the cartridge **(Figure 3.1)** is packed with the MIP and then it is conditioned with a blank solution. Then, the mixture containing the analyte and interferences is loaded into the cartridge and the separation occurred in the sorbent. During the washing process, analyte is favoured to remain in the cartridge depending on the washing solvent used. If the washing solvent is more likely to have the potential to solubilize the interferences, they will be eluted and a good separation will be obtained. Next, the analyte

is eluted using solvent which have the potential to elute all the analyte from the sorbent. The common procedure for separation using MISPE is shown in **Figure 3.2**.

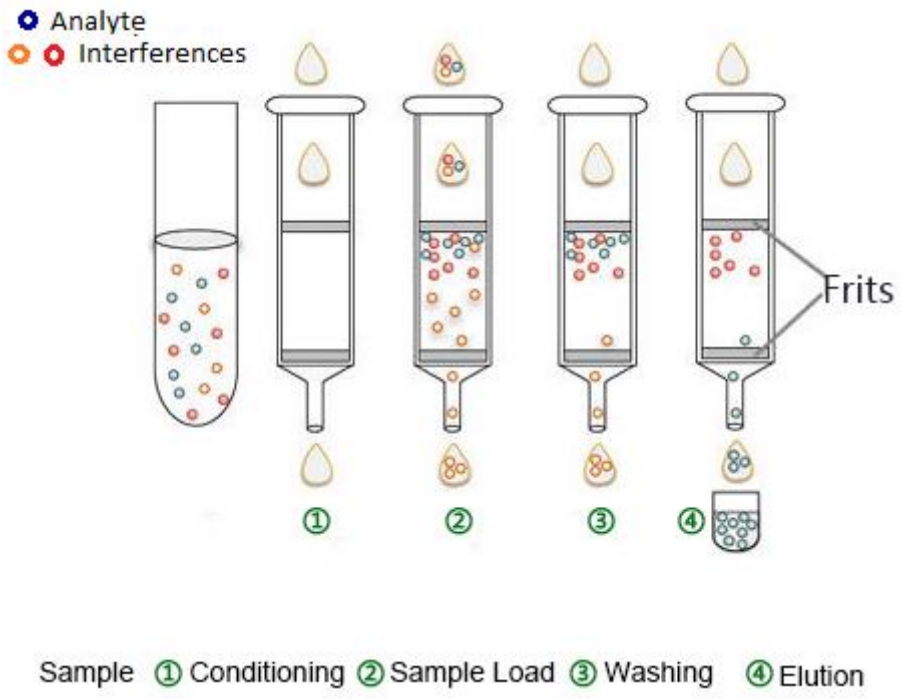


Figure 3.2 Basic MISPE procedure for separation of analytes in a mixture.

Amiri [10] investigated using MIPs as the sorbent and implemented MISPE method in order to study DCF recovery efficiency of MIPs fabricated. In addition, they observed that MIPs was a promising method in order to isolate the analyte from a mixture especially for river water samples, tap water and drinking water. The binding capacities were determined using batch mode.

The poor solubility of non-steroidal anti-imflammatory drugs especially diclofenac, indomethacin and ibuprofen makes this method suitable to be implemented in order to observe the total saturation profile.

3.1.2 MISPE in continuous-flow sorption system model

Nowadays, there are many articles reporting on the use of batch mode in order to study the rebinding properties of sorbent.[11] However, technically this method provides the knowledge in terms of homogeneity of MIPs but not about removal efficiency in real condition of continuous mode. Thus in the present work, a new approaches have been introduced by using the

continuous mode for EPPPs removal from water. Instead of clean-up purpose in order to remove the interferences in a mixture, MISPE experimental procedure can also be implemented to observe the total saturation profile by using known optimum concentration of analyte at certain volume of solution. The proposed method in the present work for the determination of the total saturation profile of analytes is shown in **Figure 3.3**. The controlled-flow of solution is loaded into the cartridge following the steps:

- First, washing and conditioning the cartridge contained with MIP to remove any impurity and to activate the functional sites of MIPs;
- Second, loading the solution containing the desired concentration of analyte;
- Third, the analyte solution is continuously loaded into the cartridge until MIP become saturated;
- Fourth, when the MIP is saturated, the excess of analyte will be loaded off from the cartridge.

Once the sorbent is saturated, the excess of analyte eluted is analysed using spectrophotometer.

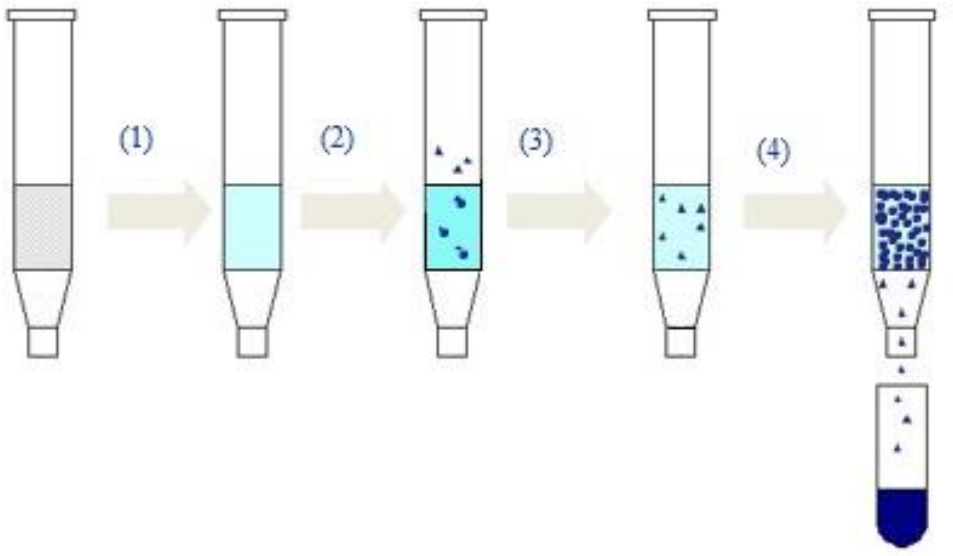


Figure 3.3 Succesive addition method via MISPE procedure (1) washing and conditioning (3) loading the analyte-contained solution (3) loading of analyte continuously and the affinity attraction occurred between MIP and analyte (4) the excess of analyte was loaded off from MIP after the saturation is measured by spectrophotometer.

In order to determine the total saturation profile using batch mode, the problem of poor solubility of analyte in water should be addressed. In the literature, most of the media used in order to determine the total saturation profile is organic solvent such as acetonitrile.[12,13] However, there are also articles reporting the use of the combination of organic solvent and water as the media of analysis e.g. methanol:water (1:1).[14] In the present, work the combination of acetonitrile with ultra high quality water will be implemented.

3.1.3 Adsorption isotherm model

An adsorption isotherm is a measure of the relationship between the equilibrium concentrations either of bound or of free component over a certain concentration range and is readily generated from equilibrium batch rebinding studies or chromatographic frontal-zone analysis.[15]

In general, binding models can be classified into two classes; homogeneous and heterogeneous. Homogeneous binding models assume that there is only one type of binding site with a single set of binding parameters. The second class are heterogeneous binding models that assume that asystem has two or more different types of binding sites in the polymer, each with a unique binding constant.[16]

The purpose of the adsorption isotherms is to relate the adsorbate concentration in the bulk and the adsorbed amount at the interface.[17] Over the years, a wide variety of equilibrium isotherm models (Langmuir, Freundlich, Brunauer–Emmett–Teller, Redlich–Peterson, Dubinin–Radushkevich, Temkin, Toth, Koble–Corrigan, Sips, Khan, Hill, Flory–Huggins and Radke–Prausnitz isotherm), have been formulated in terms of three fundamental approaches.[18]

Although batch laboratory adsorption studies provide useful data and parameters on the application of adsorbents, the data obtained from batch adsorptive system are not applicable to continuous adsorptive system, thus continuous sorption studies are needed.[19] Most research focuses on the batch adsorption model [20,21] so there is a lack of research on the study of dynamic column methods. There are a few models could be used for the continuous sorption studies such as Thomas model and Scatchard plot analysis. In the present work, Thomas model and Scatchard plot analysis will be used for the continuous sorption studies.

3.1.3.1 Thomas model

The Thomas model is another one frequently applied to estimate the adsorptive capacity of adsorbent and predict breakthrough curves. Theoretically, it is suitable to estimate the adsorption process where external and internal diffusion resistances are extremely small. The Thomas model is given by **(Equation 3.1)**:

$$ln\left(\frac{C_F}{C}-1\right)=\frac{k_{Th}q_F m}{Q}-k_{Th}C_F t \quad \text{..... Equation 3.1}$$

where k_{Th} is the Thomas rate constant, m is the mass of adsorbent in the column. With several couples of m and Q, k_{Th} and q_F values derived through a plot of $\ln[(C_F/C)-1]$ vs. t, further prediction and design is then available. **Equation 3.2** can also be expressed as

$$ln\left(\frac{C_F}{C}-1\right)=k'(t-t_1) \quad \text{..... Equation 3.2}$$

where $k'=k_{Th}C_F$ and $t_1= q_F m/(QC_F)$. The general version of **Equation 3.3** is represented as

$$ln\left(\frac{C_F}{C}-1\right)=b_o+b_1 t+b_2 t^2+\cdots=\sum_i b_i t^i \quad \text{..... Equation 3.3}$$

This equation is applied when $\ln[(C_F/C)-1]$ versus t is not in linear form. By fitting the experimental data, the corresponding parameters b_i can be calculated. Generally, it is adequately accurate to employ the former three terms. It is worth noting that q_F derived from the experiment is often conspicuously different from the value acquired by equilibrium calculation, and the bed adsorptive capacity is often determined from the dynamic adsorption.[22]

3.1.3.2 Scatchard plot analysis

The most common has been the bi-Langmuir isotherm, which has been applied using the limiting slopes analysis of curved Scatchard plots.[15] Scatchard analysis is a common model to evaluate the binding behavior of MIPs in batch rebinding experiments. Typically non-covalent binding between template and functional monomer gives a Scatchard plot with two straight

lines indicating heterogenous affinities of the binding sites in the polymer that are calculated with two association constants corresponding to the high and low-affinity binding sites.[23]

There are many articles reporting on the implementation of Scatchard plot in analysing data for saturation profile graph and MIPs are well-known to work as heterogenous sorbent.[7,10,12,13,14,24,25] The formula of Scatchard plot is showed below **(Equation 3.4)**.

$$\frac{[Bound]}{[Free]} = -\frac{[Bound]}{K_D} + \frac{B_{max}}{K_D} \quad \text{.....} \quad \textbf{Equation 3.4}$$

Where B_{max} is the maximum binding sites and K_D is the dissociation constant; [Bound], is the amount of analyte bound to polymer at equilibrium; [Free] is the analyte concentration at equilibrium.[24] The data is analysed plotting [Bound]/[Free] versus [Bound].

3.1.4 Recovery and regeneration

Since the ability of MIPs to trap and/or release the analyte depends on the elution solvent used, recovery experiments were conducted using common MISPE technique. The recovery experiment used different solvents such as ethanol or chloroform to eluate the analyte from MIP. Hence, a suitable solvent need to be chosen. Regeneration experiments were also performed in similar way to recovery experiments in order to evaluate the ability to sorbed/desorbed the analyte more than one cycle using common MISPE technique. Equation involved in recovery experimental is shown in **Equation 3.5**:

$$Percentage\ recovery = \frac{[Analyte]_f}{[Analyte]_i} \times 100\% \quad \text{.....} \quad \textbf{Equation 3.5}$$

3.2 OBJECTIVES

The objectives of the study are:

i. To investigate the homogeneity and sorption isotherm of MIP with indomethacin as a template (MIP-IDM) and MIP with diclofenac as a template (MIP-DCF) using successive total sorption study via pre-packed cartridge analysis.

ii. To characterize the functional groups of MIP with diclofenac as a template (MIP-DCF); MIP with diclofenac as a template (MIP-DCF) loaded with diclofenac; and MIP with diclofenac as a template (MIP-DCF) after 10^{th} regeneration using Fourier Transform Infrared.

iii. To characterize the surface morphology of MIP with indomethacin as a template (MIP-IDM) and MIP with diclofenac as a template (MIP-DCF).

3.3 EXPERIMENTAL

3.3.1 Reagents and equipments

Reagents:

- Indomethacin (IDM) was from Sigma-Aldrich, Spain, with 98-99% of purity. IDM was TLC grade.
- Diclofenac-Na was from Cayman Chemical, United States with purity of more than 99%.
- Ethanol with 96% purity from Scharlau Chemical, Spain.
- Acetic acid was from J. T.Baker, United States with 96% and 99.9% of purity respectively and HPLC grade.
- Acetonitrile (99% of purity) from VWR company with HPLC grade.

Equipments/Instruments:

- Grace Alltech 'Extract Clean' empty reservoirs with silica frits, 1.5 mL from Fisher Scientific, United States.
- UV double-beam spectrophotometer from UNICAM, model UV-2 200.
- Fourier Transform Infrared-Attenuated Total Reflectance (FTIR-ATR) from Bruker, Spain. The instrument was self-serviced at Servei Analitica Quimica, Universitat Autonoma de Barcelona, Spain.
- Field Emission Scanning Electron Microscopy (FESEM), model Zeiss Merlin from the Servei de Microscòpia, Universitat Autònoma de Barcelona, Spain.

3.3.2 Sorption study by successive addition method

Due to the immiscibility properties of IDM and DCF in water, total sorption capacity has been carried out in acetonitrile: water (5% v/v) media solution. The experiment was developed similar to **Figure 3.3**. 10 mg of MIP-IDM was accurately weighted and placed in a cartridge of 1.5 mL of capacity. 15 µg/mL of IDM was prepared in 2 L of acetonitrile: water (5% v/v). Then, the solution was loaded into the cartridge using the peristaltic pump at 1.67 mL/min of flow rate. The solution was collected in fractions of 5 mL until 50 mL, then fractions of 50 mL. The solution was continuously-flowing until the absorbance readings of the loaded solution reached the plateau (absorbance readings near to the absorbance of the initial concentration, meaning that no more absorption was occurring). Then, the process stopped and all solutions were collected and analysed via UV spectrophotometer. The procedure mentioned above was duplicated. The similar procedure was repeated for MIP-DCF as the sorbent. As blank, the procedure above was repeated in the absence of MIP using the same concentration in order to measure any other contribution to the elimination of the target compounds e.g. sorption in cartridge or frits. The obtained breakthrough curves were then modeled and the values of the main constants of the best fitting model determined.

3.3.3 Statistical analysis

The F-test was used to assess the significant difference in reproducibility of standard deviation between MIP-IDM for replicate 1 and replicate 2. Results with probability < 0.05 were considered to be statistically no significant different.

3.3.4 Recovery Study

10 mg of MIP-DCF was weighted and placed into a 1.5 mL cartridge. Then, 1 mL of ethanol:water (75% v/v) was loaded twice into the reservoir to activate the MIP-DCF. Next, 1 mL of water was loaded for 5 times to rinse out the ethanol residue. Then, acetonitrile:water (5% v/v) was loaded for 2 times to condition the MIP-DCF respectively. Then, 1 mL of 15 µg/mL of DCF was added twice. The solution was collected and analysed using UV spectrophotometer. Finally, 1 mL of ethanol:water (75% v/v) was loaded twice into the cartridge and the collected solution was analysed via UV spectrophotometer. The procedure was prepared in triplicates.

3.3.5 Regeneration Study

10 mg of MIP-DCF was weighted in 1.5 mL cartridge. Then, 1 mL of ethanol: water (75% v/v) was loaded twice into the reservoir to activate the MIP-DCF. Next, 1 mL of water was loaded for 5 times to rinse out the ethanol residue. Then, 1 mL of acetonitrile: water (5% v/v) was loaded twice in order to condition the MIP-DCF. Later, 1 mL of 15 µg/mL of DCF was loaded twice into the reservoir. The solution was collected and analysed using UV spectrophotometer. After that, 1 mL of ethanol:water (75% v/v) was loaded twice into the reservoir and the solution that passing through the cartridge was collected and analysed via UV spectrophotometer. The procedure above was repeated from the first step 10 times, performing so 10 cycles. The procedure was prepared in triplicates.

3.3.6 Infrared-Attenuated Total Reflectance Analysis (FTIR-ATR)

Fourier Transform Infrared – Attenuated Total Reflectance (FTIR-ATR) has been used in this study to determine the functional groups in MIP-DCF for 3 different kinds of MIP-DCF:

- original MIP-DCF.
- MIP-DCF after loading with 2 mL of DCF solution (5 µg/mL) via molecularly imprinted solid phase extraction.
- MIP-DCF after using 10 cycles.

MIP-DCF was ensure to be dried prior to be tested since were in powder form. MIP-DCF was placed on the ATR The procedure for handling FTIR-ATR was fulfilled and the results were obtained.

3.3.7 Surface morphology analysis

The MIPs were analysed by using Field Emission Scanning Electron Microscope (FESEM). The MIP particles were distributed on the carbon disc attach on the pin stubs. Then, the pin stubs were located according to the position number on the multi stubs holder. Next, the holder was located into the chamber and then analysed. The parameters were sets as follows: EHT: 1kV, Mag: 34.63KX, WD: 4.1 mm at scale 1µm. Other parameters also used were EHT: 1 kV, Mag: 16.20 KX, WD: 3.3 mm at scale 2 µm.

3.4 RESULTS AND DISCUSSION

3.4.1 Successive sorption study

By assuming the solution was homogenous, the total sorption was determined from plotting bound (mg of analyte sorbed on the MIP/g MIP) versus mg of initial analyte loaded. The graph was plotted according to the calculation made as shown in **Equation 3.6**.

$$Bound\left(\frac{mg\ DCF}{g\ MIP}\right) = \frac{[DCF]_{initial} - [DCF]_{final} - [DCF]_{blank}}{g\ MIP} \quad \text{.....} \quad \textbf{Equation 3.6}$$

where $[DCF]_{initial}$ was the initial concentration of DCF in 5% of acetonitrile and water. $[DCF]_{final}$ was obtained from the calibration curve for each final solution at 50 mL in range of solution loaded. $[DCF]_{blank}$ was obtained as the blank analysis without MIP as the sorbent to observe the retention by frits or any other factors that may contribute to the removal by non-MIPs. All the concentration in µg/mL. According to the study by Yang [26] with MIP prepared with acrylamide (AM) as the functional monomer, the observed IDM retained by MIP was 1.75 mg IDM per gram of MIP by using acetonitrile as the media of extraction.[12] In the present work the total sorption reached 700 mg IDM/g MIP or 0.02 mmol IDM sorbed in every 10 mg of MIP-IDM **(Figure 3.4 – Figure 3.5)**. In addition, the experiment also has been carried out in 1 h agitation instead of 10 h incubation as reported by Yang.[26]

Another factor that may influence the total saturation capacity is the media which content the analytes. In present work, the media used was a combination of organic solvent in water. However, in the study by Yang [26] the author used 100% of organic solvent. According to Meng Dai [25] the author observed approximately 300 mg DCF/ g MIP-DCF by using methanol:water in ratio of 1:1 as media using a MIP-DCF synthetized via precipitation using 2-VP as the functional monomer. The MIP synthetized in our work presents higher capacity in a shorter time analysis, being a non-laborious procedure.

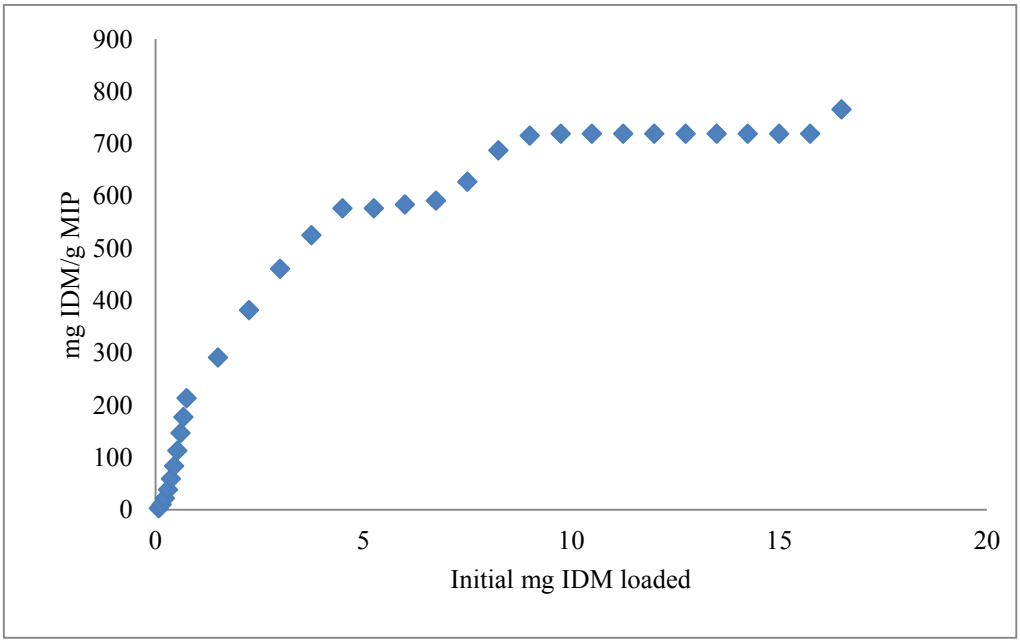


Figure 3.4 Total saturation profile of IDM. Initial concentration: 15 µg/mL IDM. Media: 5% acetonitrile: water.

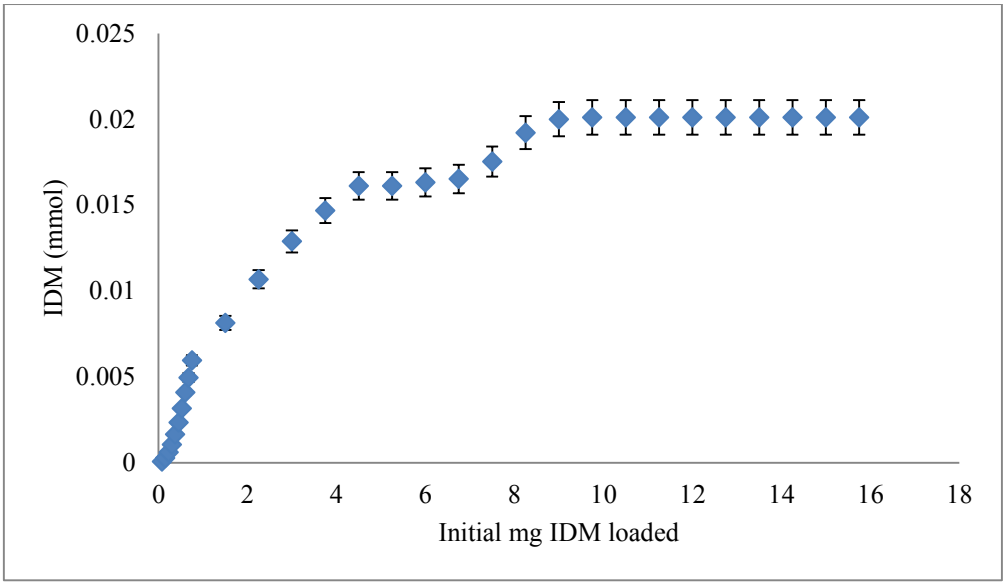


Figure 3.5 mmol of IDM sorbed into MIP-IDM. Initial concentration: 15 µg/mL IDM. Media: 5% acetonitrile: water.

In order to study the reproducibility of the sorption on MIP-IDM, F-test statistical method has been implemented in this study **(Table 3.1)**. F-test was used to compare the significant differences in standard deviation between 2 groups of replicate with 95% of confidence level or alpha value (α) equals to 0.05. Formula involved is as follows **(Equation 3.7)**:

$$F\ calculated = \frac{variance\ 1}{variance\ 2} \quad \text{.....} \quad \textbf{Equation 3.7}$$

Table 3.1 F-Test Two-Sample for Variances for reproducibility of total saturation profile for MIP-IDM as the sorbent.

	Replicate 1	*Replicate 2*
Mean	740.9	635.6
Variance	488083.21	349808.83
Observations	15	15
df	14	14
F	1.3953	
P(F<=f) one-tail	0.2707	
F Critical one-tail	2.4837	

F calculated (1.3953) < F table (2.4837); do not reject H_o, H_o is true. Hence, there is no significant differences between two groups of data that represent replicate 1 and replicate 2. Thus, the method is reproducible at 95% of confidence level.

For the removal by MIPs with DCF as the template, the total saturation obtained was 0.006 mmol DCF equivalent to 200 mg DCF/g MIP **(Figure 3.6 – Figure 3.7)**.

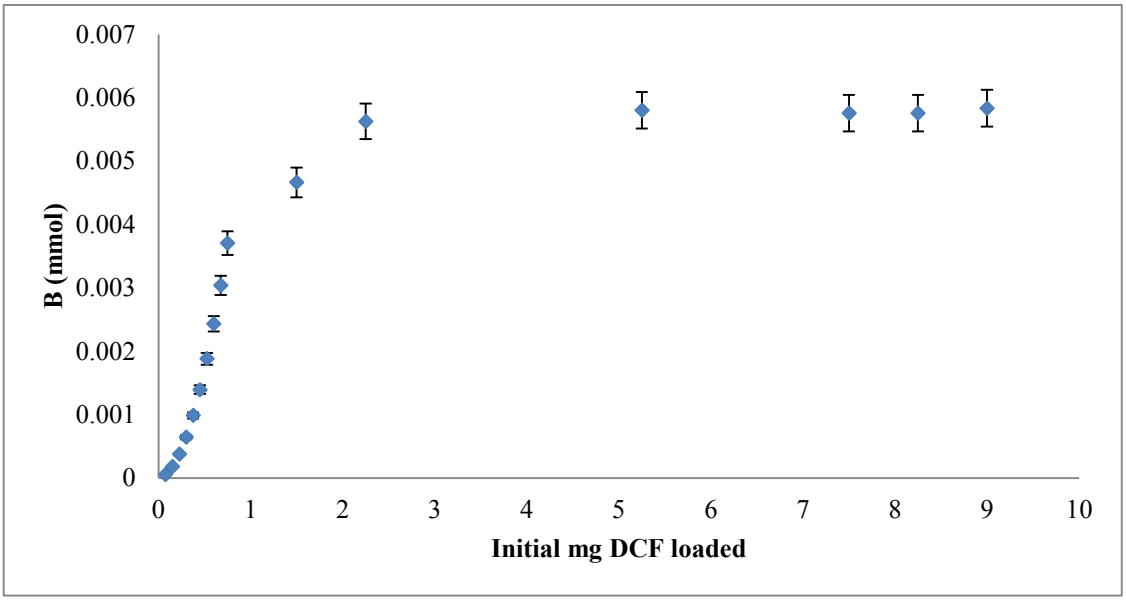


Figure 3.6 mmol DCF sorbed into MIP-DCF. Initial concentration: 15 µg/mL DCF. Media: 5% acetonitrile:water.

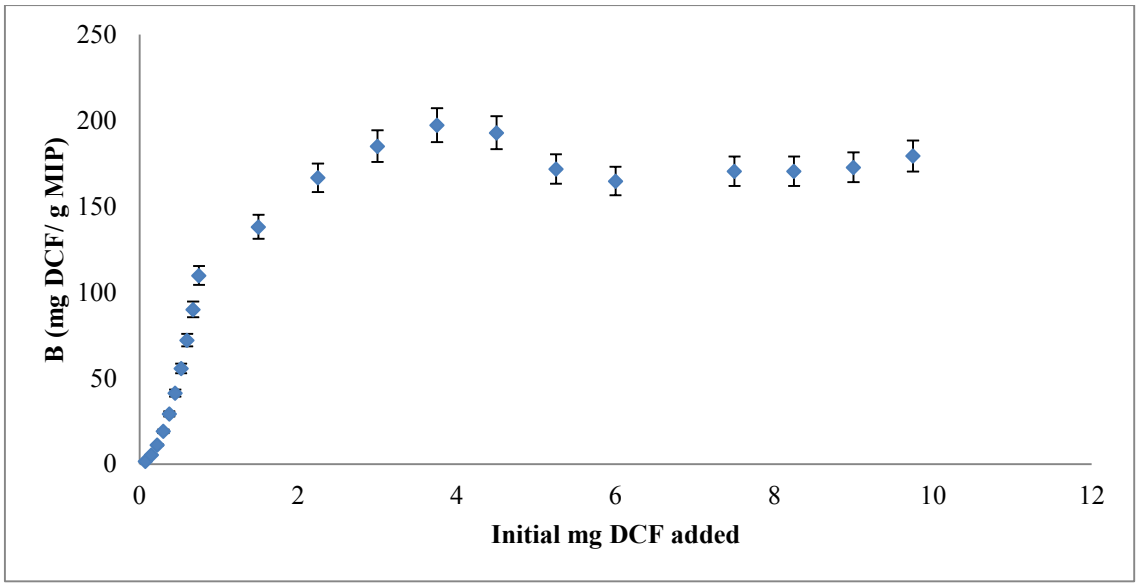


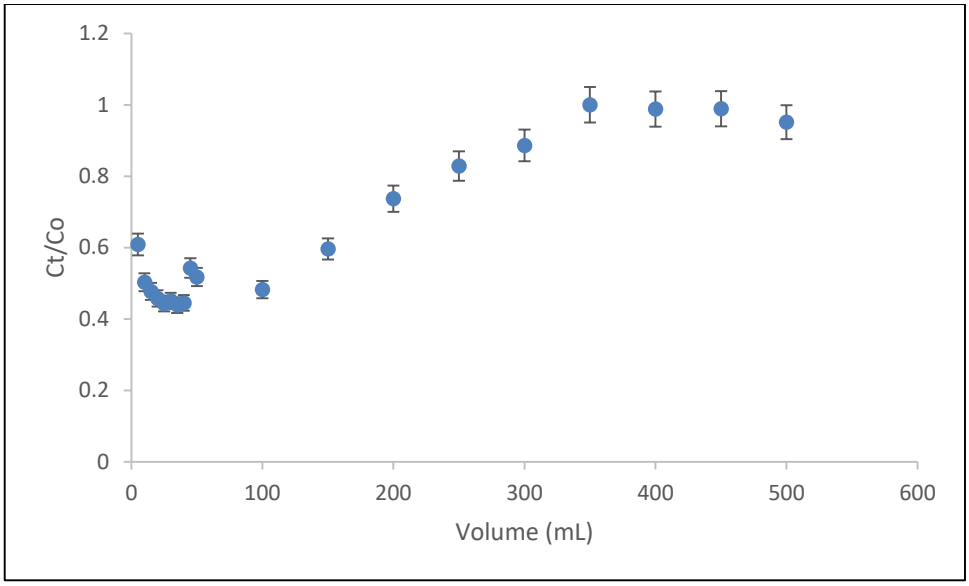
Figure 3.7 Total saturation profile of DCF. Initial concentration: 15 µg/mL DCF. Media: 5% acetonitrile:water.

As a summary, the total saturation profile was successfully developed via modified MISPE for continuous-flow sorption analysis. The sorption isotherm model also has been implemented and will be explained further.

3.4.2 Sorption isotherm models

Breakthrough in column can be identified as the amount of influent solution passing through the bed before a maximum effluent concentration is reached. The ratio C_t (effluent concentration)/ C_o (influent concentration) was plotted against the volume of the analyte solution passed and the time taken for passing the analyte solution through the cartridge. **(Figure 3.8 – Figure 3.9)**

(a)



(b)

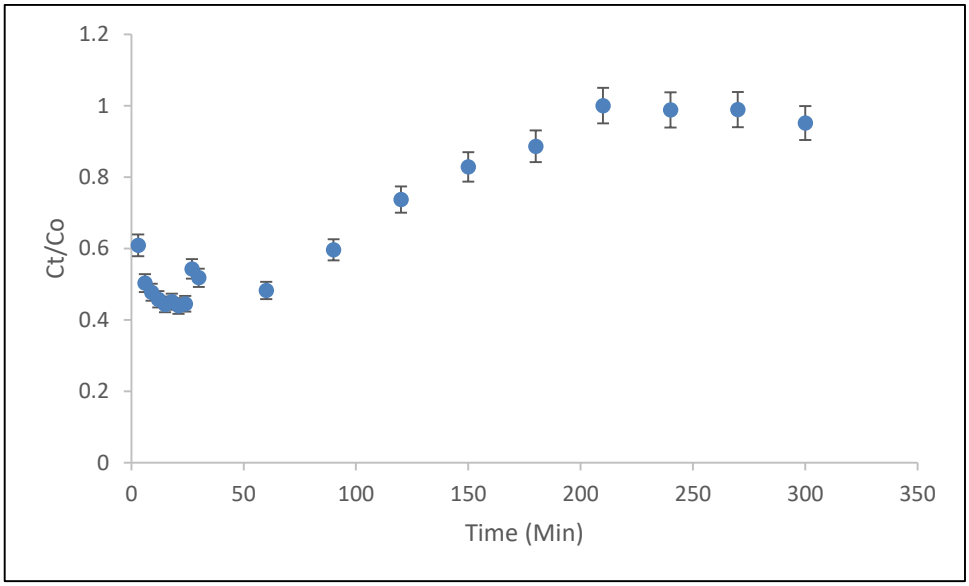
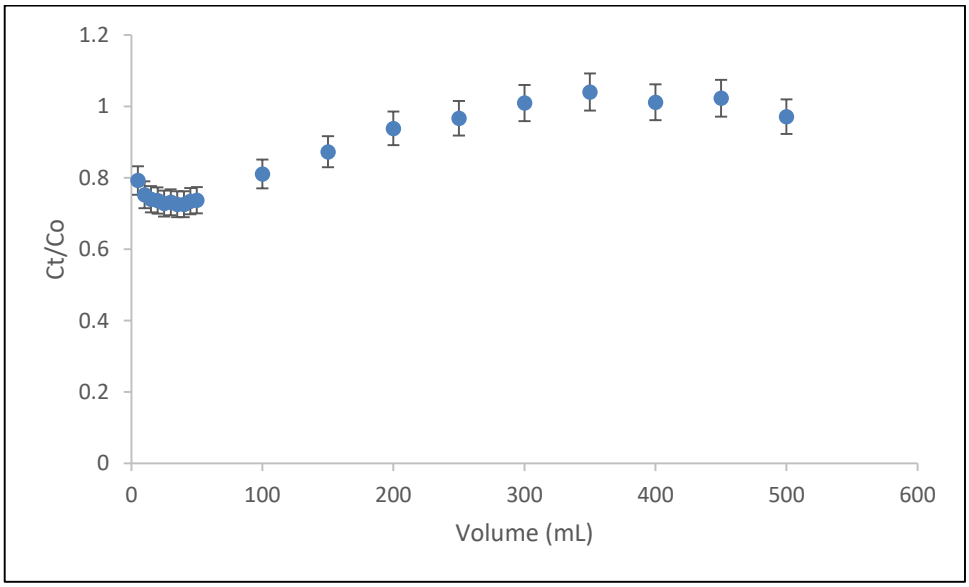


Figure 3.8 Breakthrough curves from the experimental in the packed cartridge using MIP-IDM as a sorbent of the Thomas model (a) C_t/C_o ratio versus volume (b) C_t/C_o ratio versus time.

Taking into consideration the results shown in **Figure 3.8,** the breakthrough volume and the time to sorption of IDM using MIP-IDM is similar to the kinetic study observation in which within 3 min the sorption uptake was more than 50%, thus resulting in relatively faster attainment of breakthrough. In this work, breakthrough and exhaustion are defined as outstanding when the ratios of effluent-to-influent concentration are between 45% and 99%, respectively the breakthrough point ($C_t/C_o \leq 45\%$) for IDM was 40 mL (24 min), as well as

the exhaustion point ($C_t/C_o \leq 99\%$) for IDM and DCF were 350 mL (210 min). The ratio of effluent-to-influent concentration are between 45% and 99%.

(a)



(b)

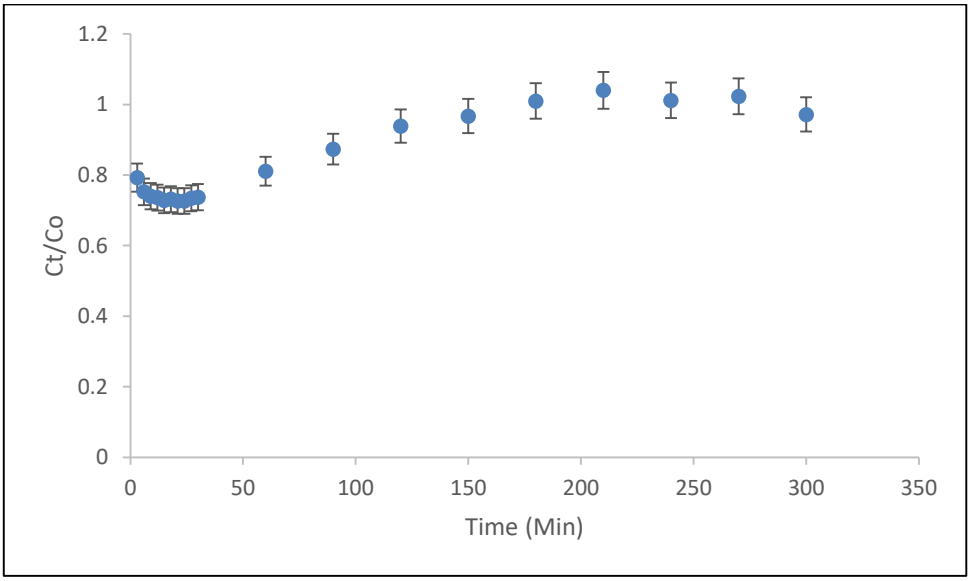


Figure 3.9 Breakthrough curves from the experimental in the packed cartridge using MIP-DCF as a sorbent of the Thomas model (a) C_t/C_o ratio versus volume (b) C_t/C_o ratio versus time.

Taking into consideration the results shown in **Figure 3.9** the breakthrough volume and the time to sorption of DCF using MIP-DCF is similar to the kinetic study observation in which within 3 min the sorption uptake was more than 80%, thus resulting in relatively faster attainment of breakthrough. In this work, breakthrough and exhaustion are defined as outstanding when the ratios of effluent-to-influent concentration are between 70% and 99%,

respectively the breakthrough point ($C_t/C_o \leq 70\%$) for DCF was 35 mL (24 min) for DCF, as well as the exhaustion point ($C_t/C_o \leq 99\%$) for DCF were 270 mL (450 min). The methodology of adsorption in fixed bed cartridge by the breakthrough curves method proved to be a good option to study the separation of NSAIDs in water.

Scatchard plot analysis for MIP-IDM as a sorbent has been carried out in this work. The binding sites of the MIPs can be estimated via Scatchard plot. Based on the theoretical equation **(Equation 3.6)**, the scatchard plot for MIP-IDM was not a single linear curve but consists of two line with different slopes. The linear regression equation for the left part of the curve as shown in **Figure 3.10** is $y = -16.18x + 0.3234$ ($R^2 = 0.97$), the unit for Q was mmol. The K_D and Q_{max} were calculated to be 0.062 mmol/L and 0.020 mmol/g of dry polymer, respectively, from the slope and the intercept of the Scatchard plot. The linear regression equation for the right part of this curve is $y = -6.4359x + 0.1601$. The K_D and Q_{max} were calculated to be 0.155 mmol/L and 0.025 mol/g of dry polymer. The result was coherent with the total saturation profile in which the plateau reached at 0.020 mmol IDM sorbed. Moreover, the fact that two lines are observed shows that MIP-IDM is a heterogeneous sorbent. This also has been agreed by Sun [13] in which the authors observed that the binding site configuration in the MIPs is heterogeneous in respect to the affinity for DCF and indicates that the binding sites could be classified into two distinct groups with different specific binding properties.

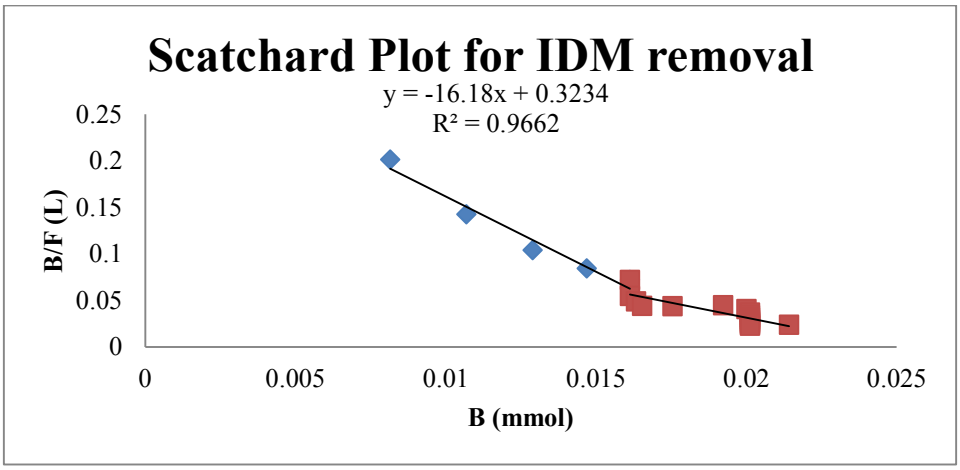


Figure 3.10 Scatchard plot analysis of the binding of IDM to the imprinted polymer. B is the amount of IDM bound (mmol) to MIP-IDM; F is the concentration of free IDM at equilibrium (mol/L).

The Scatchard plot for MIP-DCF shown in **Figure 3.11** below is straight line, indicating that the affinities of the binding sites in MIP-DCF is homogeneous among the concentrations tested. The linear regression equation in the **Figure 3.11** below is $y = -16.587x + 0.1346$ (R^2 = 0.95), the unit for B was mmol. The K_D and Q_{max} were calculated to be 0.060 mmol/L and 0.008 mmol/g of dry polymer, respectively, from the slope and the intercept of the Scatchard plot. Similar Scatchard plots were obtained with the case of Meng Dai [25] in which the authors observed one linear line in scatchard plot analysis in order to study the removal of DCF using MIP synthetised via precipitation polymerization with 2-VP as the monomer.

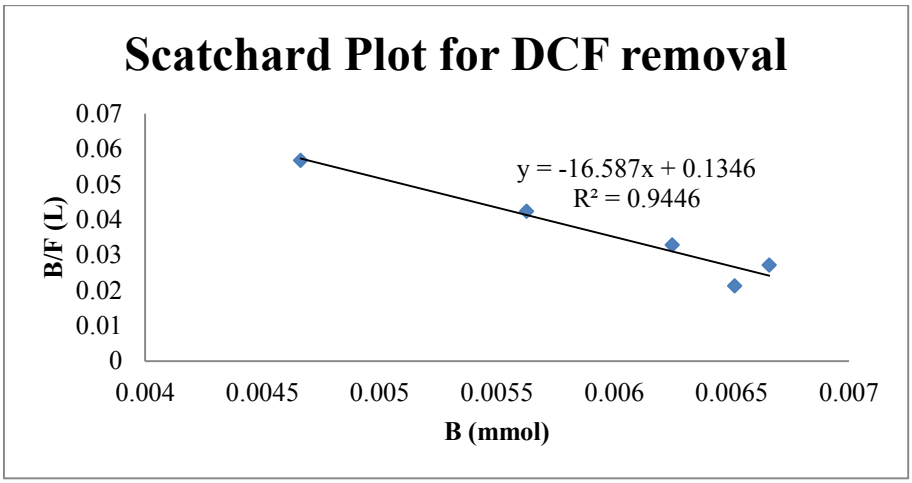


Figure 3.11 Scatchard plot analysis of the binding of DCF to the imprinted polymer. B is the amount of DCF bound (mmol) to MIP-DCF; F is the concentration of free DCF at equilibrium (mmol/L).

3.4.3 Recovery and regeneration

The recovery experimental has been conducted using molecularly imprinted solid phase extraction (MISPE) with 10 mg of MIP loaded into the cartridge in between frits as filters.The triplicate data were shown in **Table 3.2** below.

Table 3.2 % recovery of MIPs in different solution according to the template with standard deviation respectively

MIPs	**% Recovery (% ± S. D.)**
MIP-IDM	98% ± 3%
MIP-DCF	97% ± 2%

In regeneration experimental, the recovery procedure was carried out and has been repeated for 10th times in order to observed the capacity of loading by MIPs after consecutive extractions. By using MIP-IDM, the percent recovery was in average 98% until reached 10th times **(Figure 3.12)**. The ability to sorbed-desorbed by MIPs synthetized by using present method was very high and reached approximately 100% of recovery. From the result obtained, it was confirmed that the synthetised MIPs can be used as the packing media in MISPE procedure. The more number of regeneration cycle, the more efficient the MIPs to be functioned. The similar finding was obtained by Meng Dai.[21]

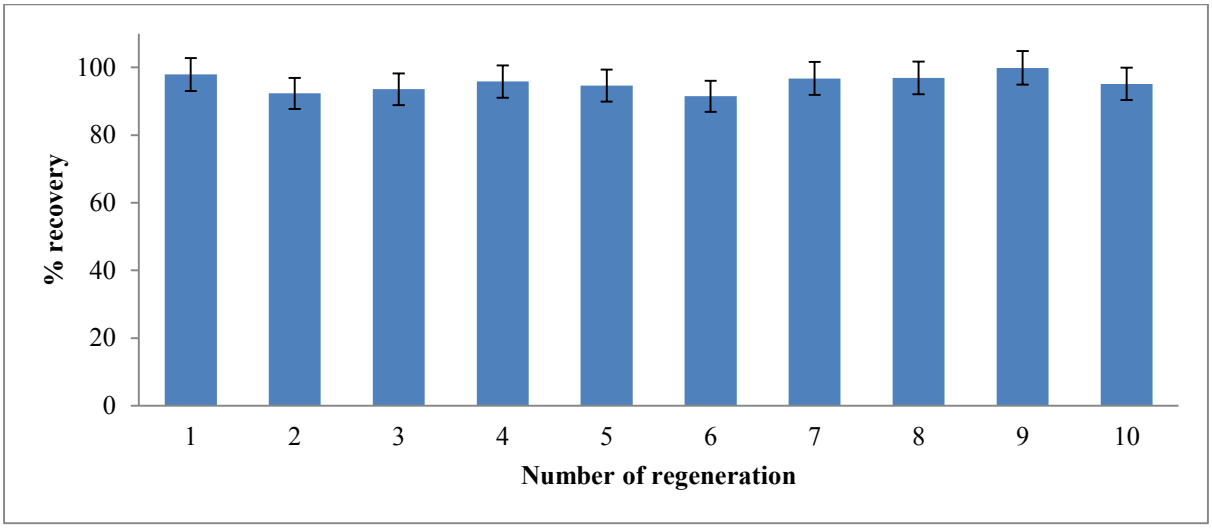


Figure 3.12 Regeneration of MIP-IDM until 10th cycle of % recovery of IDM using MIP-IDM as the sorbent via pre-packed cartridge with initial concentration at 15 µg/mL IDM, 10 mg MIP-IDM.

For the recovery by MIP-DCF, the sorption capacity was in range 9.36 µmol/g to 11.77 µmol/g for each 10 mg of MIP-DCF and this observation was in range and similar to the total sorption by batch mode which has been shown in previous chapter (2.8380 mg/g equivalent to 9.49 µmol/g with DCF initial concentration equals to 15 µg/mL). This occurred probably due to the high affinity in templates and the sorption capacity was not declined until 10th cycle of regeneration.

3.4.4 Infrared Attenuated Total Reflectance analysis

Figure 3.13 below shows the spectrum from the original MIP-DCF. As it can be observed, there is a peak at 3100 nm^{-1} showing the N-H stretch functional group that came from the active functional sites of monomer (AT) and at 1180 nm^{-1} determined as C-N stretch functional group caming also from the allylthiourea functional sites of the monomer.

In **Figure 3.14**, the spectrum of MIP-DCF after loading with DCF solution showed a broad peak at 3300 nm^{-1} corresponding to the hydroxyl (-OH) functional group coming from the DCF molecule which has been trapped into the template. There was also N-H stretch functional group at 3000 nm^{-1} which has been overlapped by –OH functional group broad peak.

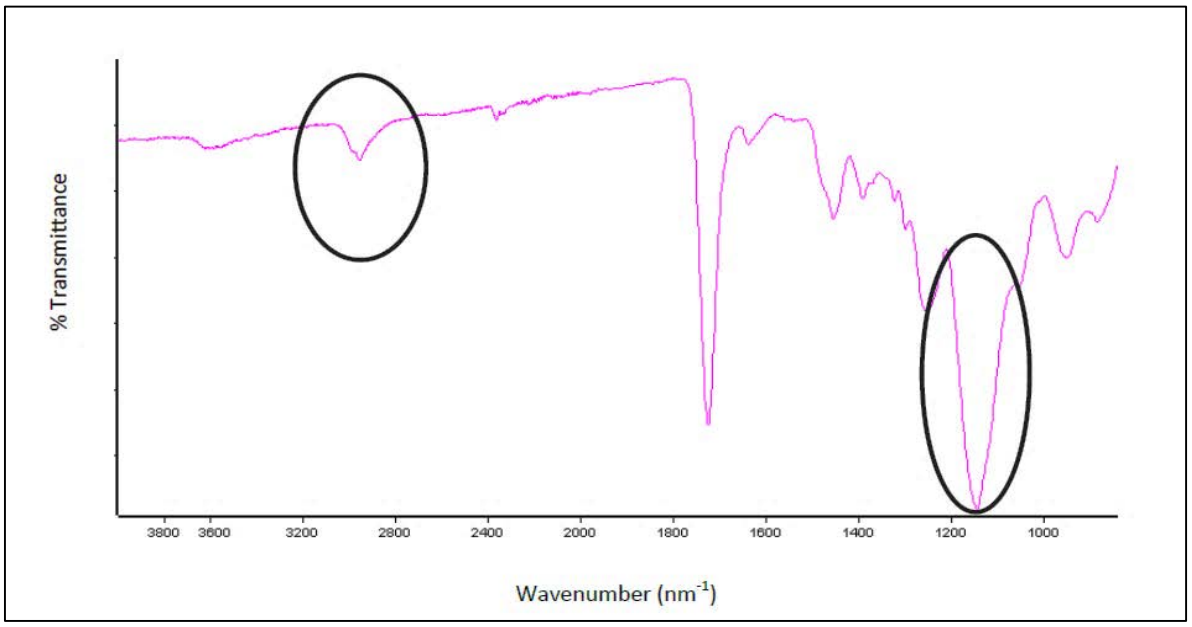


Figure 3.13 Infrared spectrum of original MIP-DCF, N-H stretch functional group was observed at 3100 nm^{-1}, C-N stretch functional group was determined at 1180 nm^{-1}

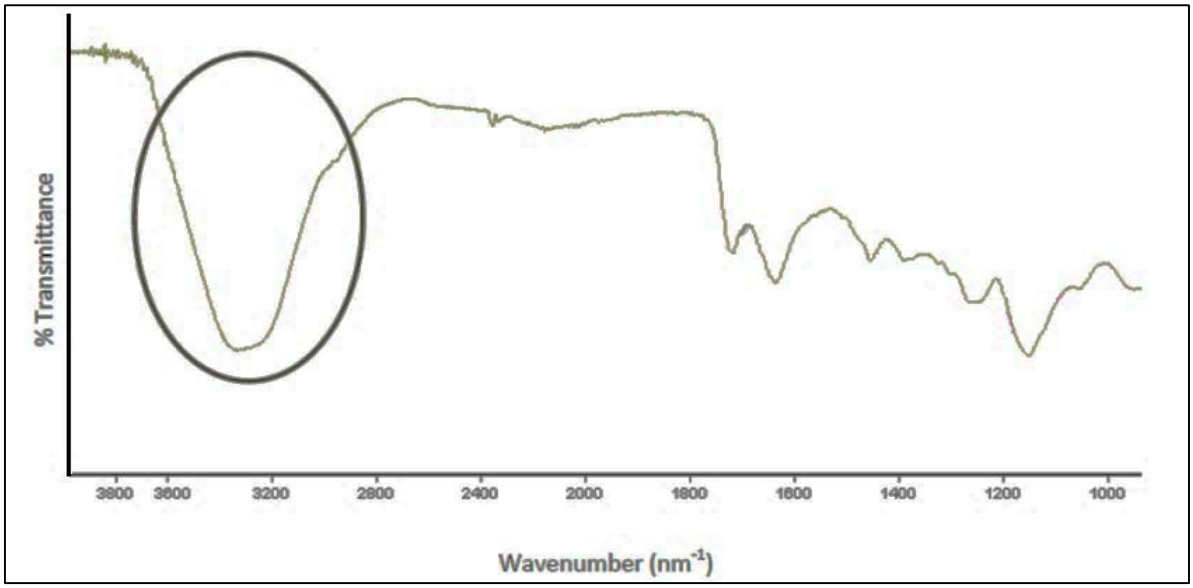


Figure 3.14 Infrared spectrum of MIP-DCF loaded with DCF, broad peak was observed at 3300 nm^{-1} due to –OH functional group from DCF bonded to MIP-DCF. Probably there also N-H functional group that might contribute in this peak.

In range of 1800 nm^{-1} to 1000 nm^{-1} the spectra pattern were differentiated and the observation showed a similar trend **(Figure 3.14)**. However, at 1650 nm^{-1}, there was slightly significant different peak in MIP-DCF loaded with DCF due to the C=C from benzene ring at DCF molecules. At 1750 nm^{-1} commonly it was from the carbonyl (C=O) functional group that came from the MIP-DCF which was consisted of crosslinked (EDGMA)-monomer-chained network.

In the next **Figure 3.15**, MIP-DCF after 10^{th} cycle of regeneration was differentiated with the original MIP-DCF. The finding showed clearly there is no changes in peaks and the MIP-DCF after 10^{th} cycle of regeneration showed highly likely as similar as it was in the original condition.

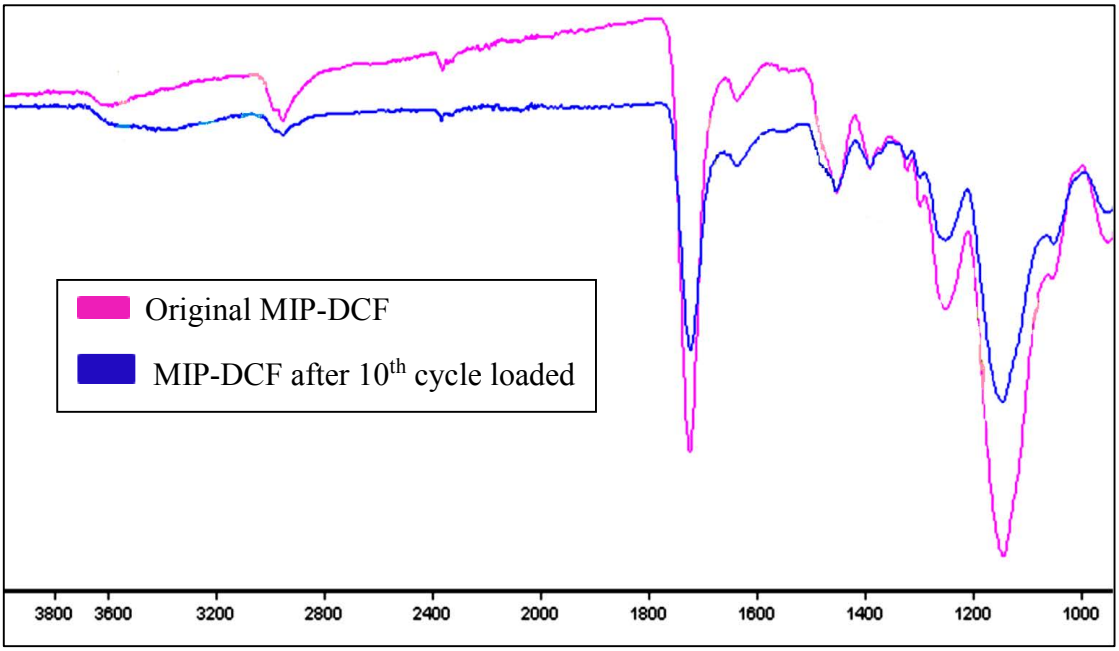


Figure 3.15 Comparison of infrared spectra between original MIP-DCF with MIP-DCF after eluted 10th cycles of regeneration.

From these results obtained, it can be concluded that the functional sites on MIP works well with the analyte (DCF). Three different kinds of MIPs have been distinguished via FTIR-ATR and the spectra obtained could be as a fingerprint in order to observe the different kinds of MIPs. The bonds formed by MIP and DCF were as followed:

- N-H stretch functional group at 3000 nm^{-1} found in original MIP
- C-N stretch bond at 1180 nm^{-1} found in original MIP
- OH broad peak at 3300 nm^{-1} found in MIP loaded with DCF
- C=O bond at 1750 nm^{-1} found in each MIPs
- C=C bond at 1650 nm^{-1} found in MIP loaded with DCF

3.4.5 Surface morphology study

Surface morphology study has been carried out in order to observed the surface structure of MIPs and NIPs. In the present work, MIPs and NIPs studied were:

- MIP with AT as the functional monomer with IDM as a template (MIP-IDM). **(Figure 3.16 & Figure 3.17)**
- NIP with AT as the functional monomer (NIP). **(Figure 3.18 & Figure 3.19)**

- MIP with AM as the functional monomer and IDM as a template (MIP-IDM-AM). **(Figure 3.20 & Figure 3.21)**
- NIP with AM as the functional monomer (NIP-AM). **(Figure 3.22 & Figure 3.23)**
- MIP with AT as the functional monomer and DCF as a template (MIP-DCF). **(Figure 3.24 & 3.26)**

It was estimated that there was no correlation between pore size observed with the removal efficiency since the interaction was occurring via chemical bonding (hydrogen bonding) between the target molecule and the active functional sites on the MIPs. Generally the pore size in MIPs were more likely homogeneous imprinted compared to NIP. In **Figure 3.16 & Figure 3.17**, the pores of MIP-IDM were almost homogenous and smaller comparing to NIP in **Figure 3.18 & Figure 3.19**.

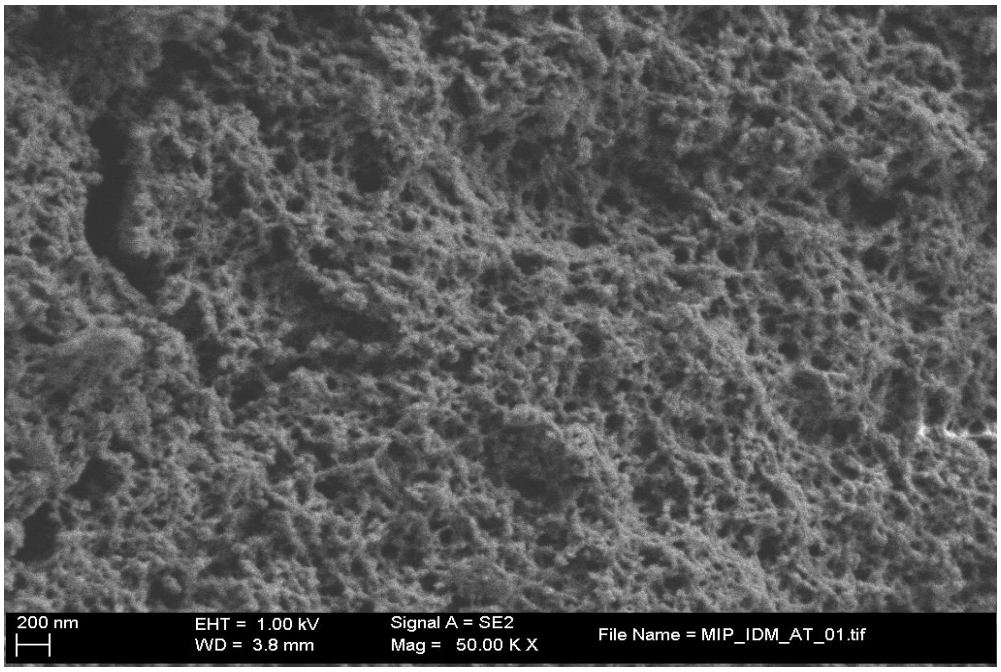


Figure 3.16 SEM profiles for MIP-IDM with AT as the functional monomer at magnificence 50 kV; EHT at 1.00 kV and scale 200 nm.

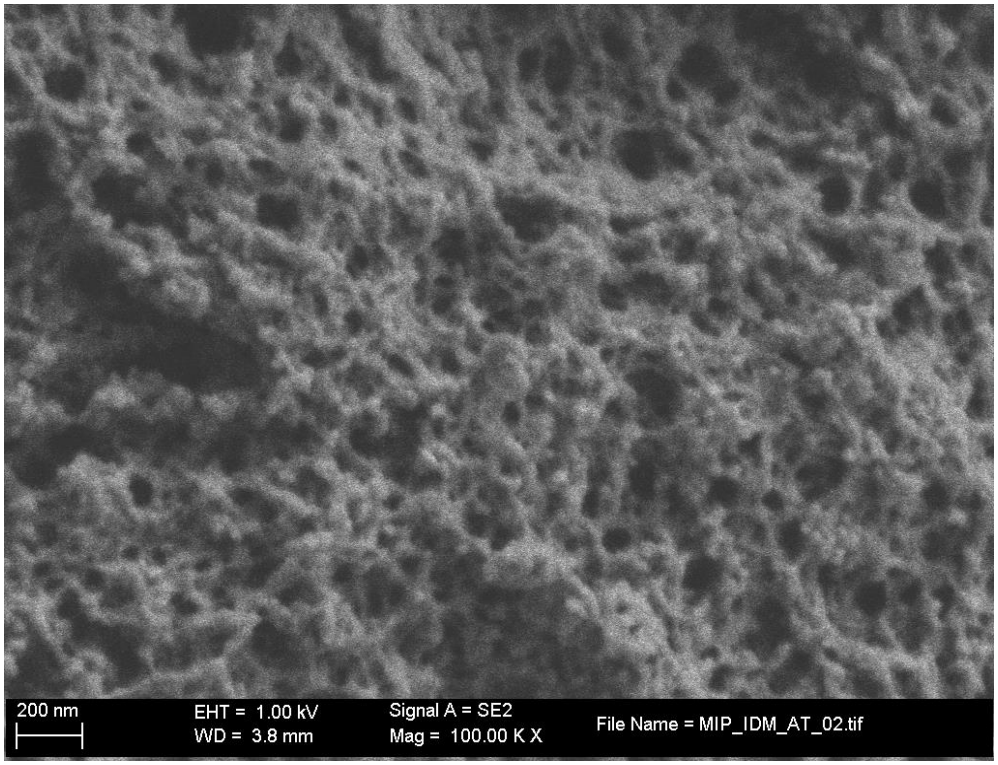


Figure 3.17 SEM profiles for MIP-IDM with AT as the functional monomer at magnificence 100 kV; EHT at 1.00 kV and scale 200 nm.

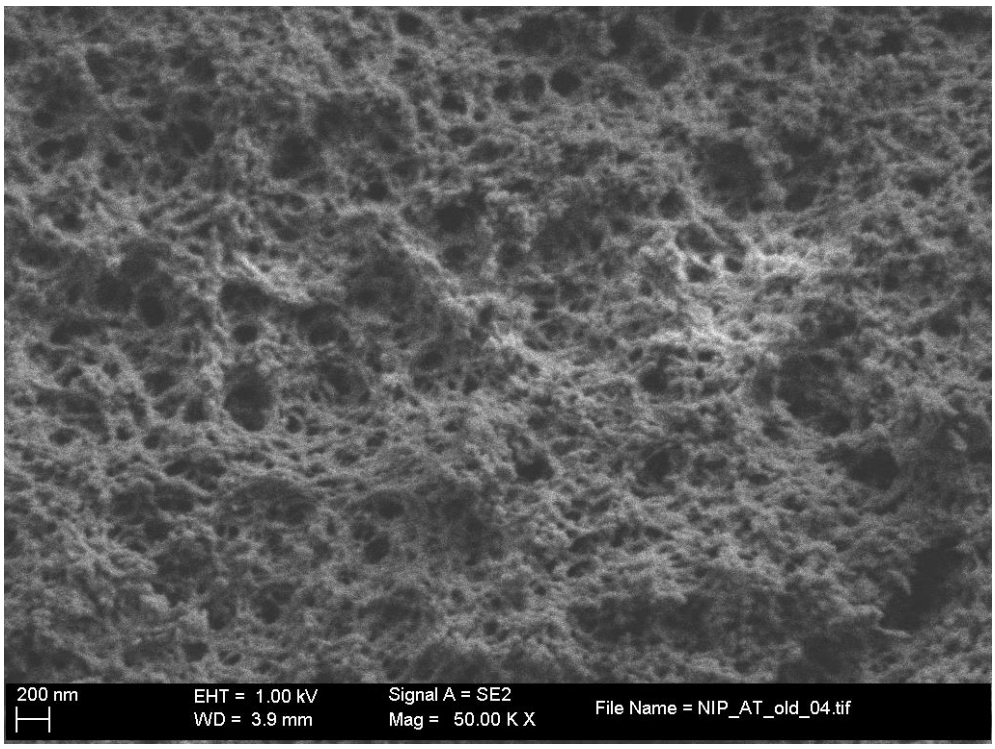


Figure 3.18 SEM profiles for NIP with AT as the functional monomer at magnificence 50 kV; EHT at 1.00 kV and scale 200 nm.

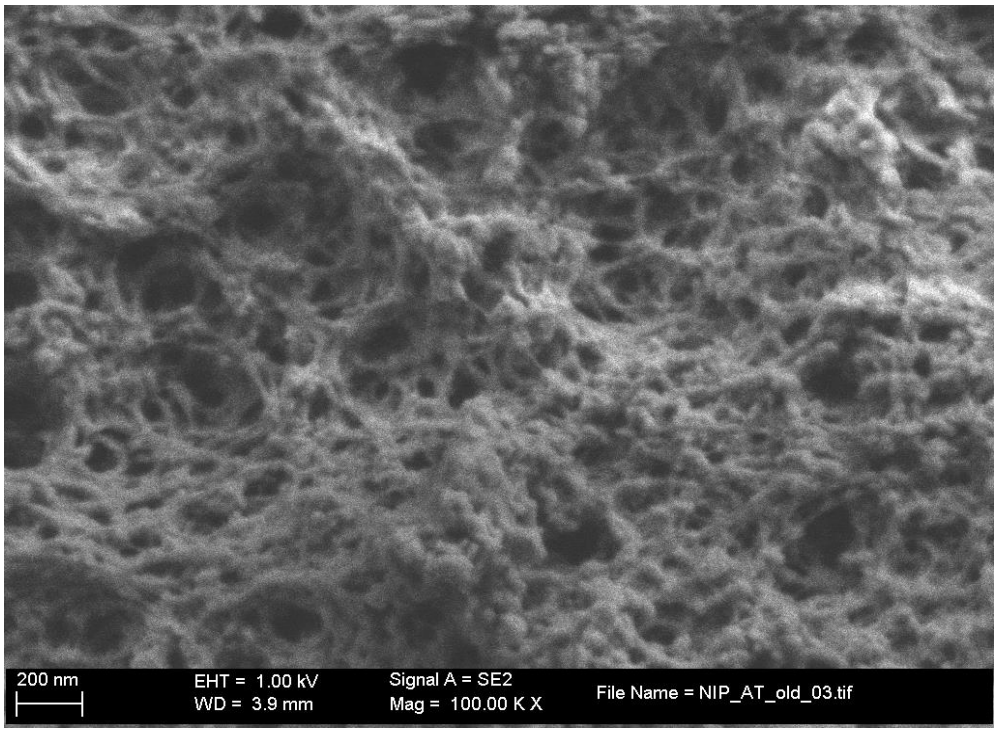


Figure 3.19 SEM profiles for NIP with AT as the functional monomer at magnificence 100 kV; EHT at 1.00 kV and scale 200 nm.

The morphology of MIP-IDM-AM **(Figure 3.20 & Figure 3.21)** and NIP-AM **(Figure 3.22 & Figure 3.23)** are shown below respectively. From the observation, pores of MIP-IDM-AM were smaller than pores in NIP-AM. In terms of comparison between monomers (AT and AM), observation was approximately similar between MIP-IDM-AT and MIP-IDM-AM since both MIPs were crushed and sieved prior to be used for EPPPs removal from aqueous medium.

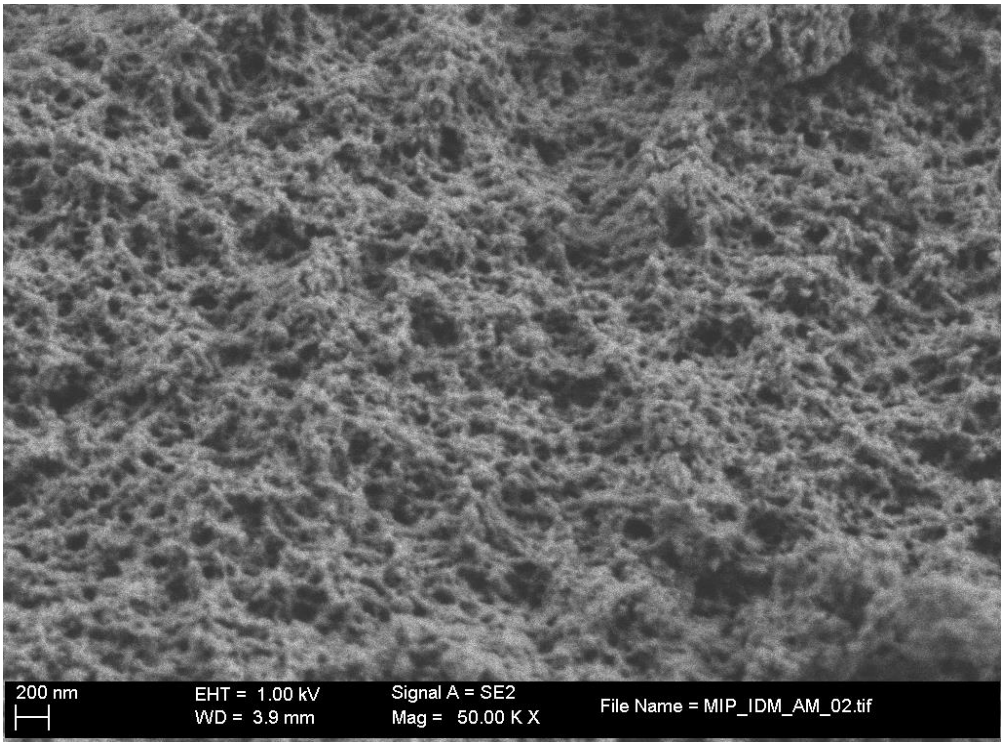


Figure 3.20 SEM profiles for MIP-IDM-AM and NIP at magnificence 50 kV; EHT at 1.00 kV and scale 200 nm.

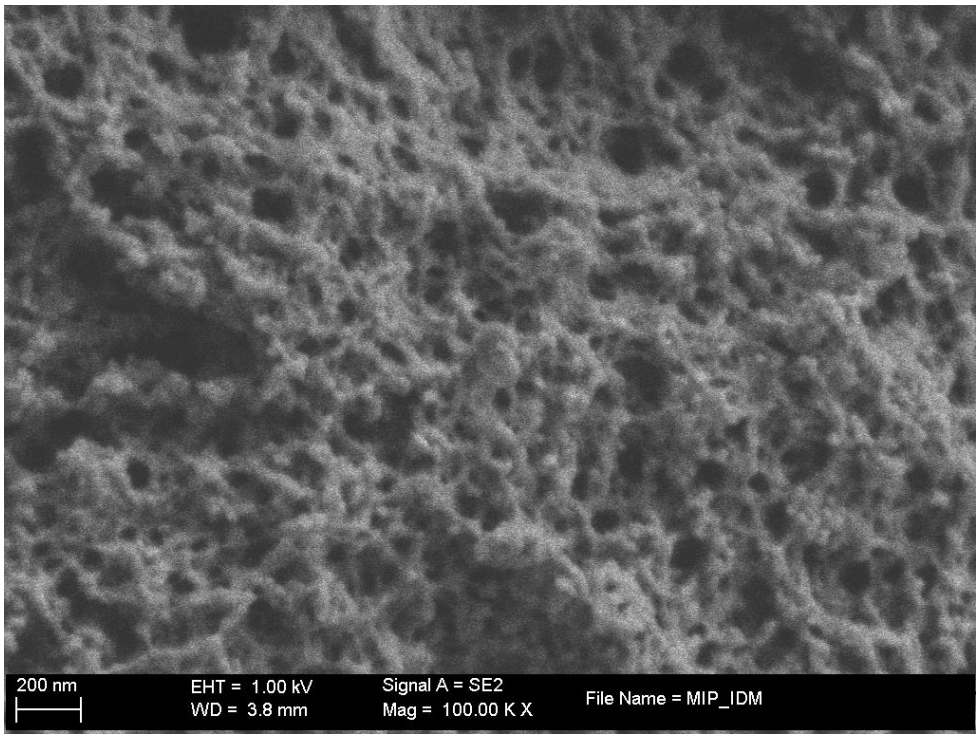


Figure 3.21 SEM profiles for MIP-IDM-AM at magnificence 100 kV; EHT at 1.00 kV and scale 200 nm.

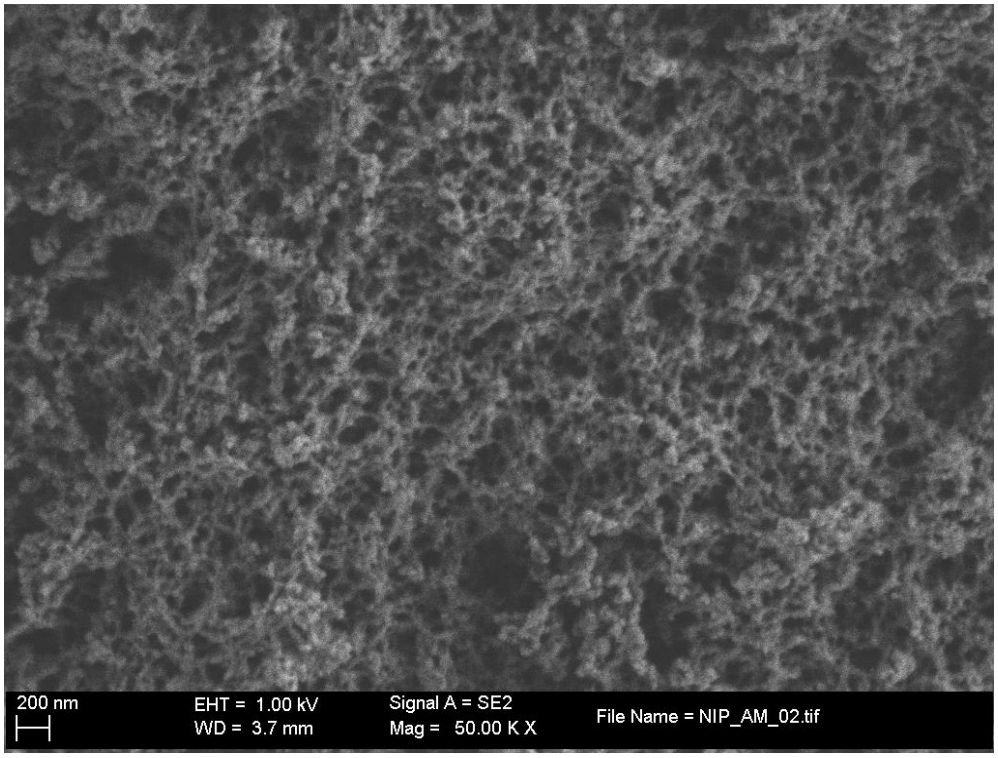


Figure 3.22 SEM profiles for NIP-AM at magnificence 50 kV; EHT at 1.00 kV and scale 200 nm.

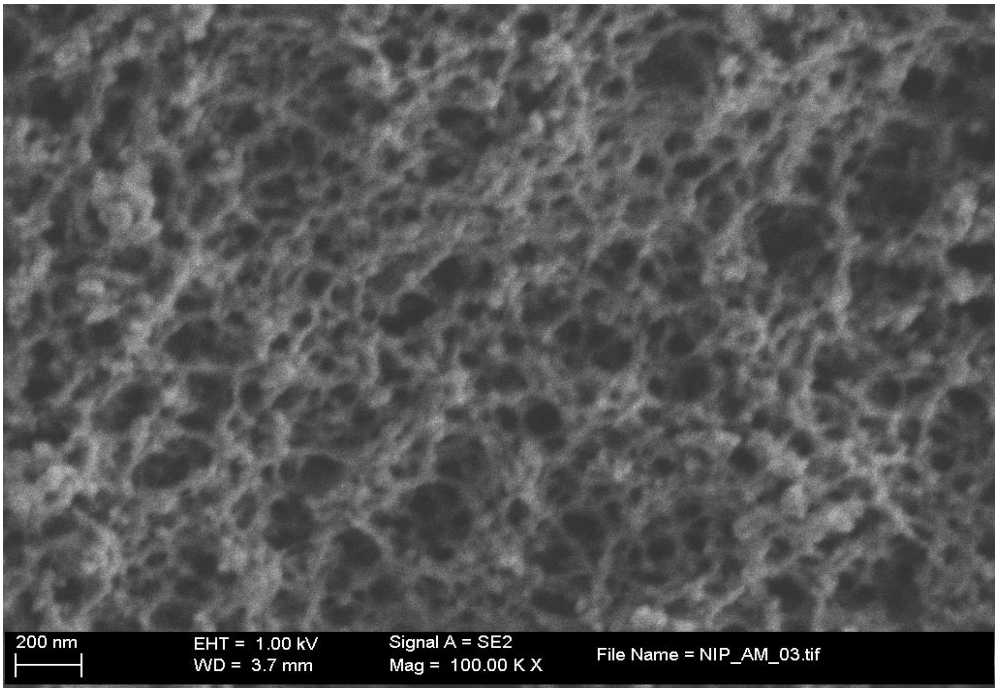


Figure 3.23 SEM profiles for NIP-AM at magnificence 100 kV; EHT at 1.00 kV and scale 200 nm.

For MIP-DCF, there is no observable difference between MIP-IDM and MIP-DCF because it has been synthetised using similar procedure. **(Figure 3.24 – Figure 3.26)**.

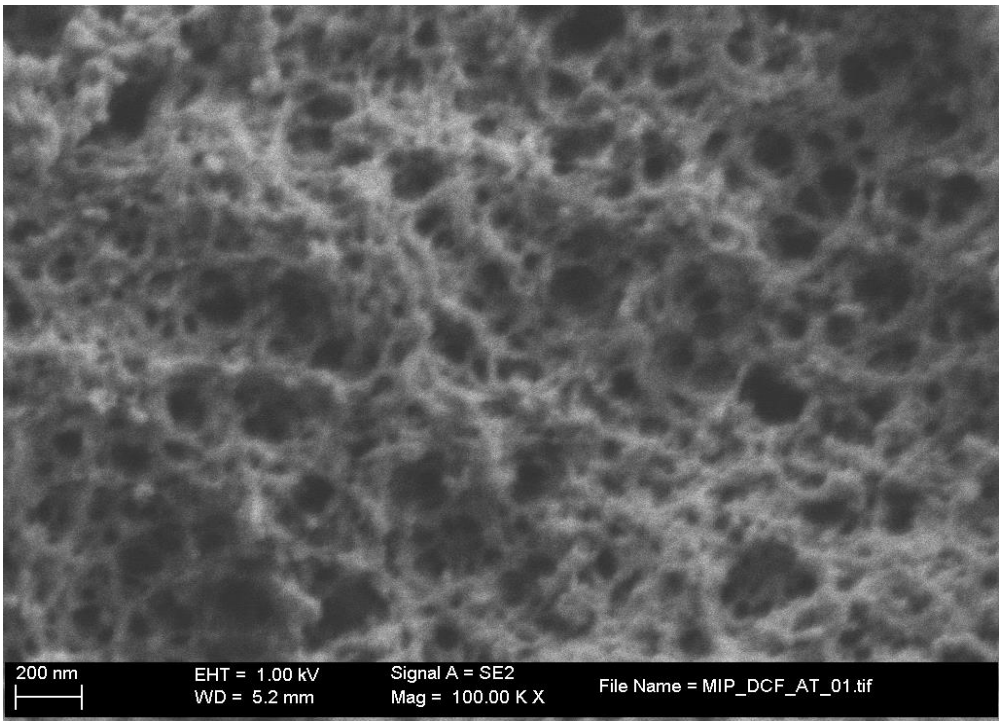


Figure 3.24 SEM profiles of MIP-DCF at 100KX scales magnified.

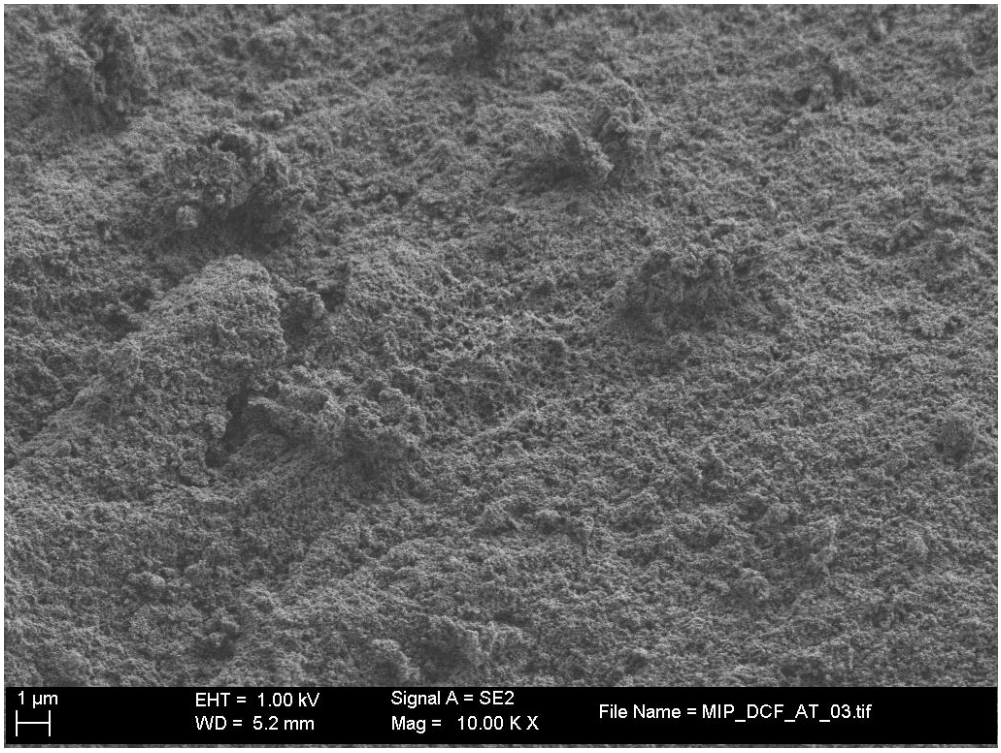


Figure 3.25 SEM profiles of MIP-DCF at 10KX scales magnified.

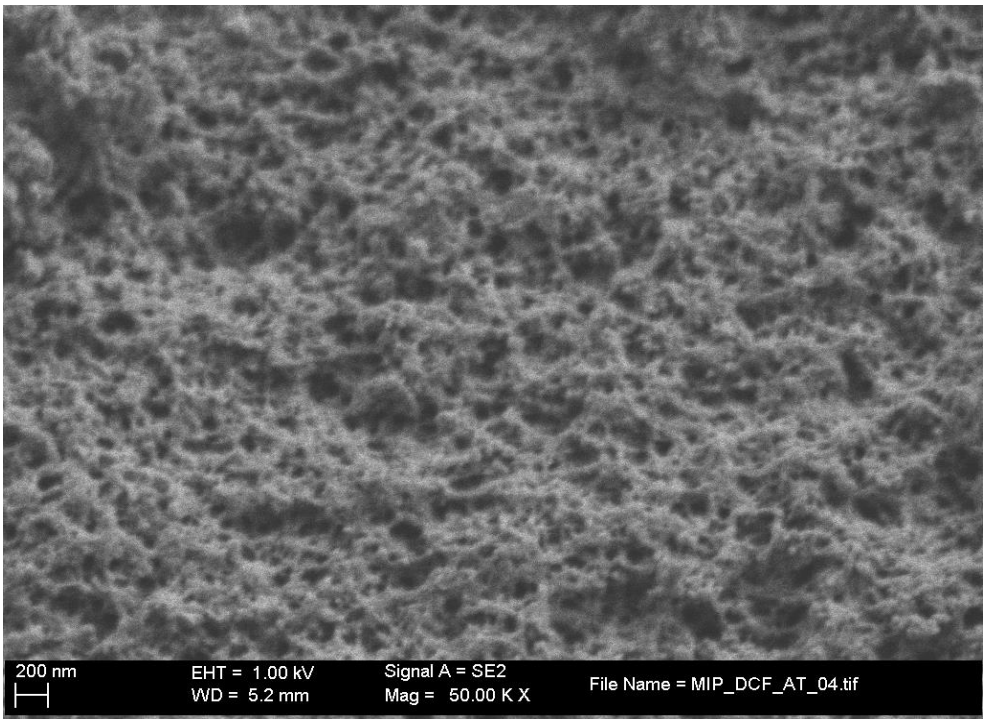


Figure 3.26 SEM profiles of MIP-DCF at 50KX scales magnified.

3.5 CONCLUSION

As conclusion, all the objectives were successfully achieved. It can be concluded that:

- Bulk polymerization is a good method approach in terms of total capacity loaded since the template was well-templated in the cross-linked polymer.

- MIP-DCF possess to have homogeneous properties whereas MIP-IDM possess to have heterogeneous properties due to the reaction occurred between N-H functional group on the monomer crosslinked polymer with N-H functional group at the centre of DCF molecule.

- The interaction between analyte and functional site on MIP was depending on the active functional sites AT which was very selective towards N-H functional group and because of that the MIP-DCF was choosen for the application procedures.

- From the infrared spectra, the ability of MIP to be used more than once have been proven. Each functional group in MIP has been determined which were N-H stretch functional group at 3000 nm^{-1} found in original MIP, C-N stretch bond at 1180 nm^{-1} found in original MIP, OH broad peak at 3300 nm^{-1} found in MIP loaded with DCF, C=O bond at 1750 nm^{-1} found in each MIPs and C=C bond at 1650 nm^{-1} found in MIP loaded with DCF. The spectra obtained could be as a fingerprint in order to distinguish among three kinds of MIPs tested.

- The eluted MIP spectra was similar to the original MIP which shows that the functional site of eluted MIP still can work on removal although it was regenerated until 10^{th} cycles.

- For the morphology study, the synthetised MIP was highly likely to have homogenous cavities compared to NIP.

3.6 REFERENCES

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Chapter 4

Analytical methods for application of Molecularly Imprinted Polymer Using Selective Functional Monomer for Diclofenac Recovery from Water and Wastewater

Chapter 4

Analytical methods for application of Molecularly Imprinted Polymer Using Selective Functional Monomer for Diclofenac Recovery from Water and Wastewater

4.1 INTRODUCTION

Molecularly imprinted polymers (MIPs) are synthetic materials with artificially generated recognition sites able to specifically rebind a target molecule in preference to other closely related compound.[1] The imprinting process usually starts with the interaction between a functional monomer and the selected template in a selected solvent that acts as the porogen.[2] Figueiredo and co-workers reviewed that MIPs have potential in wide application especially in aquatic environmental issues.[2] In addition, MIP has been widely used as selective sorbent in solid phase extraction (SPE) or so-called molecularly imprinted solid phase extraction (MISPE).[1]

There are two approaches in order to investigate the application of MIP as a sorbent in real wastewater samples such as clean-up process (offline analysis) and direct detection of pharmaceuticals separation (online analysis).

Martín had reviewed that MISPE is a promising method which can be done via offline and online protocols.[3] In off-line MISPE, a small amount (15–500 mg) of imprinted polymer is packed into polyethylene cartridges. Then, after the conditioning, loading and washing steps, analyte are eluted, ideally free of co-extractives, and the elution extract is further analysed by chromatographic techniques.[3] MISPE via offline protocols, has been implemented in most of the sample analysis using chromatography since it is an obligatory procedure in order to remove any impurity and interferences from a real samples.

On-line MISPE procedures overcome some of the drawbacks associated with off-line MISPE for instance, there is dramatically reduced sample manipulation between the steps of pre-concentration and analysis, so loss of analyte and risk of contamination are reduced, and detection limits and reproducibility are improved.[4] However, to date, the online protocols using MIP as a stationary phase in a pre-packed column equipped to chromatography in environmental application has not been reported.

In the present work, there are three separation methods which has been performed:

- continuous-flow mode via MISPE online detection using ultraviolet (UV) spectrophotometry detection;
- NSAIDs separation method using commercial C18 column equipped to HPLC with UV as detector
- MIP as the stationary phase in column equipped to HPLC with UV as a detector.

In the different sections of the present chapter, all the experimental design using MIPs as the sorbent for removal and recovery study will be elaborated.

4.1.1 Continuous-flow mode via MISPE coupled to UV spectrophotometer

Spectrophotometry, a relatively inexpensive and easy handling technique with good precision and accuracy of analysis, offers the practical and economic advantages over the other techniques but it lacks the required selectivity and sensitivity for the determination of low level of analyte in complex matrices.[5] Most of the emerging contaminants can be detected under UV directly. In the present study, the spectrophotometry has been used as the detector using continuous flow mode and applied to the analysis of wastewater. However, the continuous-flow mode via MISPE was likely depending on the normal pressure/ambient. To date, there was no study on continuous-flow mode via MISPE with AT as the functional monomer coupled to UV spectrophotometer.

4.1.2 DCF recovery using conventional C18 column equipped to HPLC

A simple column chromatography was first introduced by Russian botanist Mikhail Tswett (1872–1919).[6] High Performance Liquid chromatography (HPLC) has been used in many types of separation study involving emerging contaminants for instance DCF, IDM and IBU.[7] **(Figure 4.1)**

Figure 4.1 High performance liquid chromatography.

The advantage of HPLC is that compounds present in liquid samples do not need to be volatilized as in gas chromatography (GC) where the compounds need to be derivatized prior to run the analysis. The derivatization is needed in GC to prevent the peak tailing and thus to improve detection limits by the peak shaping.[8] Moreover, the more derivatized cycles into the sample preparation, the more outliers can be obtained.[9] Commonly, GC analysis require high temperature which lead to racemization or decomposition of some compounds.[10] Second advantage of HPLC is that high resolution can be obtained. However, still, HPLC performance depends on few parameters such as types of mobile phase, injection volume, flow rate and temperature.

The schematic diagram of HPLC analysis is shown in **Figure 4.2**. The HPLC analysis consist of mobile phase reservoirs, solvent proportioning valve, pump, pulse damper, loop injector, guard column, column and detector. In HPLC, the sample carrier is in liquid form and is called mobile phase. The solvent proportioning valve is important to control the flow and

portion for each mobile phase. The function of pump is to ensure the mobile phase flows according to the flow rate which has been programmed before analysis. Loop injector is the place to receive the liquid sample for injection. Guard column is an inexpensive column used to protect a more expensive analytical column. Column has a stationary phase in the inner part and is used to separate analytes from the mixture.

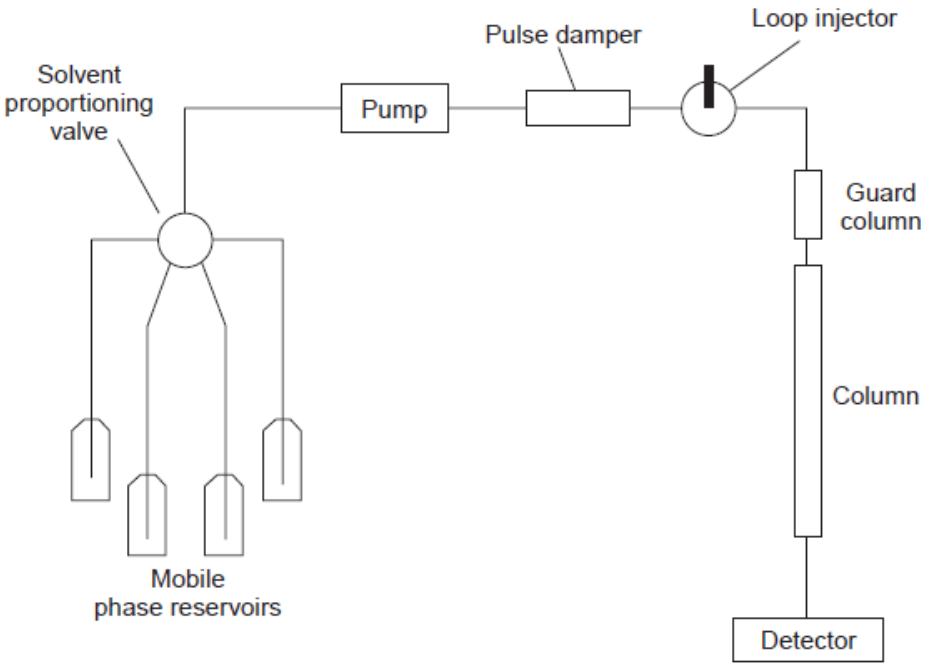


Figure 4.2 Schematic diagram of high performance liquid chromatograph.[6] Reprinted with permission.

The common column used to separate the emerging contaminants from samples is C18 column. C18 column is very good in separating polar and non-polar analytes. In the literature, many studies use C18 column and acidified water mobile phase combined with organic solvents to separate any emerging contaminants including DCF.[7,11,12] In a review article written by Poole noted that commercial stationary phase for SPE based on silica and bonded silicas have been used for a wide range of analytes.[13] Early problems with batch-to-batch variation in analyte recovery inhibited their use but these have been addressed by manufacturers. One of the biggest problems was the presence of residual silanols on the most popular reversed phase materials.

The components in a mixture can be separated according to the retention time, t_R. Retention time, t_R is the time at which each component is eluted from the column and provides signal in

the detector after being injected. Different components will have different retention times. However, peak shifting and peak broadening usually occurred during analysis.[14] The output for chromatography is called chromatogram. The chromatogram is a graph of intensity versus retention time, t_R.

In prior to implement MIP using real wastewater samples, blank analysis is a compulsory need to be done. All the measures have been done in order to certify the quality of the measurements. In addition, after blank analysis has been estimated, a series of standard solutions need to be prepared in order to validate the analysis. In this study, MIP has been used as a sorbent for WWTP samples analysis. Commonly, river water samples content many type of other components or interferences. Hence, to study the DCF recovery in river water samples, HPLC is the best instrument to be used. In order to use the HPLC, the description of quality assurance and quality control will be introduced. The parameters such as limit of detection (LOD) and limit of quantification (LOQ) will be determined.

4.1.2.1 Sampling point

The Ebro river basin is located in the North East of Spain, and it is the largest river of the Iberian Peninsula in terms of flow discharge **(Figure 4.3)**. Ebro river generates the Ebro Delta, a relevant wetland area (320 km^2) in the western Mediterranean region with a high ecologic value. The most important economic activity in the basin is agriculture (vineyards, cereals, fruit, corn and horticulture along the river and rice production in the delta). In the last century, the river flow has decreased approximately 40% due to land use change (reforestation and increase of irrigated agriculture) as well as precipitation decrease. Although it is not densely inhabited (approximately 2.8 million inhabitants), almost half of the population is concentrated in big cities. Industrialized regions are mainly situated in the North and central part around big cities of Zaragoza, Pamplona and Lleida. The Ebro River receives urban contamination coming from the effluent discharges of many wastewater treatment plants (WWTPs).[15]

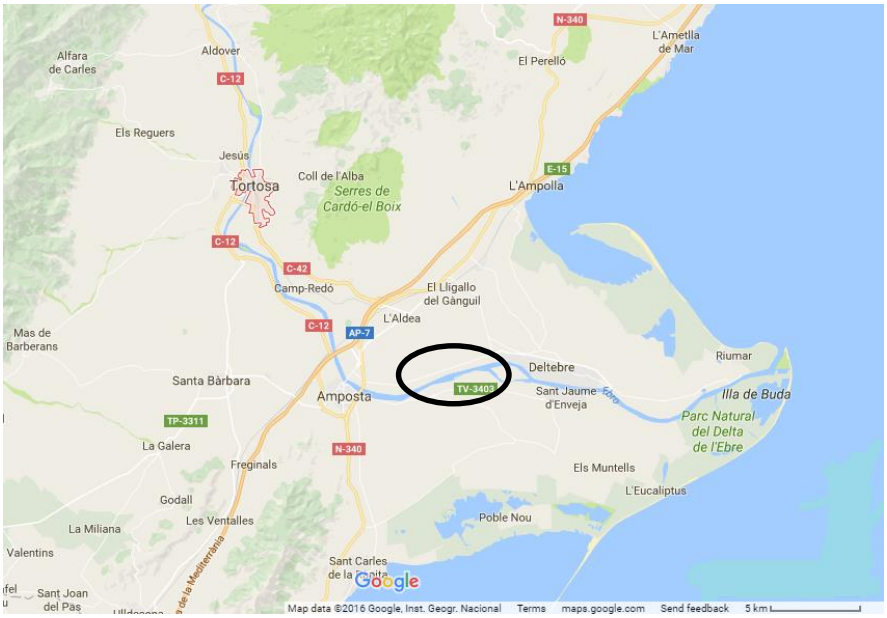


Figure 4.3 Map of Ebro river, Spain, showing WWTP was located after the Amposta city near towards the Deltebre.[16]

4.1.3 MIP as the stationary phase in HPLC application

Instead of using MIP in a batch removal processes, MIP also can be used directly as chromatographic stationary phases which generally provide a quicker and easier method for analyzing the binding properties of MIP.[17] For the chromatographic evaluation, the MIPs are sized to particles in the micron particle-size range (20–25 µm), and packed into stainless steel HPLC columns.[18] According to the previous results elaborated in *chapter 2* and *chapter 3*, the removal of DCF has been done by using MIP-DCF with the particles size from 28 µm to 100 µm. However, in order to prepare the packed column with MIP-DCF in a column, the size of particles must be lower than 28µm due to the high back pressure occurred in the column while mobile phase is flowing.

Regarding the choices of mobile phase, for the C18 column, the combination of 0.1% until 1% acetic acid of phosphate acid in high quality water with acetonitrile is the optimum for DCF and IBU since many previous studies use this kind of combination as the mobile phase.[7] Acetonitrile is among the most commonly used organic solvents in reversed-phase liquid chromatography, mainly due to its miscibility with water, acceptable UV absorptivity, and low viscosity. But a problem has raised recently, since acetonitrile is obtained as a co-product in the production of acrylonitrile, the pharmaceutical, food, environmental, and chemical

industries are experiencing an unprecedented shortage in acetonitrile. This shortage has resulted in a sharp price increase which is projected to remain high even after the production returns to normal. The on-going acetonitrile shortage has forced chemists to either reduce their solvent usage by using shorter, narrower columns packed with small particles, or, replace acetonitrile with more readily available solvents such as methanol.[19] The idea on using ethanol as the mobile phase was suggested by Brettschneider.[20]

Recently, there are an increasing number of articles preparing and using MIP as a sorbent in removing DCF for clean-up method via MISPE in real wastewater samples.[21]. The functionalized MIP working as stationary phase in the separation of DCF from other compounds is quite challenging since to date, there is no direct method or online method which can separate DCF from other compounds in a real wastewater which contained with many other micropollutants. In the present work, the use of MIP-DCF as a stationary phase has been proposed. As mobile phase, knowing the problem of acetonitrile, together with the fact that acetonitrile could damage the MIP-DCF, it has been chosen a combination of acidified water with ethanol.

4.2 OBJECTIVES

The objectives of the present study are:

i. To develop online detection flow analysis using UV spectrometry for three components in mixture (DCF, IDM and IBU)

ii. To calculate the recovery of DCF until 10^{th} cycles of reuse of MIP, in natural water using HPLC equipped with UV detector

iii. To pack a HPLC column with MIP-DCF as the stationary phase and implement it to an HPLC-UV equipment to separate DCF and IBU in a mixture.

4.3 EXPERIMENTAL

4.3.1 Reagents, samples and equipments

Reagents:

- Indomethacin (IDM) from Sigma Aldrich with 98-99% of purity.
- Diclofenac-Na was from Cayman Chemical with purity of more than 99%.
- Ethanol with 96% purity from Scharlau.
- Acetonitrile from VWR with 99.9% of purity. Acetonitrile was HPLC grade.
- Acetic acid from J.T. Baker.
- Ibuprofen (IBU) were from VWR with 99% of purity Methanol were from VWR Chemicals with 99.5%.

Samples:

The water samples were obtained from the inlet and outlet of WWTP located at the south of the Ebro river, in the city named Amposta. The samples were collected before and after the Amposta WWTP. The illustration is shown in the **Figure 4.4**. In order to do the sampling, opaque glass bottles were used to preserved the quality of water samples. The sampling date was recorded and labelled. The water samples were located in the fridge at 4^{o}C. There was no pre-treatment done during the sampling.

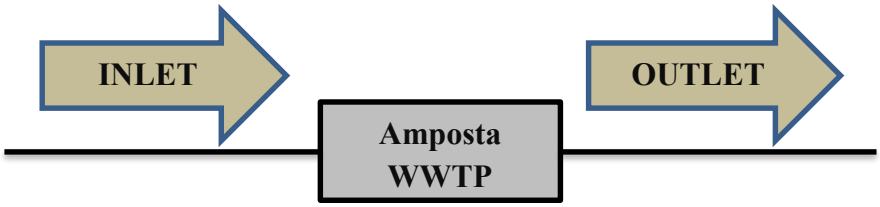


Figure 4.4 The inlet and outlet of Amposta WWTP.

Equipments/Instruments:

- Grace Alltech 'Extract Clean' empty reservoirs with silica frits, 1.5 mL from Fisher Scientific. Plastic tubes with engaged holder.

- Mortar grinder from Retsc
- Water bath from E. Gabarro A-G.
- UV double-beam spectrophotometer from UNICAM, model UV-2200.
- High Performance Liquid Chromatography coupled to Ultra Violet detection (HPLC-UV) from Thermo Elemental Spectrasystem.
- HPLC Syncronis C18 column (longitude 250mm, 5mm) Thermo Scientific.
- For column packing, a stainless steel column (15 cm, 2cm external diameter, 0.5 cm internal diameter) was used in this study.
- Syringe with needles.
- Glass column with column holder from Prague.

4.3.2 Continuous-flow mode MISPE coupled to UV spectrophotometer

First, a mixture of IDM, IBU and DCF was prepared in 0.017 mmol/L each one in 250 mL volumetric flask. Next, 10 mg of MIP was weighted and located into the reservoir in between two silica frits. The experimental equipment consists of 5 parts as shown in Figure 4.5 and Figure 4.6:
(1) mixture solution or sample,
(2) peristaltic pump to flow the solution into the system at 1.67 mL per minute of flowrate.
(3) reservoir which contained the MIPs. A detailed picture is shown in **Figure 4.7**.
(4) spectrophotometer which the quartz cell cuvettes was used and connected using tubes as shown in Figure **4.8**.
(5) waste container to collect the solution after passing through the cuvettes. The software used was set to determine at 220 nm, 260 nm and 280 nm. The reading were taken for time interval 1 minute until 15 min. The plastic tubes were used as the connector between (1) and (2), (2) and (3), (3) and (4), (4) and (5).

In prior to load the mixture solution, the blank solution was prepared and loaded into the system. After the experiment has been done, the results obtained were calculated using Equation 3.7. The procedure was repeated for two times. The spectrophotometer was selected for continuous flow mode in which the maximum wavelength for DCF, IDM and IBU were inserted into the software program. The average value for each minute was used in the calculation.

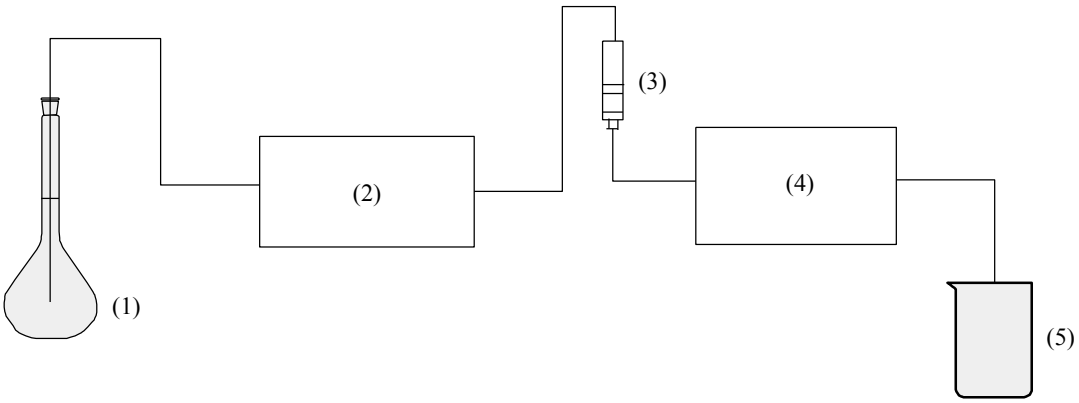


Figure 4.5 Experimental set up diagram for continuous mode online coupled to UV spectrophotometer (1) solution contained with 0.017 mmol/L of DCF, IBU and IDM mixture, (2) peristaltic pump, (3) cartridge contained with 10 mg MIP, (4) spectrophotometer, (5) waste container.

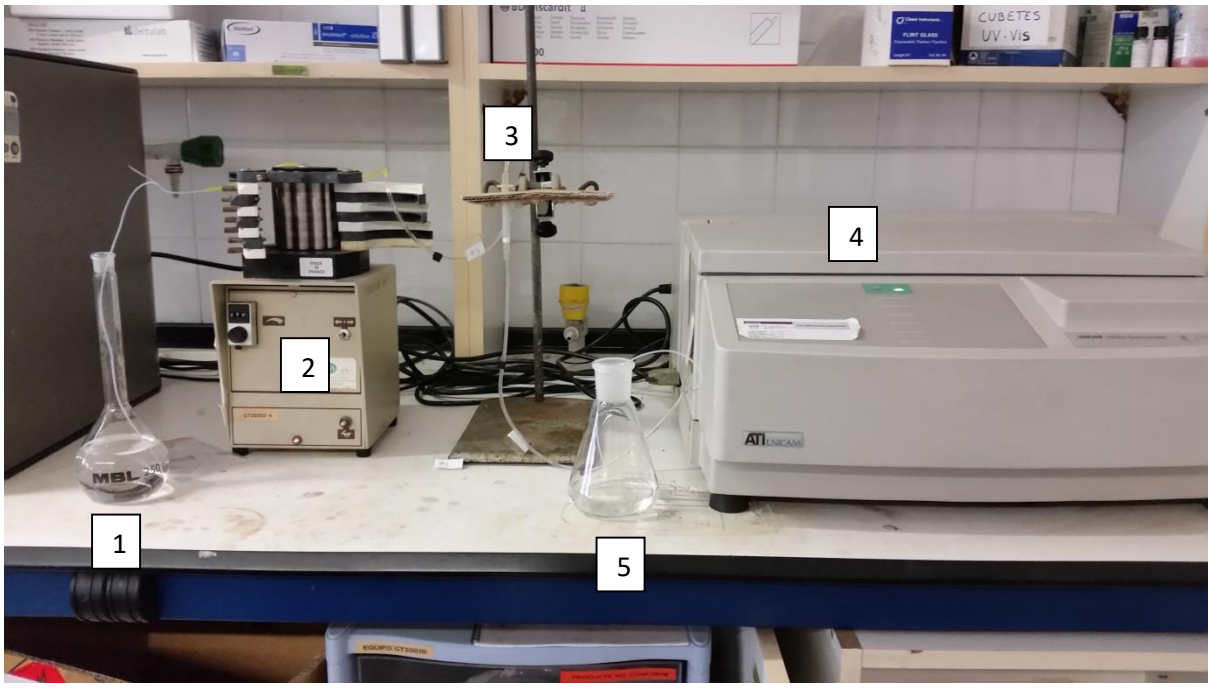


Figure 4.6 Experimental set-up for continuous-flow mode combined with MIP packed cartridge via spectrophotometry detection. 1: standard solution or sample, 2: peristaltic pump, 3: cartridge contained with 10 mg MIP, 4: spectrophotometer, 5: waste container.

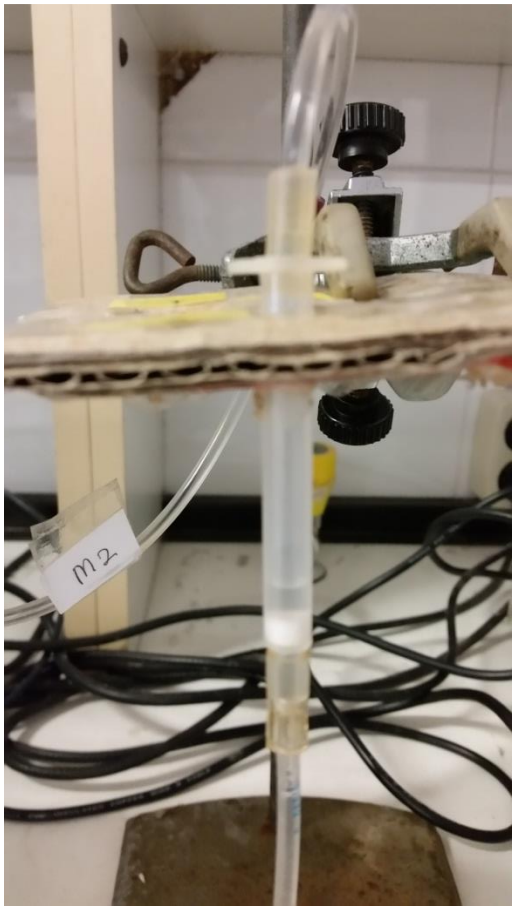


Figure 4.7 The detailed picture of the MIP inside the packed cartridge with tubing connected at inlet and outlet of the cartridge.

Figure 4.8 Tubing cuvettes which has the inlet and outlet connected via tubes from the cartridge to waste container and located inside the UV spectrophotometer.

4.3.2.1 Target NSAIDs compound

The target NSAID compounds in this study were diclofenac (DCF), indomethacin (IDM) and ibuprofen (IBU).

4.3.3 Offline MISPE for DCF recovery using HPLC-UV equipped with C18 column

Firstly, deionized water and a series of standard solutions were prepared in order to have a blank analysis and optimize the system, respectively. The multiple-point standardization has been implemented in this study. The standard solutions were then filtered using syringe filter (0.22 μm pores size) and poured into vials for HPLC-UV detection.

Water samples from the river just before and after the Amposta WWTP were prepared in duplicates. The samples were filtered using vacuum pump equipped with Buchner funnel. The filtered solutions were then divided into two portion of solutions to prepare 250mL of un-spiked and spiked solutions. The spiked solution was prepared by adding 12.5 mL of acetonitrile and 0.5 mg/L of DCF whereas to the un-spiked solution 12.5 mL of acetonitrile were added. The solutions were filtered using syringe filters and poured into HPLC vials. . The samples were then analyzed by HPLC

4.3.3.1 Clean-up analysis via MISPE

Firstly, 10 mg MIP-DCF was accurately weighted and located in the cartridge as previously explained. 5 mL of EtOH: water (75%) was passed through the cartridge. Then, the water sample solution was loaded on the column with controlled flow rate at 1.00mL min^{-1}. Then, the solution was collected and an aliquot of the solution (1.5 mL) was taken in order to be analysed by HPLC-UV. Next, 5 mL of EtOH: water (75% v/v) was poured into the column and then was collected in order to recover the amount of DCF sorbed. After that, the solution was placed into the vial and analyzed by HPLC-UV. The method for analysing DCF via HPLC-UV was gradient mode. The mobile phase used was 1% Acetic acid (HOAc) in water and acetonitrile for C18 column. Analysis was carried out at room temperature (20^{o}C). The detector used was photodiode array (PDA) UV detector. The analysis has been carried out in

duplicates. The removal efficiency was calculated for each collected time using **Equation 4.1**. The initial concentration was assumed to be samefor each minute.

$$Removal\ efficiency = \frac{Initial\ concentration - Final\ concentration}{Initial\ concentration}\ X\ 100\% \quad \cdots \quad \textbf{Equation 4.1}$$

The recovery experiment in continuous flow mode was performed in the same way as for the removal process but after the removal process finished, 250 mL of a mixture of ethanol and water (75%) was flowed into the system with the same flow rate for another 15 min. The absorbance reading was obtained and calculated using the **Equation 4.2**.

$$Recovery = \frac{Final\ concentration}{Initial\ concentration\ from\ each\ pre\ cycle*}\ X\ 100\% \quad \cdots\cdots\cdots \quad \textbf{Equation 4.2}$$

*The initial concentration was calculated based on the previous cycle for example, the final concentration for first cycle would be the initial concentration for second cycle.

4.3.4 Online analysis using MIP-DCF as packing for HPLC

4.3.4.1 Preparation for packing MIP-DCF inside column

In order to pack a column with the MIP-DCF, MIP-DCF was crushed and sieved until the diameter of particles was approximately between 5 µm and 10 µm. Then, 25 mL of 5% ethanol in high quality water was prepared and mixed with 1 gram of MIP-DCF in order to prepare the slurry solution. The slurry solution was then leaved for overnight after shaking vigorously.

The excess solution was then removed until the final volume of approximately 5 mL. A glass column with an aluminium filter placed at one end was packed with the MIP-DCF as shown in **Figure 4.9** below. To do so, a reservoir is connected to an HPLC pump (in direction of upside down) and the column to the reservoir. The mobile phase was flowed in the column with the column connected to the reservoir. The slurry solution was poured into reservoir using a long needle of a syringe. Then, the HPLC pump was started to pump at 1.5 mL/min of flow rate (the pressure was control to be lower than 782 psi). Next, the column was re-connected to the reservoir and the flow was continued for maximum of 2 h. Next, the column with reservoir were disconnected from the HPLC pump and they were turned in the direction

of gravitation and left overnight. After that, the column was disconnected from the reservoir. Next, the column was finished by installing a porous stainless steel, a teflon ring, a stainless steel ring and finally a teflon fitting as shown in **Figure 4.10**. The flow direction of mobile phase was same with the loading of slurry and marked at the column body to avoid wrong direction for the whole analysis.

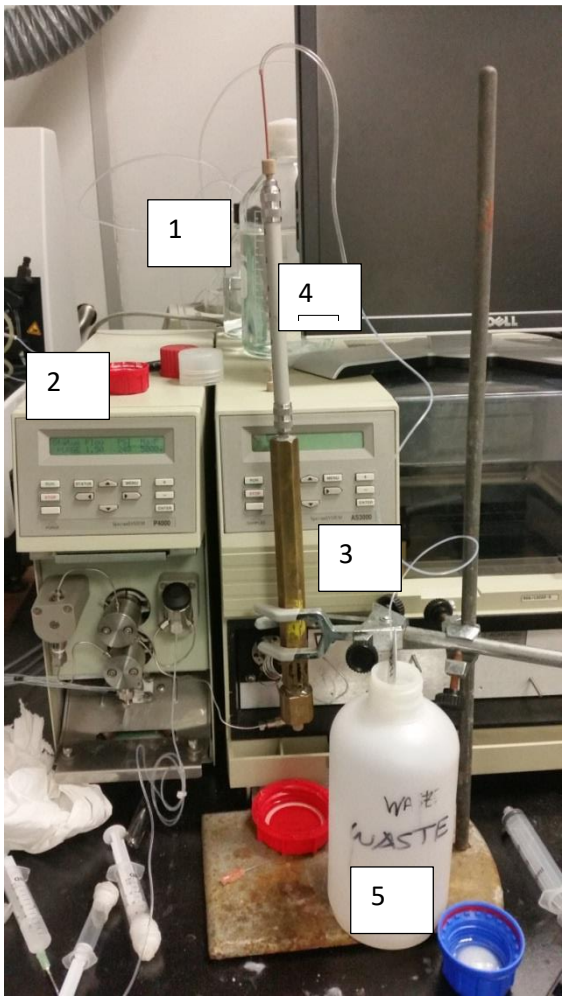


Figure 4.9 Experimental setting of MIP-DCF packing method in the glass column, (1) Mobile phase reservoir, (2) HPLC pump, (3) MIP reservoir, (4) holder containing the column, (5) waste reservoir.

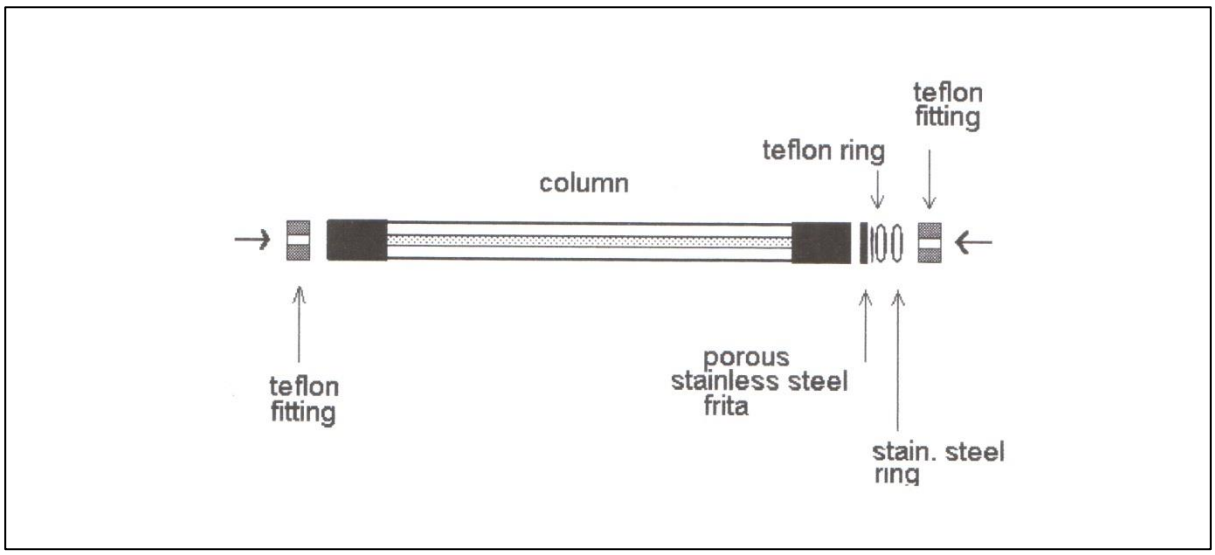


Figure 4.10 The end-column installation for MIP-DCF packed column using porous stainless steel, teflon ring, stainless steel ring and teflon fitting.

4.3.4.2 Preparation of mixture solution

The target NSAID compounds in this study were diclofenac (DCF) and ibuprofen (IBU). 0.5 mg of DCF and IBU for each were weighted and mixed with 5 mL of ethanol. Then, high quality water was added into the mixture solution. The solution was wrapped using aluminium foil. 1.5 mL of solution was filtered using syringe filer with mesh size at 0.22 μm. The filtered solution then was poured into vial. Then, the vial was located in the sample compartment. Then, the program was started.

4.3.4.3 Morphology study

The morphology surface of MIP-DCF after sieving was characterized using Field Emission Scanning Electron Microscope (FESEM). MIP-DCF particles were distributed on the carbon disc attached on the pin stubs. Then, the pin stubs were located according to the position number on the multi stubs holder. Next, the holder was located into the chamber and then analysed.

4.3.4.4 Recovery analysis via online MIP-DCF as packing media

The recovery analysis was done by preparing the mixture solution contained with DCF and IBU with each concentration at 5 µg/mL respectively. The peak obtained in the chromatogram was then interpreted.

4.4. RESULTS AND DISCUSSION

4.4.1 Quantitative analysis of DCF, IDM and IBU using MIP-packed column via UV spectrophotometry

First, calibration was performed in mixed stock solutions of the three NSAIDs studied which are DCF, IDM and IBU, using five standards in the concentration range of 0.1 - 25 µg/mL in the UV equipment. From the **Table 4.1** below, the data shown for three NSAIDs which are DCF, IDM and IBU. Results obtained for calibration equation, limit of detection (LOD) and limit of quantification (LOQ) are shown in **Table 4.1**.

Table 4.1 The calibration data for each target molecule using UV spectrophotometry detection.

NSAIDs	λ max. (nm)	Regression linear equation	LOD (µg/mL)	LOQ (µg/mL)
DCF	280	$y = 22.04x + 0.09$ $r^2 = 0.986$	0.2193	0.5213
IDM	260	$y = 22.45x + 0.10$ $r^2 = 0.984$	0.2183	0.4944
IBU	220	$y = 48.92x + 0.14$ $r^2 = 0.993$	0.2270	0.4229

Following the experimental described in section 4.3.2, the removal of the compounds was determined. As can be seen in **Figure 4.11** and **Figure 4.12** below, the average efficiency removal analysis for continuous-flow mode equipped with UV spectrophotometer showed approximately 65%% of removal for DCF and IDM whereas 60% for IBU **(Equation 4.1)**. In contrast to the results obtained in batch mode where the removal reached almost 100% for DCF and IDM, due to the different procedure process used. Since the MIP used was

synthetized using a template, it was expected to be selective to the molecule used as template. Yet, this approach shows that from the mixture solution, the three components (DCF, IDM and IBU) can be removed from the water using the continuous flow mode. The vivid data were shown in **Table 4.2** for MIP-IDM and **Table 4.3** for MIP-DCF.

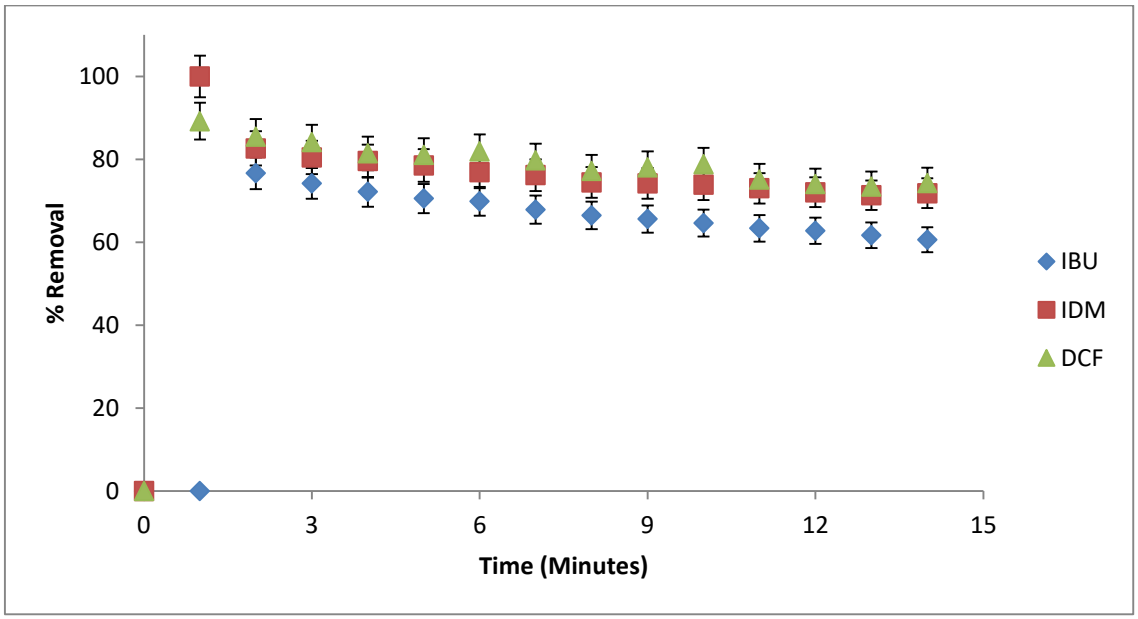


Figure 4.11 Efficiency removal of mixture of IDM, IBU and DCF by MIP-IDM template, in continuous-flow mode with flow rate 1.67 ml/min at room temperature.

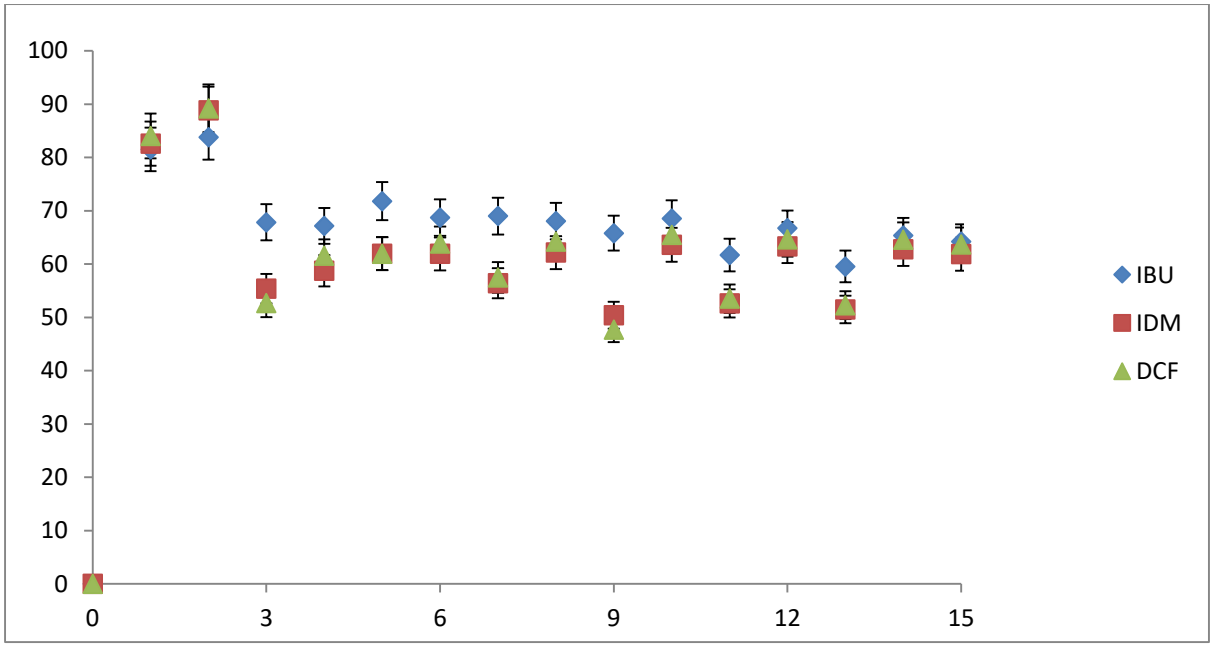


Figure 4.12 Efficiency removal of mixture of IDM, IBU and DCF by MIP-DCF template, in the continuous-flow mode with flow rate 1.67 ml/min at room temperature.

Table 4.2 % removal for IBU, IDM and DCF using MIP-IDM as sorbent at 1 minute.

	IBU (220 nm)	IDM (260 nm)	DCF (280 nm)
Final concentration (µg/mL)	0.465	0.00	0.050
Initial concentration (µg/mL)	0.465	0.945	0.466
% removal	0	100	89

Table 4.3 % removal for IBU, IDM and DCF using MIP-DCF as sorbent at 1 minute.

	IBU (220 nm)	IDM (260 nm)	DCF (280 nm)
Final concentration (µg/mL)	0.759	1.173	1.110
Initial concentration (µg/mL)	4.103	6.743	6.952
% removal	82	84	83

There were few problems encountered during experiment, first there was always air bubbles trapped into the cuvette what can provide inaccurate results during the absorption reading.. The pattern produced between MIP-IDM and MIP-DCF were not similar during the first minute. Within the first minute, the MIP shows significant separation for both MIP-IDM and MIP-DCF. Based on the results observed, the continuous-flow mode using UV spectrophotometer was successful in terms of removing the NSAIDs from water. However,, the method can be improved by using HPLC detection analysis which more precise and higher sensitivity compared to spectrophotometer.

4.4.2 Offline EPPPs detection via conventional C18 column equipped to HPLC-UV detector using different matrix samples

4.4.2.1 Blank analysis

In order to optimize the analysis, blank analysis is necessary to be done first. After washing the MIP-DCF using 2 mL of ethanol:water, 5 mL of mili-Q water and 5 mL of blank solution (5%ACN:mili-Q water, 1% acetic acid in water), were passed through the MIP and analyzed by the HPLC-UV detector. The blank analysis was performed in duplicate. The mobile phase for the present work was a combination of acetonitrile and acidified water. The ratio of volume of acetonitrile was shown in **Table 4.4** below.

Table 4.4 Parameters setting for HPLC separation using C18 column.

Time (min)	Acidified water (%)	Acetonitrile (%)	Flowrate (mL/min)
0	50	50	1.00
5	42	58	1.00
6	42	58	0.50
30	5	95	0.50

4.4.2.2 Analysis of DCF from natural water via HPLC

The calibration of DCF in the C18 column was performed in 5% (v/v) ACN:water. , The limit of detection (LOD) was 2.31 µmol/L whereas limit of quantification (LOQ) equals to 7.01 µmol/L. Range for the calibration curve for 5%(v/v) acetonitrile/water was between 5 mmol/L to 10 mmol/L.

The recovery method by MIP-DCF followed was developed in *chapter 3* and implemented in the samples from the river spiked with 0.5 mg/L of DCF and un-spiked. In prior to be tested using water samples, deionized water was used in order to optimize the analysis. During the first regeneration, the percent recovery was 97%. However, for the second regeneration onwards, the percent recovery was reduced and the analysis need to be optimized.

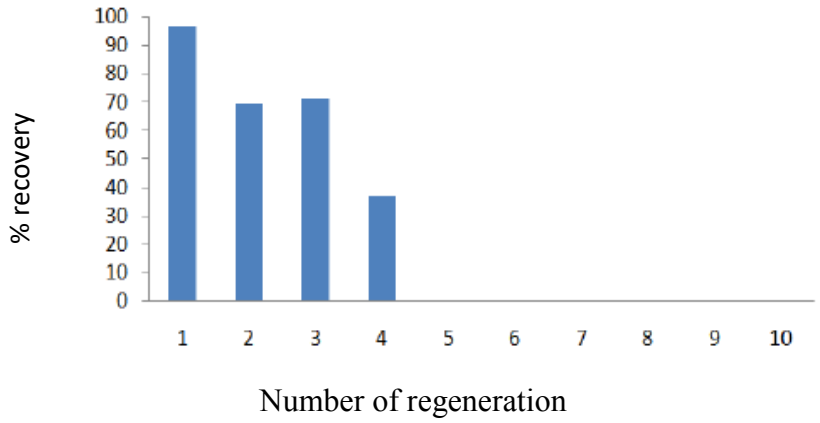


Figure 4.13 % recovery of DCF spiked in water (before the method optimized) using MIP-DCF as a sorbent using HPLC separation.

However, recovery of DCF was performed consecutively for 10 times in order to evaluate the good performance of MIP-DCF as explained in *chapter 3*). As shown in **Figure 4.14** and **Figure 4.15**, the DCF nmol recovered was low and fluctuated between experiments due to many disturbance components in WWTP water samples during removal analysis by using MIP-DCF as a sorbent. The recovery analysis calculation followed the **Equation 4.2**. Another reason for the fluctuation in each result is because different WWTP water samples was used. Although it came from the same source, still the components can be different. Similar finding was obtained by using outlet water samples. (**Figure 4.16** and **Figure 4.17**).

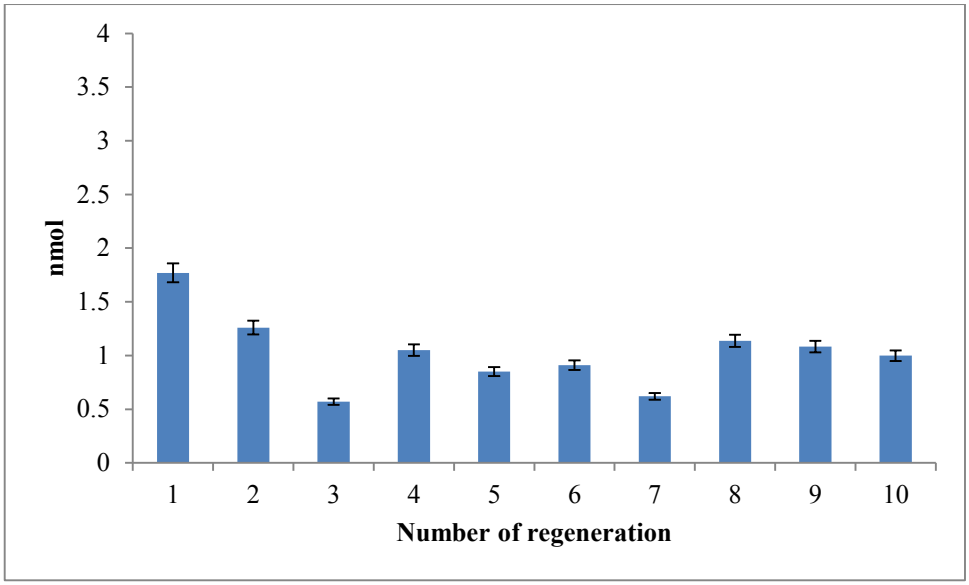


Figure 4.14 nmol recovery of inlet WWTP samples for first replicate until 10th regeneration with average recovery: 1.0 ± 0.3 nmol. Initial concentration DCF spiked: 1.7 µmol/L.

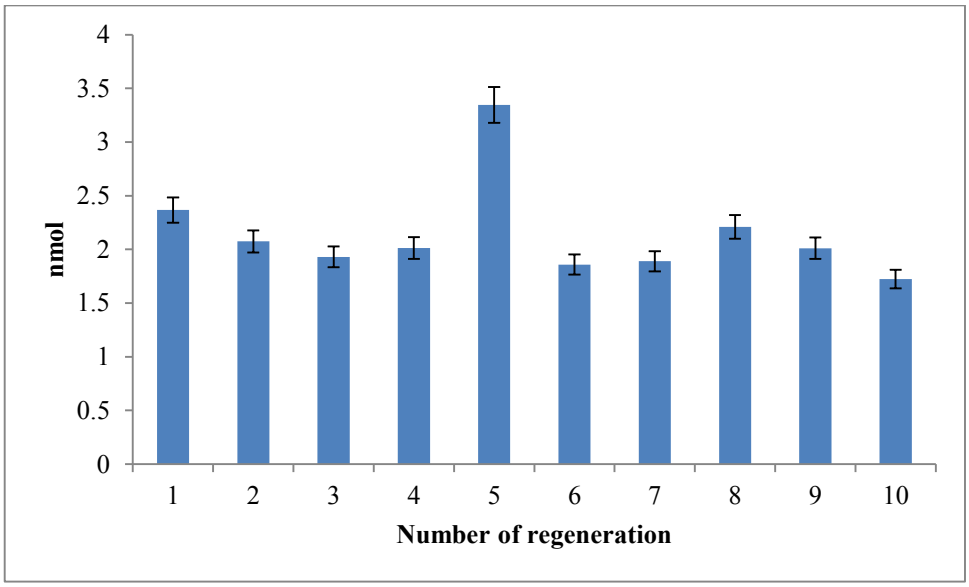


Figure 4.15 nmol recovery of inlet WWTP samples for second replicate until 10th regeneration with average recovery: 2.1 ± 0.5 nmol, initial concentration DCF spiked: 1.7 µmol/L.

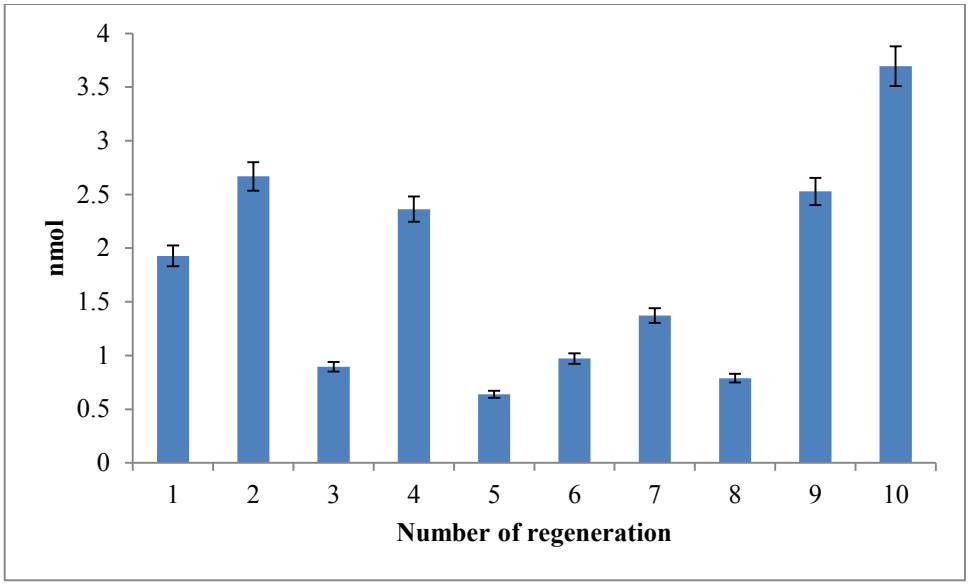


Figure 4.16 nmol recovery of outlet WWTP water samples for first replicate until 10th regeneration with average nmol recovery: 1.8 ± 1.0 nmol, initial concentration DCF spiked: 1.7 µmol/L.

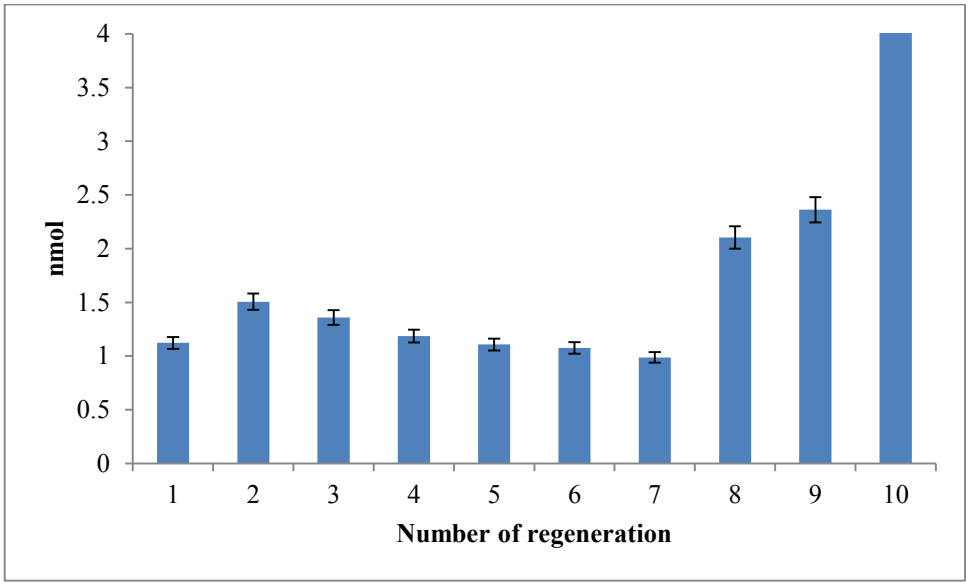


Figure 4.17 nmol recovery of outlet WWTP water samples for second replicate until 10th regeneration with average nmol recovery: 1.9 ± 1.5 nmol, initial concentration DCF spiked: 1.7 µmol/L.

From the results obtained, the efficiency recovery of DCF was approximately higher in outlet WWTP water samples compared to inlet WWTP water samples because it was expected the presence of other micropollutants in water before passing through WWTP However, this

conventional WWTP was not enough to eliminate the emerging contaminants such as DCF from the water. Refer to **Table 4.5**.

Table 4.5 Recovery (nmol) with standard deviation (S.D.) of DCF for inlet and outlet Amposta WWTP

	recovery (nmol)	S. D.
Inlet WWTP	1.6	0
Outlet WWTP	1.8	1

The recovery in the present work was done using WWTP water samples showing high sorption capacity, approximately three times more than the previous study which the experiment conducted in aqueous media using similar MISPE method, but different synthetised MIP. Hence, MIP-DCF synthetized in the present work shows significant improvement compared to previous study.

In the present work, the efficiency recovery in the inlet and outlet WWTP water samples were approximately lower compared to synthetic aqueous media **(Table 4.6)**, as expected due to the presence of other compounds in natural or waste waters in the matrix. Similar finding was observed by Sun.[22]

Table 4.6 Efficiency recovery of DCF by MIP-DCF for 1st cycle.

	Ave. % recovery	S. D.
miliQ water	84.53	15.66
Inlet WWTP	36.85	22.41
Outlet WWTP	42.11	14.95

In present work, mobile phase used for the HPLC was acidified water (1% acetic acid in water) and acetonitrile. Acetonitrile has been used as the main mobile phase for C18 column in many DCF separation analysis using HPLC.[23,24] Acetonitrile was categorized in organonitriles group which has been classified as priority pollutants due to the carcinogenic and mutagenic character.[25] Further experimental procedure has been developed to improve the method in order to decrease the consumption of acetonitrile as the mobile phase. The new mobile phase for DCF separation via HPLC coupled to UV detector has been introduced in the next sub-chapter.

4.4.3 Online analysis using MIP-DCF as packing for the HPLC column

The calibration of DCF in 75% (v/v) ethanol:water. The calibration curve obtained had R^2 value equals to 0.9623. Limit of detection (LOD) was 6.7 µmol/L whereas limit of quantification (LOQ) equals to 20.4 µmol/L. Range for the calibration curve was between 0.05 mmol/L and 5 mmol/L for 75%(v/v) ethanol/water. The 75% (v/v) ethanol/water has been used for further experimental due to the dissolution properties of MIP-DCF synthetised in acetonitrile. Moreover, ethanol is preferred to be used due to safety issues comparing to acetonitrile, being also less expensive.

4.4.3.1 The MIP-DCF column packing

The glass column with inner diameter of 3 mm and 15 cm long was successfully packed with MIP-DCF **(Figure 4.18)**. Next, the packing process was started by running the mobile phase through the glass column. The content of the column can be seen through the holes of the column holder so the movement of the MIP while packing the columns can be seen **(Figure 4.19)**. The mobile phase used in this study was a mixture of ethanol and water. The uniqueness of this study was that the mobile phase was not an acidified solvent. The similar procedure which used non-acidified solvent has been shown by Nuria.[26]

Figure 4.18 Successful MIP-DCF packed in the glass column with inner diameter 3 mm and 15 cm long.

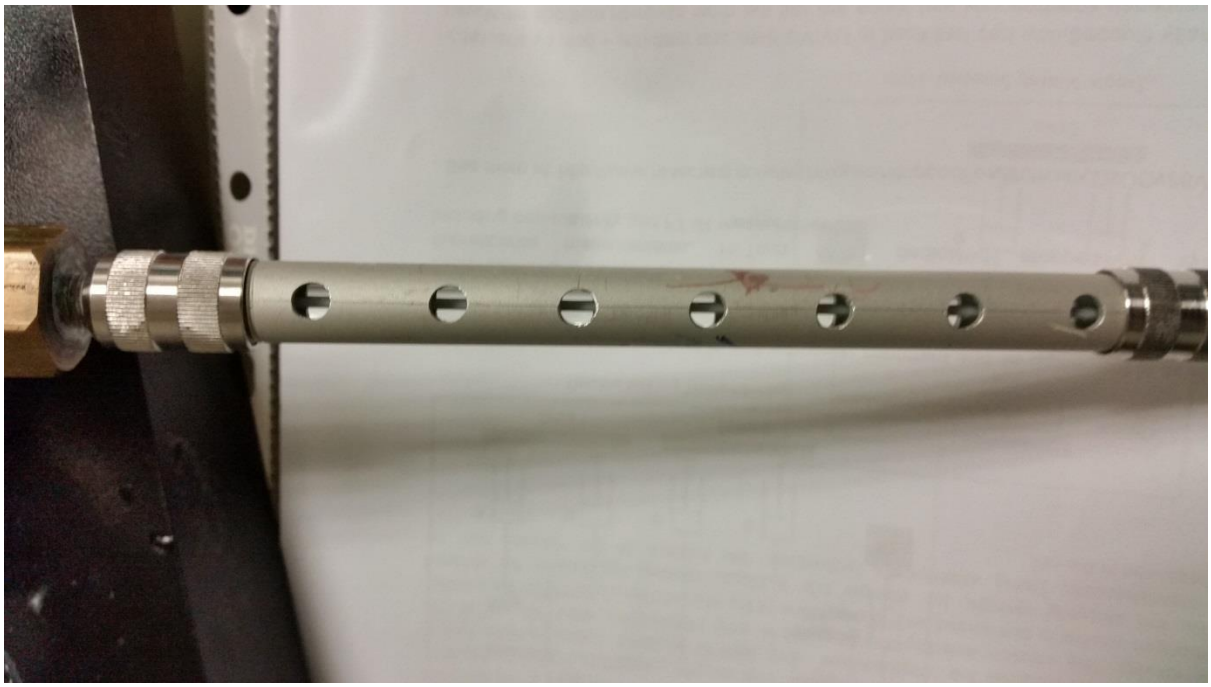


Figure 4.19 The column holder in which the MIP particle movement while packing can be seen through the holes.

4.4.3.2 Morphology study of MIP-DCF for column packing purpose

MIP-DCF after sieving within range 5µm to 10µm was then analysed using Field Emission Scanning Electron Microscope (FESEM) as shown in **Figure 4.20**. At 1.00 KX of magnification power, the MIP-DCF particles was highly homogenous in terms of diameter of particle size. The morphology of one MIP-DCF particle is shown in **Figure 4.21**. According to the findings obtained, MIP-DCF was shown to be very porous sorbent and the cross linked polymer can be seen clearly in the **Figure 4.22**.

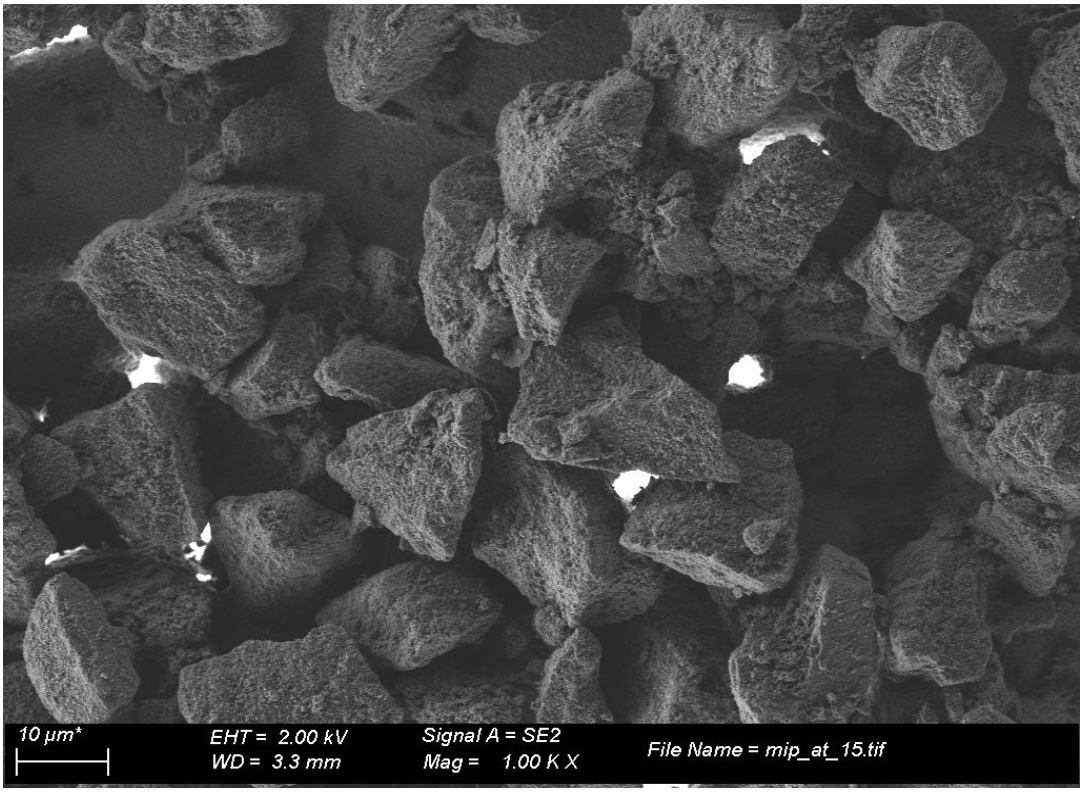


Figure 4.20 Surface morphology of MIP-DCF at 1.00 KX of magnification power, EHT: 2.00 kV.

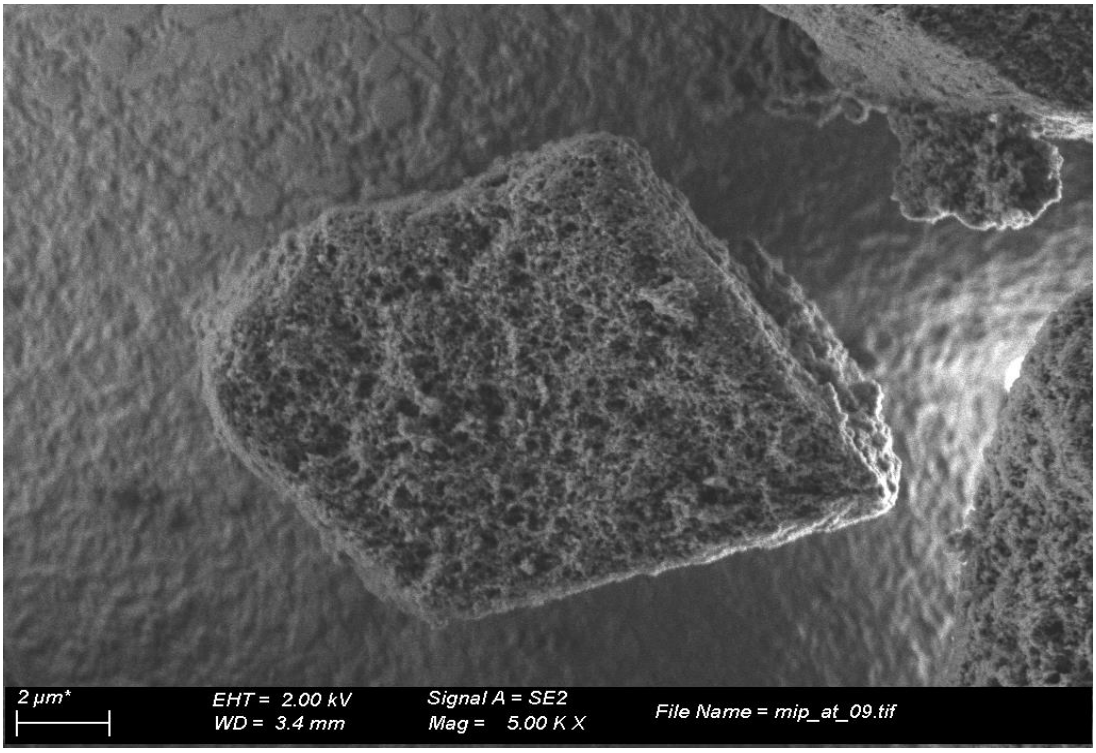
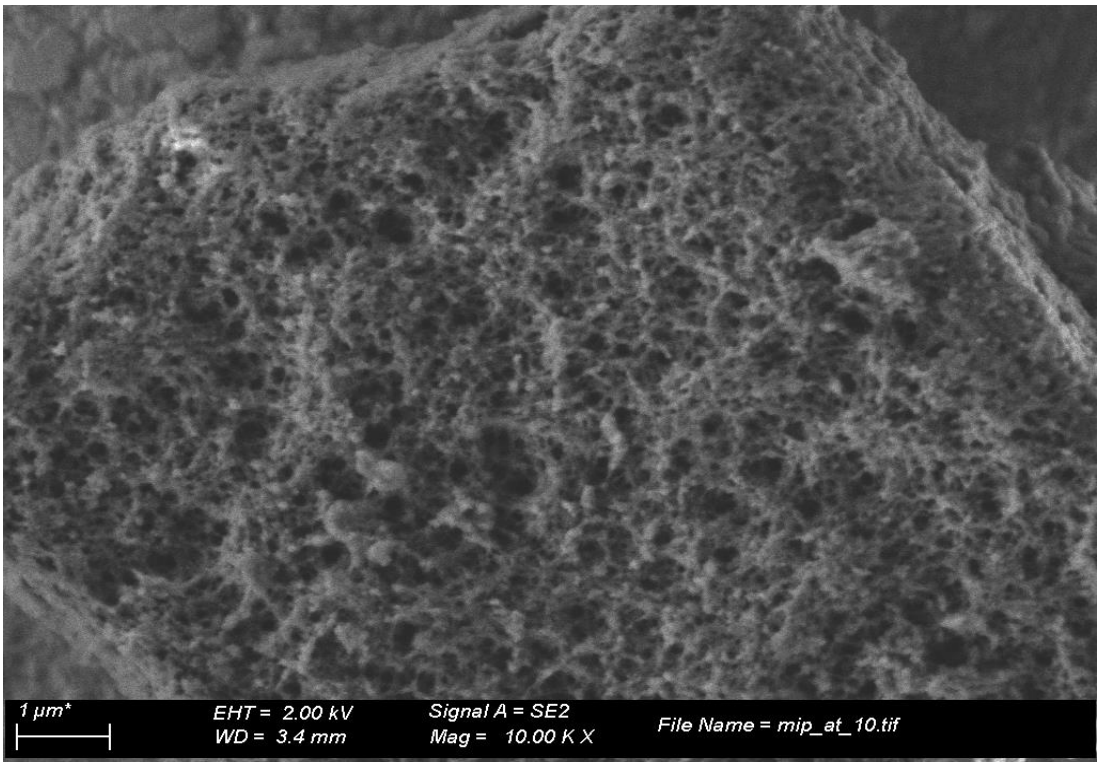
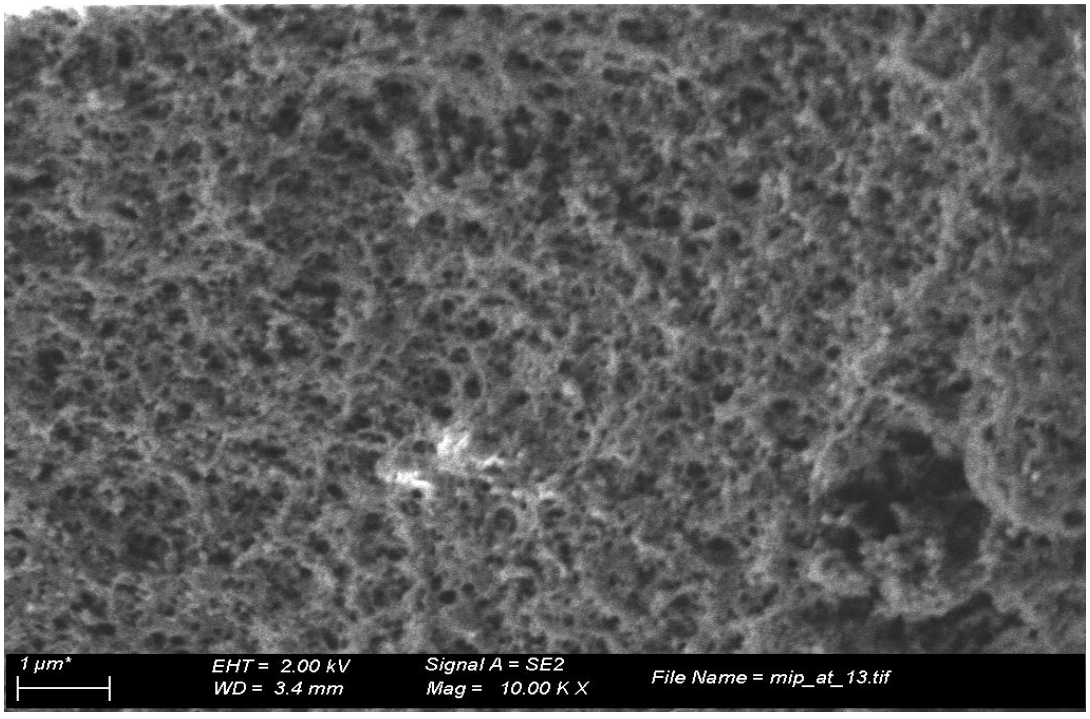


Figure 4.21 Surface morphology of MIP-DCF for single particle at 5.00 KX of magnification power, EHT: 2.00 kV.



(a)



(b)

Figure 4.22 Surface morphology of MIP-DCF for single particle at 10.00 KX of magnification power, EHT: 2.00 kV (a) & (b).

4.4.3.3 Separation between DCF and IBU using MIP-DCF as packing media via online analysis

The analysis was done in reversed phase and gradient mode until 15 minutes per analysis. The mobile phase were ethanol and water. The gradient for HPLC separation was optimized to finally find the one shown in **Table 4.7**. The pressure of column was reached maximum at 1700 psi per analysis. The injection volume was 100 µL.

Table 4.7 Gradient optimized for HPLC separation using MIP pre-packed column.

Time	Water (%)	Ethanol (%)	Flowrate (mL/min)
0	95	5	0.5
15	25	75	1.0
16	25	75	0.5

For the blank analysis, the blank solution was injected with the specified method and no peak was observed.

5 µg/mL of DCF solution was prepared and filtered. Then, the solution was poured into into small vials for HPLC analysis. From the **Figure 4.23**, DCF peak was observed at 7.0 minutes of retention time, T_R.

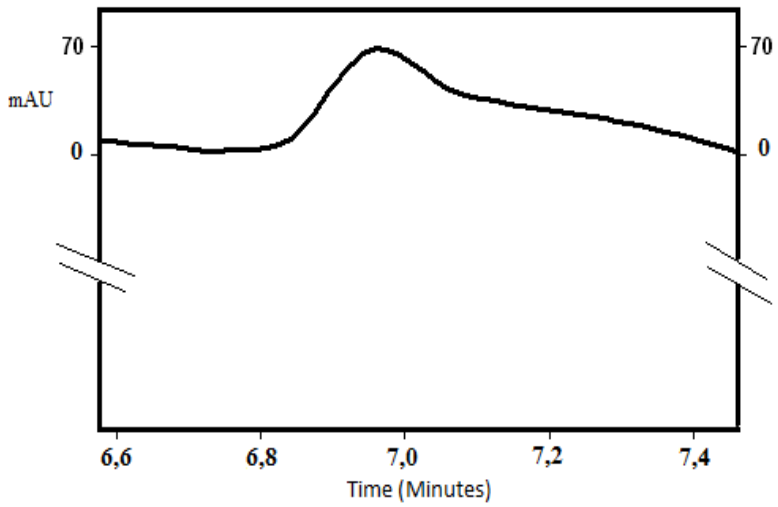


Figure 4.23 HPLC chromatogram of DCF using MIP-DCF as the stationary phase packed in column at 7.0 minutes of retention time.

DCF has absorbance maximum at 273 nm that tails well over 300 nm.[27] The spectral observed for DCF detection is shown in **Figure 4.24**.

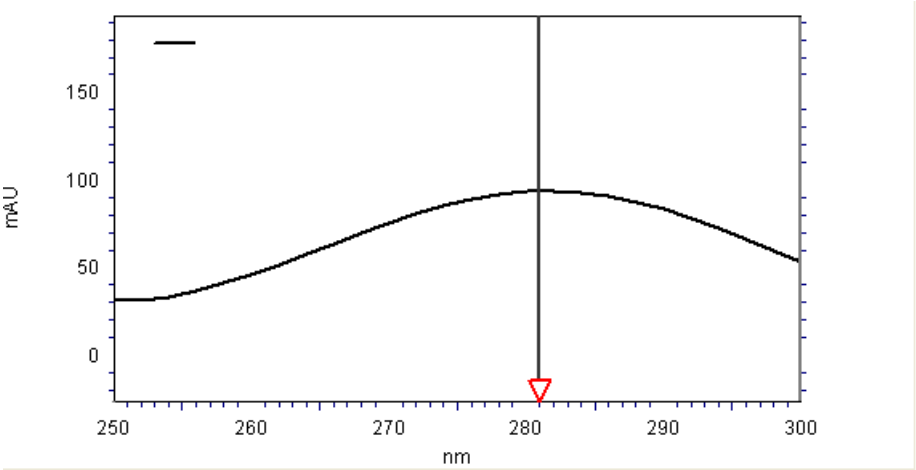


Figure 4.24 Spectral of DCF detection using MIP-DCF as stationary phase with initial concentration at 5 µg/mL at 280 nm of maximum wavelength (λ_{max}).

By using commercial C18 column, DCF peak was observed at 19 minutes of retention time **(Figure 4.25)** $t_R$which was longer compared to MIP-DCF as stationary phase.

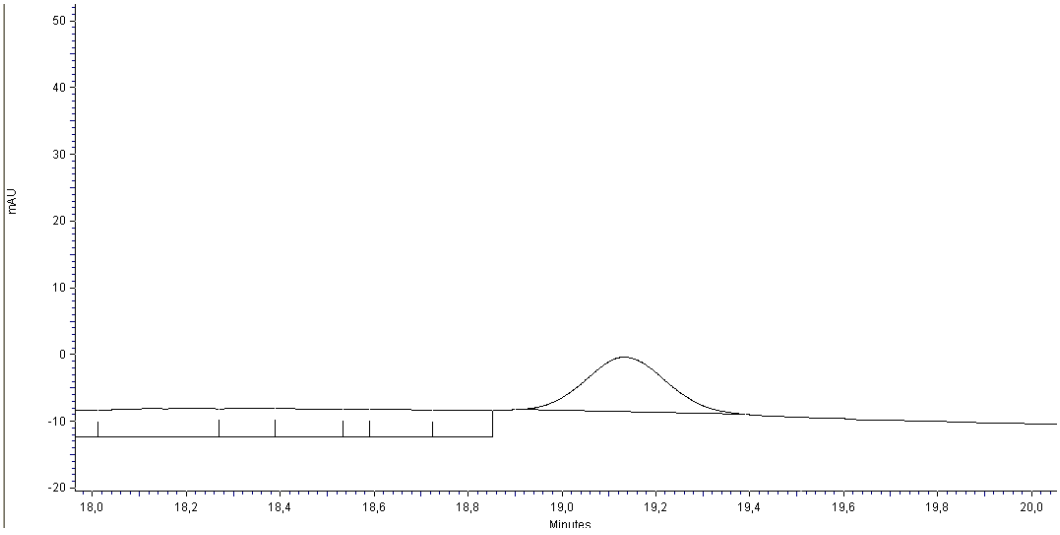


Figure 4.25 Chromatogram of DCF using a commercial C18 column in aqueous media.

When using the HPLC-DAD at 280 nm of maximum wavelength with the synthetised MIP-DCF as the stationary phase, the separation of IBU and DCF was successfully observing peaks at 5 minutes and 7 minutes of retention time respectively as clearly shown in **Figure 4.26.**

The photo stability of IBU is explained by its UV-visible spectrum, which exhibits a maximum at 223 nm and shows little absorbance in the wavelength region of the solar spectrum.[27]

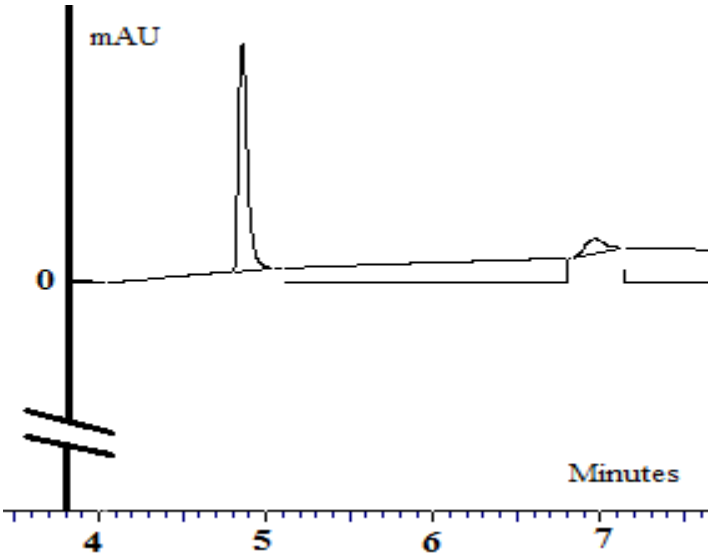


Figure 4.26 Separation of IBU and DCF qualitatively with initial concentration 5 µg/mL with MIP-DCF as stationary phase at 5.0 minutes and 7.0 minutes respectively.

The mixture involved were IBU and DCF with 5 µg/mL of concentration per each. Percent recovery of DCF in mixture was approximately at 75% calculated by **Equation 4.3**.

$$\%\, recovery = \frac{[DCF]\ in\ mixture}{Initial[DCF]} \times \ 100\% \qquad \textbf{Equation 4.3}$$

In other previous study conducted by Sun et al [22] for the separation of DCF from raw influent wastewater, the polymer chromatographic evaluation was done in isocratic mode and ACN/H_2O (60:40, v/v) with 0.1M acetic acid was used as the mobile phase at a flowrate of 0.8mL/min.[22] The separation of DCF took more than 20 minutes to observe the peak.

The concept of particle movement in a column is shown in **Figure 4.27.** DCF and IDM (represented in orange and green points respectively) are separated in the column due to the higher interaction of DCF with the MIP. From the chromatogram in **Figure 4.26**, sharp peak

is observed for IBU indicating that the movement of particles was smooth and does not fully resolved in the stationary phase. However, the broad peak observed for DCF is probably due to the strongest interaction of the active functional group on DCF molecules with functional sites of MIP-DCF via hydrogen bond . The statement mentioned was also agreed by Wenzel.[28]

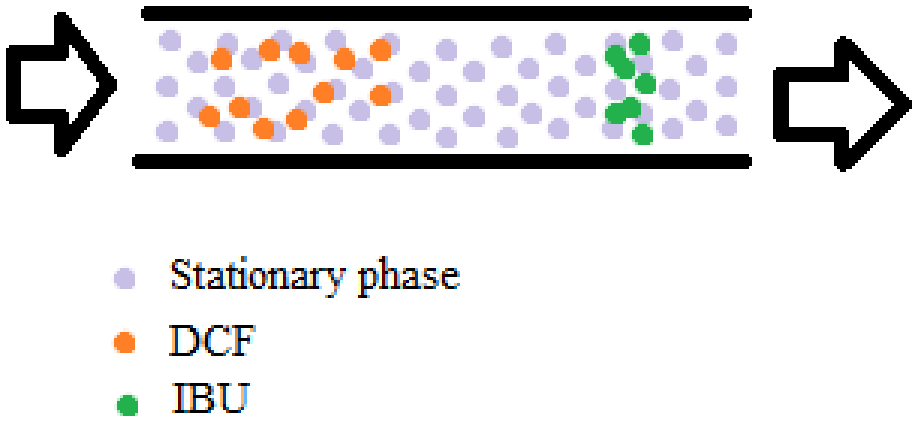


Figure 4.27 DCF molecules tends to remain longer in the column compared to IBU due to the strong hydrogen bonding between MIP-DCF and DCF molecules.

4.5 CONCLUSION

In the last decade, several researchers have begun to study the use of MIPs for water and wastewater treatment.[29,22,30,31,32] Many kinds of treatment methods via MIPs have been developed. In the present study, three approaches of developing analysis methods have been studied. Generally, the objectives of study were successfully achieved.

- The first approach of treatment method involved the use of MISPE in continuous-flow mode coupled to spectrophotometry detection. In this approach, the results obtained shows that the mixture solution contained with three components (DCF, IDM and IBU) can be removed from water using continuous flow mode with high efficiency removal. By using direct detection via spectrophotometry, the separation among mentioned pharmaceuticals were poor especially when there is more than two components in a mixture solution. This is the challenging in removing the organic micropollutants from water.

- In the second approach, the developed method was an offline analysis of DCF separation using river water samples from Ebro river, Spain via commercial C18 column equipped to HPLC with UV detector. From the findings obtained, the recovery analysis was disrupted by other micropollutants and the interferences which present in water samples are the major factor on the declining of MIP-DCF ability. In addition, the preparation of MIP-DCF via bulk polymerization caused the imprinted sites being broken. Hence, it is suggested that the preparation of MIP-DCF can be improved via precipitation polymerization in future.

- The third approach is the development of a methodology where the MIP-DCF was packed into an HPLC column. As the results, the analysis shows that the separation between IBU and DCF was complete and within shorter time than using commercial C18 column. In addition, in this study the mobile phase used was ethanol and water instead of acetonitrile usually used for commercial C18 column. Hence, the present finding shows that the recovery analysis was environmental-friendly and economic.

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Chapter 5

Conclusions

Chapter 5

CONCLUSIONS

As a summary, three scopes of work have been constructed in this study which were; firstly, the synthesis of MIPs (MIP-DCF and MIP-IDM) and MIPs characterization in batch mode was succeeded. Secondly, MIPs characterization via molecularly imprinted solid phase extraction (MISPE) including total saturation capacity study. Thirdly, analytical methods for application of molecularly imprinted polymer using selective functional monomer for diclofenac recovery from water and wastewater were constructed. For each scope of work, it can be concluded that;

5.1 Synthesis and Characterization of Molecularly Imprinted Polymer Using Selective Functional Monomer for Diclofenac and Indomethacin Removal in Aqueous Media via Batch Mode

In the first scope of work, there were three objectives of study which were successfully achieved.

The main objective was to synthetize new molecularly imprinted polymers (MIPs) with DCF and IDM as the template using allylthiourea (AT) as the monomer individually. Non-imprinted polymer (NIP) also was synthetized using similar approach.

- After trying different approaches, the new synthetized MIPs via bulk polymerization using AT as a functional monomer was successful prepared.
- The binding sites study showed more than 90% of efficiency removal for both MIP-DCF and MIP-IDM.
- The NIP showed poor removal of target molecule meaning that the retention of the MIPs was due to the well-template of target molecule during the polymerization.
- MIPs with AT as the functional monomer were more attracted to DCF instead of IDM because the N-H functional group located at the center of DCF molecule was bonded to the functional sites of MIPs
- DCF has a smaller molecule size and is easily trapped into the imprinted cavities located in MIP-IDM.

- The selectivity study shows that the synthetised MIP was possessed towards DCF instead of IDM due to the N-H functional group located at the center of DCF instead of IDM compound which favor react to the active functional sites of the MIP. In addition, the size of DCF molecules was smaller than IDM molecules. Hence, DCF was favor to be trapped into the cavities.
- The removal of DCF and IDM using MIP synthetised in the present work required less time for the removal of 90% of the compound and is less laborious to characterize.
- The experimental results obtained in pre-polymerization study was consistent to the suggested scheme reaction of MIP synthesis.

5.2 Characterization of Molecularly Imprinted Solid Phase Extraction using Selective Functional Monomer for Removal and Recovery of Diclofenac and Indomethacin from Aqueous Media

In this scope of work, the molecularly imprinted solid phase extraction (MISPE) has been developed and all the objectives were successfully achieved. It can be concluded that:

- The total saturation profile was successfully calculated via MISPE method.

- MIP-DCF shows homogeneous properties whereas MIP-IDM shows heterogeneous properties due to the reaction occurred between N-H functional group on the monomer crosslinked polymer with N-H functional group at the center of DCF molecule.

- The interaction between analyte and functional site on MIP was depending on the active functional sites AT which was very selective towards N-H functional group and because of that the MIP-DCF was chosen for the application procedures.

- When there were more than one active functional sites on the MIPs, it was determined that the MIPs will have high affinity towards the analyte in sorption and desorption process.

- From the infrared spectra, the ability of MIP to be used more than once have been proven. Each functional group in MIP has been determined which were N-H stretch

functional group at 3000 nm^{-1} found in original MIP, C-N stretch bond at 1180 nm^{-1} found in original MIP, OH broad peak at 3300 nm^{-1} found in MIP loaded with DCF, C=O bond at 1750 nm^{-1} found in each MIPs and C=C bond at 1650 nm^{-1} found in MIP loaded with DCF.

5.3 Analytical methods for application of Molecularly Imprinted Polymer Using Selective Functional Monomer for Diclofenac Recovery from Water and Wastewater

Third scope of the present work was the application of MIPs using spectrophotometry and High Performance Liquid Chromatography (HPLC). Recently, several researchers have begun to study the use of MIPs for water and wastewater treatment. In present study, there are three approaches of treatment methods. Generally, the objectives of study were achieved successfully.

- The first approach of treatment method involved with MISPE in continuous-flow mode coupled to spectrophotometry detection. In this approach, the results obtained shows that the mixture solution contained with three components (DCF, IDM and IBU) still cannot be separated and need more advance method development in doing so.

- In the second approach, the treatment method involved was offline analysis of DCF separation using river water samples from Ebro River, Spain via commercial C18 column equipped to HPLC with UV detector. From the findings obtained, the recovery analysis was disrupted by other micropollutants and the matrix of the river water samples are the major factor on the declining of MIP-DCF ability in isolation of DCF from the matrix. In addition, the preparation of MIP-DCF via bulk polymerization caused the imprinted sites to be broken. Hence, it is suggested that the preparation of MIP-DCF can be improved via precipitation polymerization in future.

- The third approach is the treatment method involved with the MIP-DCF packing into column and online recovery analysis of mixture using column with MIP-DCF as the packing media equipped to HPLC-UV. As the results, the recovery analysis shows an important finding which was the separation between IBU and DCF within shorter time

than using commercial C18 column. In addition, in this study the mobile phase used was ethanol and water compared to commercial C18 column. In C18 column, the mobile phase used was acetonitrile and acidified water. Hence, the present finding shows that the recovery analysis was environmental-friendly and economic.

APPENDICES

Note:

Manuscript 1 entitled “Modelization of the Diclofenac and Indomethacin Recovery by Molecularly Imprinted Polymer” was submitted to the *International Conference Young Chemists 2017 Special Issue in Journal of Physical Sciences*.

Manuscript 2 entitled “Diclofenac Removal from Water by Molecularly Imprinted Polymer” will be submitted to the *Science of the Total Environment*.

Manuscript 1

Modelization of the Diclofenac and Indomethacin Recovery by Molecularly Imprinted Polymer

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Modelization of the Diclofenac and Indomethacin Recovery by Molecularly Imprinted Polymer

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ABSTRACT: *Pharmaceutical compounds in surface waters are an emerging environmental concern due to their biological activity and consequently provide a new challenge for drinking water and wastewater treatment systems. In this work, Diclofenac (DCF) and Indomethacin (IDM) have been used as target molecules or templates in order to be removed by means of Molecularly Imprinted Polymers (MIP). MIP-DCF and MIP-IDM were synthesized and tested using two mathematical models such as Lagergren and Thomas models. In addition, functional groups of the active functional sites and the target molecules have been also studied by using Fourier Transform Infrared spectroscopy. Both MIPs followed the Lagergren pseudo first order kinetic model. Comparing the original MIP-DCF, MIP-DCF after loading with DCF and MIP-DCF after 10 times of regeneration, differences in their active functional sites were found. In conclusion, MIP-DCF and MIP-IDM work as good sorbents to recover these emerging pharmaceuticals persistent pollutants from water.*

Keywords: Molecularly imprinted polymer, MIP, Diclofenac, Indomethacin

1. INTRODUCTION

Few decades ago, Emerging Persistent Pharmaceutical Pollutants (EPPPs) have been introduced as one type of recalcitrant pollutant sources in water.[1] Recent studies have demonstrated that, despite the relatively low concentrations of pharmaceuticals in the environment (typically in sub-parts-per-billion levels), pharmaceuticals are of great concern due to their potential long-term adverse effects on humans and wildlife.[2]

Nowadays, there are many methods used for the recovery of pharmaceuticals, such as photocatalysis[3] and sorbents, but present some drawbacks, e.g. after photocatalysis the by-product generated may be more harmful than the parent compounds. There are many kinds of sorbents that have been studied, but not all the sorbents have the potential to recover the target molecules. In this work, Molecularly Imprinted Polymers (MIPs) as a separation method is proposed and

characterized. The polymeric matrices obtained using the imprinting technology, are robust molecular recognition elements able to mimic natural recognition entities.[4] MIPs are also good sorbents because of their porosity and their robustness properties. In this study, the removal of Diclofenac (DCF) and Indomethacin (IDM) have been carried out using MIPs, synthetized via bulk polymerization with allylthiourea (AT) as the functional monomer and using DCF or IDM as template (MIP-DCF or MIP-IDM, respectively). DCF and IDM were detected using spectrophotometry.

To study the properties of the sorbents, MIPs have been tested in batch mode and continuous flow mode. In the batch mode, Lagergren model was used to describe the adsorption properties. However, in the continuous flow mode, Thomas model was used for the breakthrough study. On the other side, the functional groups can be used as a fingerprint for certain MIPs to show if the active functional sites work. However, there are few articles reporting on this parameter which give important information about MIP development. FTIR-ATR has been used to observe the different types of MIPs and to study their functional sites.

2. EXPERIMENTAL

2.1 Materials

Indomethacin (IDM) was purchased from Sigma-Aldrich, Spain, with 98-99% of purity (TLC grade). Diclofenac-Na was from Cayman Chemical, United States with purity of more than 99%. Ethanol with 96% purity from Scharlau Chemical, Spain. Acetic acid was from J. T.Baker, United States with 99.9% of purity, respectively, and HPLC grade. Acetonitrile (99% of purity) from VWR company with HPLC grade. Grace Alltech 'Extract Clean' empty reservoirs with silica frits, 1.5 mL from Fisher Scientific, United States.

2.2 Synthesis of MIP-DCF and MIP-IDM

Synthesis of MIPs has been done through polymerization reaction. The process started by mixing the template and allylthioura in acetonitrile Then, Ethylene Glycol Dimethacrylate (EGDMA) and Azobisisobutyronitrile (AlBN) were added followed by a degassing procedure (via sonication or nitrogen gas purge). Polymerization occurred for 24 h in a water bath at 60 ^{o}C, and the prepared polymer was crushed and located into the Soxhlet extractor with methanol as a solvent in order to remove the template.

2.3 Recovery Study

10 mg of MIP-DCF were weighted and placed into a 1.5 ml cartridge. Then, 1 mL of ethanol:water (75% v/v) was passed through it twice to activate the MIP-DCF. Next, 1 mL of water was added for 5 times to rinse out the ethanol residue. Afterwards, acetonitrile:water (5% v/v) was added twice for conditioning the MIP-DCF. Later, 1 mL of 15 µg/mL of DCF was added twice. The eluted solution was collected and analysed using an UV-vis spectrophotometer. After that, 1 mL of ethanol:water (75% v/v) was loaded twice into the cartridge and the collected solution was also analysed by UV spectrophotometry. The procedure was done in triplicate. The percentage of recovery was calculated using Equation 1, where C_i and C_f represent the analyte concentration for initial and final respectively.

$$\% \text{ Recovery} = \frac{C_f - C_i}{C_f} \times 100 \quad (1)$$

2.4 Kinetic study

10 mg of MIP-DCF were individually weighted and placed in 5 mL tubes. 2 mL of 5 mg/L DCF solution were poured into the tubes [5] and were covered using aluminum foil. After 120 min of agitation, the solutions were filtered using a 0.22 µm pore size syringe filter. The solutions were then analysed by UV spectrophotometry at 280 nm. The procedure was performed in triplicate. The same methodology was followed for MIP-IDM using IDM solution.

2.5 Breakthrough experiment – Continuous flow mode

Due to the immiscibility properties of DCF in water, total sorption capacity has been carried out in acetonitrile: water (5% v/v) media solution. 10 mg of MIP-IDM was accurately weighted and placed in a cartridge of 1.5 mL of capacity. 15 µg/mL of IDM was prepared in acetonitrile: water (5% v/v). Then, the solution was loaded into the cartridge using a peristaltic pump at 1.67 mL/min of flow rate. The eluted solution was collected in fractions of 5 mL until 50 mL, then, the fractions collected were of 50 mL. The solution was continuously-flowing until the absorbance measurements of the eluted solution reached a value similar to the absorbance of the initial concentration, meaning that no more absorption was occurring. At this point, the process was stopped and all solutions were collected and analysed in the UV spectrophotometer. The procedure mentioned above was done in duplicate. The same methodology was repeated for MIP-DCF as the sorbent and in the absence of MIP, in order to measure any other contribution to the elimination of the target compounds i.e. sorption in cartridge or frits. The obtained breakthrough curves were then modeled and the main constants values of the best fitting model were determined.

2.6 Fourier Transform Infrared analysis

Attenuated Total Reflectance Fourier Transform Infrared spectroscopy (ATR-FTIR) has been used in this study to determine the functional groups in MIP-DCF for 2 different kinds of MIP-DCF: original MIP-DCF, MIP-DCF after loading with 2 mL of DCF solution (5 µg/mL) via molecularly imprinted solid phase extraction.

3. RESULTS AND DISCUSSION

3.1 Recovery study

The percentage of recovery of IDM and DCF using MIP-IDM and MIP-DCF were 98± 3% and 97 ± 2% respectively. For the regeneration experiment, the recovery procedure was repeated for 10 times (consecutive extractions) in order to observe the loading capacity of MIPs. The ability to sorb-desorb for the synthetized MIPs were not significantly decreased after 10 cycles reaching almost 100% of recovery.

3.2 Lagergren Model Application

From the kinetics, the 100% removal was obtained starting at 3 min of removal in batch mode. It is well accepted that two-parameter adsorption kinetic equations (Lagergren first-order and second-order) are useful tools to describe the adsorption properties of a sorbent.[6] The Lagergren pseudo first-order equation can be linearly expressed as Equation 2, where Q_e and Q_t are the adsorption capacities (mg/g) of MIP at equilibrium and at time t, respectively; k_1 is the first order rate constant. Figure 1 shows that both MIPs fitted to the Lagergren pseudo first order model. For further experimental procedure, MIP-DCF was used as a sorbent.

$$\text{Ln}\,(Q_e - Q_t) = \text{Ln}Q_e - k_1 t \quad (2)$$

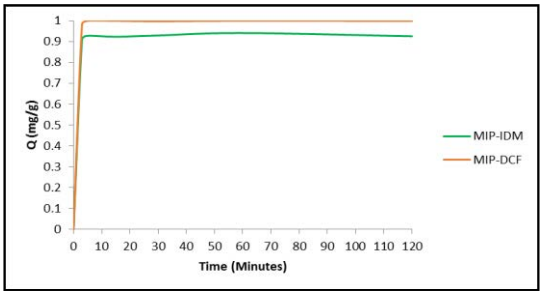

Figure 1: Adsorption capacities (Q) of MIP-IDM and MIP-DCF in front of time following Lagergren pseudo first order model plot.

3.3 Breakthrough study for MIP-DCF

Breakthrough in column can be identified as the amount of influent solution passing through the bed before a maximum effluent concentration is reached. The ratio C_t (effluent concentration)/ C_o (influent concentration) is plotted against the volume of the analyte solution loaded and also the time as seen in Figures 2a and 2b. They show that the breakthrough volume and the sorption time of DCF using MIP-DCF is similar to the kinetic study observation where within 3 min the sorption uptake was higher than 80%, thus resulting in relatively faster attainment of breakthrough. Breakthrough and exhaustion are defined as outstanding when the ratios of effluent-to-influent concentration are between 70% and 99%, respectively. In this work, the breakthrough point was reached at 35 mL (24 min) and the exhaustion point at 270 mL (450 min). The methodology of adsorption in fixed bed cartridge by the breakthrough curves method proved to be a good option to study the separation of DCF in water.

3.4 Fourier Transform Infrared analysis

FT-IR spectrum in Figures 2(a) and 2(b) show that the functional sites on MIP work well with the analyte (DCF). The different kinds of MIPs have been differentiated via FTIR-ATR and the spectra obtained could be used as a fingerprint in order to distinguish the different MIPs. N-H stretch functional group at 3000 nm^{-1} and C-N stretch bond at 1180 nm^{-1} found in original MIP (Figure 2(a)); OH broad peak at 3300 nm^{-1} found in MIP loaded with DCF (Figure 2(b))

due to –OH functional group from DCF bonded to MIP-DCF. Probably there is also N-H functional group that might contribute in this peak; C=O bond at 1750 nm^{-1} found in all MIPs; and C=C bond at 1650 nm^{-1} found in MIP loaded with DCF.

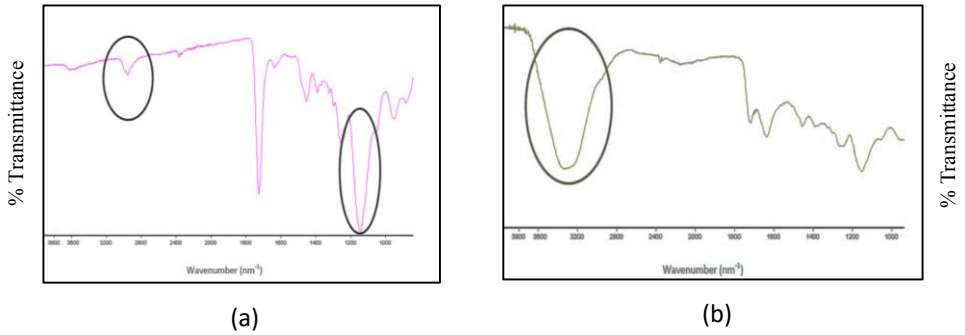

Figure 2: Infrared spectrum of (a) original MIP-DCF and (b) MIP-DCF loaded with DCF.

4. CONCLUSION

In conclusion, MIP-DCF and MIP-IDM have shown to be highly effective for the removal of the pharmaceutical compounds DCF and IDM. MIP is a promising analytical method for the removal of pharmaceuticals from water.

5. ACKNOWLEDGEMENTS

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Manuscript 2

Diclofenac Removal from Water by Molecularly Imprinted Polymer

DICLOFENAC REMOVAL FROM WATER BY MOLECULARLY IMPRINTED POLYMER

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Abstract

Environment Persistent Pharmaceutical Pollutants (EPPPs) have been introduced as one type of recalcitrant pollutant sources in water. In this study, the removal of a non-steroidal anti-inflammatory drugs Diclofenac (DCF) has been carried out using Molecularly Imprinted Polymer (MIP), synthetized via bulk polymerization with allylthiourea (AT) as the functional monomer and using DCF as template (MIP-DCF). The detection has been performed by UV spectrophotometer. From the kinetic study in batch mode, approximately 100% of removal is observed, with an initial concentration of 5 mg L^{-1} of DCF within three minutes, agitated at 25ºC and in the range of pH 3-7. From the total adsorption study using a cartridge pre-packed with 10 mg of MIP a high adsorption capacity of 160 mg DCF/g MIP was obtained. The Scatchard plot also has been determined showing the profile for the homogenous process of adsorption similar to that obtained by previous study. In order to study the morphology of MIPs, scanning electron microscopy (SEM) has been used. All experiments were carried out in triplicate. In order to observe the chemical reaction occurred between monomer and template, the pre-polymerization for MIP-DCF has also been studied by using ^{1}H NMR. The shift in the signal observed has been identified with the interactions between amine of AT group with carboxylic acid on DCF. As conclusion, the developed MIP works as a good adsorbent in DCF removal. The molecularly imprinted technology has shown to be a promising technology for the removal of pharmaceuticals from water.

Keywords: Molecularly imprinted polymer, diclofenac, persistent pollutant

1.0 Introduction

Organic micropollutants are typically released into the environment via wastewater discharges and land application of bio-solids and contaminate the groundwater and surface waters that are used as drinking water resources.[1] Even though the concentration was at trace amount, it gives adverse effect to human health especially when micropollutants was found after the wastewater plant treatment release the treated water for human's need such as in drinking water and tap water. Emerging Pharmaceutical Persistent Pollutant consist of organic matters which persistent towards the conventional wastewater treatments. The best way to vanish them was to degrade using advanced oxidation process. However, there are a few problems may arise due to the toxin from the by-product compounds after the degradation process occurred. These conventional wastewater treatments were still practiced and applicable until now. However, there are also other methods that apply recovery techniques to separate the compounds from water. This method has been used present works as the price of drugs in market and the pharmaceutical production is remaining increase. Therefore, it was an opportunity for EPPPs to be recovered and can be re-using instead of producing more of it.

A recent study suggests that wide-spread veterinary use of diclofenac (DCF), an anti-inflammatory drug, is indirectly causing the near extinction of vultures in South Asia.[2] DCF is one of the pharmaceutically active compounds most frequently detected in the water-cycle. It is considerably stable under normal environmental conditions and the most probable decomposition pathway for its onsite elimination is photodecomposition.[3] Previous researches shows that before the consumption of DCF, the diet cycle of fish species such as bream, roach and stickleback were not disturbed. These kinds of fish species were now abundant at the lowland river which contain with most of organic compounds especially EPPP. Andrew et al. (2013) [4] claimed that the loss of organic chemical emissions to water contended might cause certain fish species to come under pressure.[4] It is true that the changes in sewage effluent system might cause the lowland river to be disturbed.[5]

Molecularly-imprinted polymers (MIPs) are synthetic materials with artificially generated recognition sites being able to rebind a target molecule specifically in preference to other closely-related compounds.[6] More recently, molecularly imprinted polymers (MIPs) are attracting widespread attention due to their prominent selectivity properties. MIPs are

synthetic polymers possessing specific cavities designed for target molecules.[7] To the best of our knowledge, DCF [8–10] removal via MIP has been synthetized in many ways previously including bulk polymerization and precipitation polymerization. However, recent development shows that analytical method via MIP has been improved in terms of total sorption capacity via precipitation polymerization method. However, precipitation polymerization required high volume of porogen solvent during polymerization in order to produce the microspheres particles. In this study the differences in solvent volume also will be discussed.

The basic procedure for characterization of MIP also will be discussed further including the kinetics, total sorption capacity, pH effect, media effect, morphology study and selectivity studies. In previous work, selectivity study was conducted in single solution as disruptor and to the best of our knowledge it does not shows any selectivity properties although it was almost 0% of disruptor removal.

The main objectives of present work are (1) to synthetize successive new molecularly imprinted polymer using indomethacin and diclofenac individually as a template with 1-allylthiourea as the monomer via bulk polymerization, (2) to characterize the MIPs properties such as: binding properties, the influence of media, the influence of different pH solutions and the selectivity properties in binary mixture via batch mode for MIP with diclofenac as a template and (3) to study the pre-polymerization of complex formed between functional monomer and templates.

2.0 Materials & Instruments

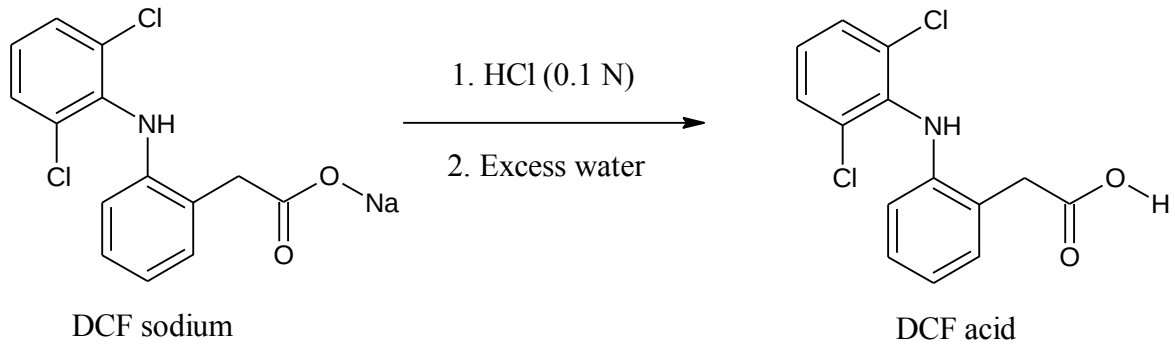
Reagents: Diclofenac-Na and ibuprofen was from Cayman Chemical with purity higher than 99%. Indomethacin (IDM), 1-allylthiourea (AT), acrylamide (AM) and ethylene glycol dimethacrylate (EGDMA) were from Sigma Aldrich with 98-99% of purity. TLC grade. 2,2-azobisisobutyronitrile (AIBN) was from Acros Organic with 98% of purity. Acetic acid (TLC grade) and hydrochloric acid were from J. T. Baker with 96% and 37-38% of purity respectively. Sodium hydroxide from Panreac with purity of 98%. Chloroform and acetonitrile (HPLC grade) were from VWR Chemicals with 99.5%-99% of purity.

Acetonitrile-d_3 from Sigma Aldrich, Spain with 99.9% purity and analytical grade. Methanol from VWR company with 99.8% purity and HPLC grade.

Equipments/Instruments: Water bath from E. Gabarro A-G. Soxhlet extraction apparatus set from VWR company. Centrifuge from Orto Alresa model Digicen. Mortar grinder from Retsch. UV double-beam spectrophotometer from UNICAM, model UV-2 200. 400 MHz Nuclear Magnetic Resonance (^{1}H NMR) from Merlin, Germany. The instrument was self-serviced at *Servei de Ressonància Magnètica Nuclear* (SeRMN), Universitat Autònoma de Barcelona, Spain.

2.1 Synthesis of new MIP using DCF as template

In order to synthetize the MIP-DCF, firstly the synthesis of DCF acid from DCF salt was required. The following procedure is the synthesis of DCF acid from DCF sodium salt. DCF sodium was dissolved in water at a concentration of 7 mg/mL. When the DCF sodium was completely dissolved, it was acidified with an equal molar amount of hydrochloric acid (HCl). This solution was stirred using a magnetic stir bar and plate for 10 min. Since the DCF acid was not dissolved in water, it was immediately precipitated out of solution.[18] The free acid of DCF was a suspension in water. Figure 1 shows the chemical reaction. The mixture was filtered using 0.45 μm filter paper and a vacuum apparatus. The filtrate was washed with dilute HCl (0.001 N) and excess amounts of water to remove any sodium chloride and unreacted DCF sodium. The powder was allowed to dry under a hood, collected and stored in a clear glass vial and prepared for next purpose.[19]



DCF sodium DCF acid

Figure 1 Chemical reaction scheme for the transformation of DCF acid to DCF salt.

A new procedure for preparing molecularly imprinted polymer was used based on reference (17): Diclofenac (DCF, 1.0 mmol), 1-allylthiourea (AT, 4.0 mmol), ethylene glycol dimethacrylate (EGDMA, 20 mmol), and 2,2-azobisisobutyronitrile as an initiator (AIBN, 0.12 mmol) were dissolved in 4.0 mL of acetonitrile.[13] The solution was sonicated and sparged with N_2 for 5min.[10] Then, the sealed solution was polymerized at 60°C in a water bath for 24 h. Successive washing steps with methanol/acetic acid (9:1, v/v) were performed in a Soxhlet apparatus.[14,15,16] The final washing step used 25 mL of methanol for three cycles and later was centrifuged at 5300 rpm for 3 min to remove acetic acid residual. The supernatant of methanol was analysed using UV spectrophotometer in order to confirm that there was no more template eluting from the polymer particles. The polymer was dried at 60 °C under vacuum overnight and stored at room temperature.[14] Next, the polymer was ground using automated mortar for 5 min and sieved to yield a particle size between 28 μm and 100 μm. The process was repeated until the size of particles was in the selected range. The polymer particles then were dried at room temperature overnight. The non-imprinted polymer (NIP) with absence of template was synthetises using the similar procedure as mentioned above but without the addition of template.

2.2 Kinetic study

10 mg of MIP-DCF and NIP were individually weighted and placed in 5 mL tubes. 2 mL of 5 mg/L DCF solution were poured into the tubes[14] and were covered using aluminium foil. After the solutions were agitated for the required time up to 120 min, the solutions were filtered using the syringe filter (mesh size: 0.22 μm). The solutions were then analysed by UV spectrophotometer. The absorbance value according to the selected wavelength (λmax) was determined. The procedure was performed in triplicate. The similar method as above was repeated for MIP-IDM and NIP using IDM solution. The percentage of removal (% removal) was calculated using the **Equation 1.0** with $[Analyte]_i$ is the initial concentration of analyte and $[Analyte]_f$ is the final concentration of analyte. The concentration was obtained from calibration curve of absorbance versus concentration. The calculation was used for all the experiments relates to percentage of removal.

$$\%\, removal = \frac{[Analyte]_i - [Analyte]_f}{[Analyte]_i} \times 100\% \quad \text{..... Equation 1.0}$$

2.3 Total adsorption study

Batch mode: DCF solutions were prepared between 1 mg/L until 25 mg/L due to its limit of solubility in 5% of acetonitrile/water at room temperature. Different concentration solutions of 2 mL DCF were poured into 5 ml tubes.10 mg of MIP-DCF and NIP were individually weighted in 5 mL tubes. The solutions were agitated for 1 h and covered using aluminium foil. Then, the solution was filtered using the syringe filter (0.22 μm). Finally, solutions were analysed by UV spectrophotometer.

Continuous flow mode: Due to the immiscibility properties of IDM and DCF in water, total sorption capacity has been carried out in acetontrile/water (5% v/v) media solution. 10 mg of MIP-IDM was accurately weighted and placed in a cartridge of 1.5 mL of capacity. 15 μg/mL of IDM was prepared in 2 L of acetontrile/water (5% v/v). Then, the solution was loaded into the cartridge using the peristaltic pump at 1.67 mL/min of flow rate. The solution was collected in fractions of 5 mL until 50 mL, then fractions of 50 mL. The solution was continuously-flowing until the absorbance readings of the loaded solution reached the plateau (absorbance readings near to the absorbance of the initial concentration, meaning that no more absorption was occurring). Then, the process stopped and all solutions were collected and analysed via UV spectrophotometer. The procedure mentioned above was duplicated. The similar procedure was repeated for MIP-DCF as the sorbent. As blank, the procedure above was repeated in the absence of MIP using the same concentration in order to measure any other contribution to the elimination of the target compounds e.g. sorption in cartridge or frits.

2.4 Effect of pH

10 mg of MIP-DCF was individually weighted in 5 mL tubes. 2 mL of 15 ppm DCF solutions were prepared in different pH range from 3 to 12. The solutions were added to the tube and agitated for 1 h then were filtered using the syringe filter (mesh size: 0.22μm). The solutions were analysed by UV spectrophotometer.

2.5 Morphology study

The MIPs were analysed by using Field Emission Scanning Electron Microscope (FESEM). The MIP particles were distributed on the carbon disc attach on the pin stubs. Then, the pin stubs were located according to the position number on the multi stubs holder. Next, the holder was located into the chamber and then analysed. The parameters were sets as follows: EHT: 1kV, Mag: 34.63KX, WD: 4.1 mm at scale 1μm. Other parameters also used were EHT: 1 kV, Mag: 16.20 KX, WD: 3.3 mm at scale 2 μm.

2.6 Pre-polymerization study via ^{1}H NMR

A non-covalent molecular imprinting approach was followed to prepare the MIP and NIP. Molecular recognition of the template molecule by imprinted polymers is based on the intermolecular interaction between the template molecule and functional groups in the polymer. Thus, to study the interaction between the template molecule and the monomer in pre-polymerization complexes it is important to predict the recognition mechanism of the imprinted polymer.

In the present work, the interaction between template and monomer in pre-polymerization complexes was studied by using ^{1}H NMR spectroscopy which is now being widely used within the field of molecular imprinting.[8] To do so, a series of samples were prepared with a fixed concentration of template (DCF), 0.05 mol/L, and varying concentration of monomer (AT), 0.10 mol/L (Mixture 1), 0.30 mol/L (Mixture 2) and 0.50 mol/L (Mixture 3) in acetonitrile-d_3. ^{1}H NMR spectra were acquired at room temperature.[10]

3.0 Results and Discussion

3.1 Kinetic and sorption capacity studies

For DCF removal by MIP-DCF, the kinetic experiment shows important different between MIP-DCF with NIP. According to the result obtained, more than 90% removal by MIP-DCF whereas approximately less than 30% removal by NIP (Figure 2). The binding is mainly due to MIP-DCF has been well-templated in the cross-linked polymers. All the experiments have been done in a mixture solution of acetonitrile and water (5% in volume ratio).

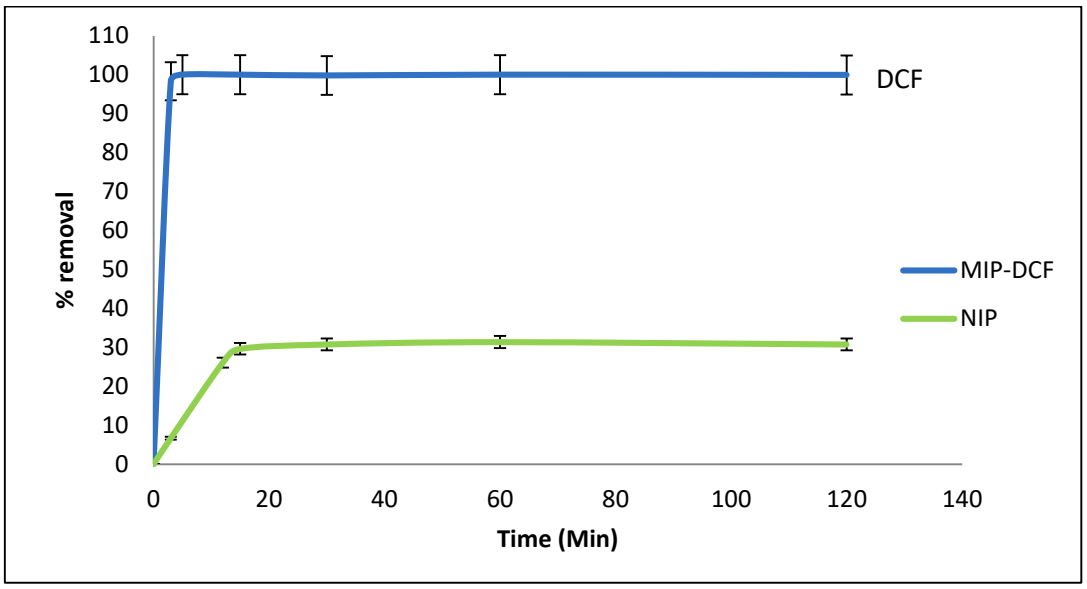


Figure 2 Sorption kinetic curve of MIP-DCF and NIP with initial DCF concentration of 5 µg/mL via batch mode. 10 mg sorbent (NIP, and MIP-DCF), volume solution: 2 mL, room temperature, extraction media: acetonitrile/water (5% v/v).

In order to characterize the total sorption or saturation profile of MIPs, the analysis of the sorbed amount for different initial concentrations of IDM or DCF via batch mode have been done. However, the solubility of target molecule in water is limited. Thus, in this study the use of a mixture of acetonitrile and water (5%v/v) has been used to dissolve the pharmaceutical compounds.

One significant research has been developed by Sun and co-workers.[10] They develop a MIP founding an adsorption capacity of 19.1 µmol/g which is equal to 5.6564 mg/g by using bulk polymerization method. This method is a common method in preparation of MIP. However, it was time-consuming and the purity might be disputed since the MIP need to be crushed and sieved in order to have the particles size in certain range. However, as long as the bulk polymerization can produce high affinity polymers and selective, it still can be considered as a good approach.

As it is seen, the total sorption capacity, Q increases uniformly as the initial concentration also increases. The regression linear line for the total sorption is obtained being $y = 0.1956x - 0.0795$ with R^2 equals to 0.9999 for DCF removal, being y the Q and x the concentration of

pharmaceutical. The removal by MIP is mainly due to the specific interaction created by the template and not by the polymer itself. However, the removal by NIP with capacity loaded almost zero because NIP does not have imprinted properties and NIP itself could not remove the analyte from aqueous media. The removal of IDM using NIP show significant differences compared to MIP with approximately zero sorption of capacity. The same trend has been observed for the DCF removal by MIP-DCF as shown in Figure 3.

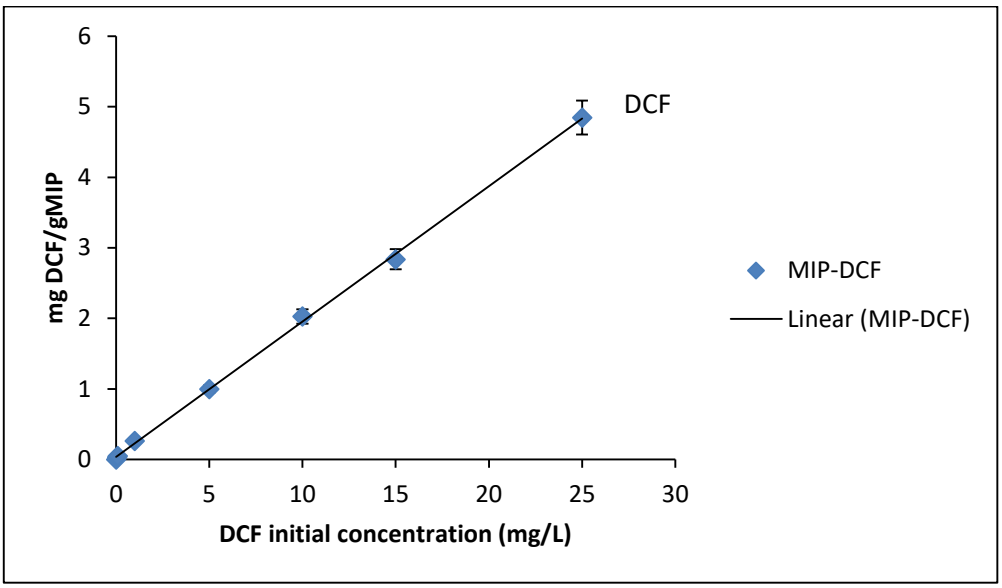


Figure 3 Sorption capacity (mg/g) for DCF removal by MIP-DCF with initial concentration 5 µg/mL within 60 min agitation. Solution volume: 2mL, Extraction media: acetonitrile/water (5%), room temperature.

Since the total sorption of target molecule using batch mode did not reach the plateau, other analysis method was suggested via molecularly imprinted solid phase extraction (MISPE). For the removal by MIPs with DCF as the template, the total saturation obtained was 0.006 mmol DCF equivalent to 200 mg DCF/g MIP (Figure 4 – Figure 5).

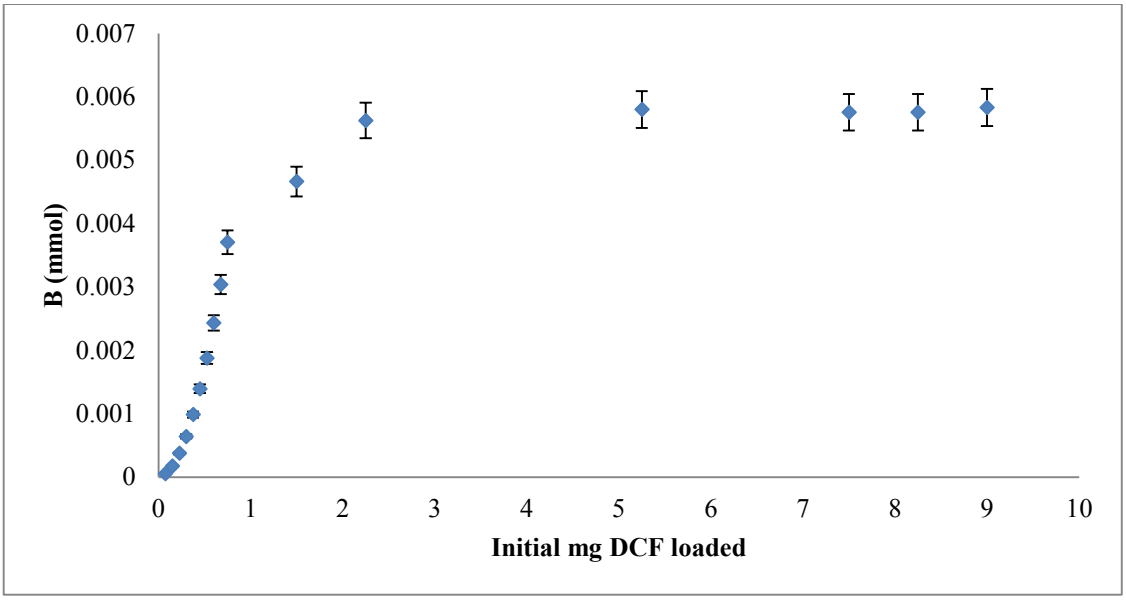


Figure 4 mmol DCF sorbed into MIP-DCF. Initial concentration: 15 µg/mL DCF. Media: 5% acetonitrile:water.

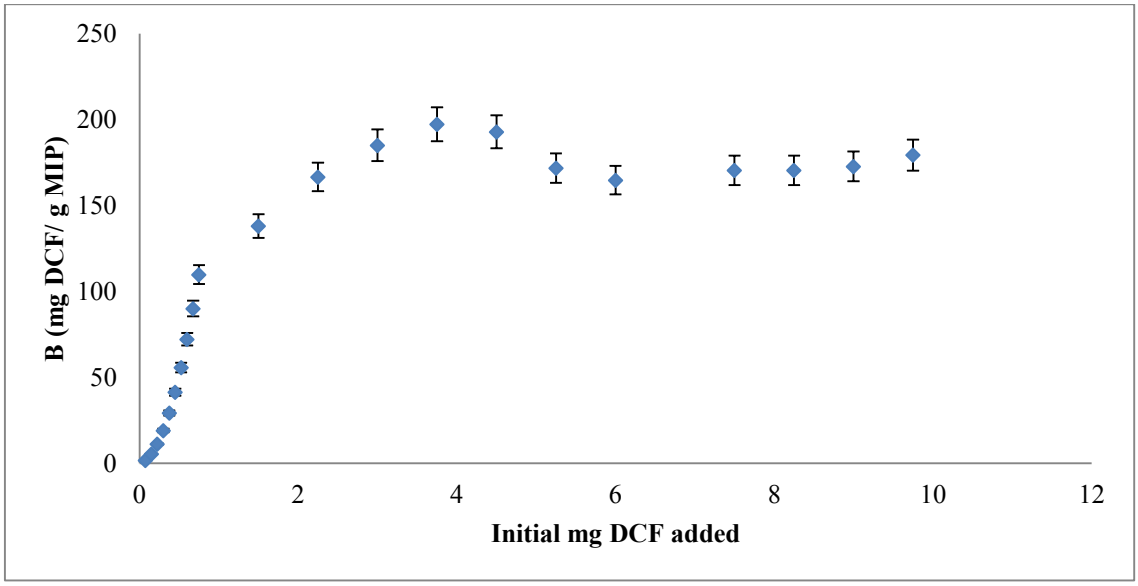


Figure 5 Total saturation profile of DCF. Initial concentration: 15 µg/mL DCF. Media: 5% acetonitrile:water.

3.2 Influence of pH

pH commonly affects the process of removal if the target molecule contains acidic or/and basic functional sites. According to the study carried out by Asiabi [6] MIPs fabricated tends to isolate the target molecule under neutral pH. However, it was dependent on the target molecule and the functional sites in the cross-linked polymer (MIPs). In the present study, the

pH range from 5 to 8 was studied. From the obtained results, (Figures 6 – Figure 7), the efficiency removal was approximately 90% for both MIP-IDM and MIP-DCF. Dai and co-workers found a similar pattern comparing with the MIP-IDM synthetised in the present study wherein the removal efficiency works well.[8] The efficiency removal value was calculated using Equation 1.0.

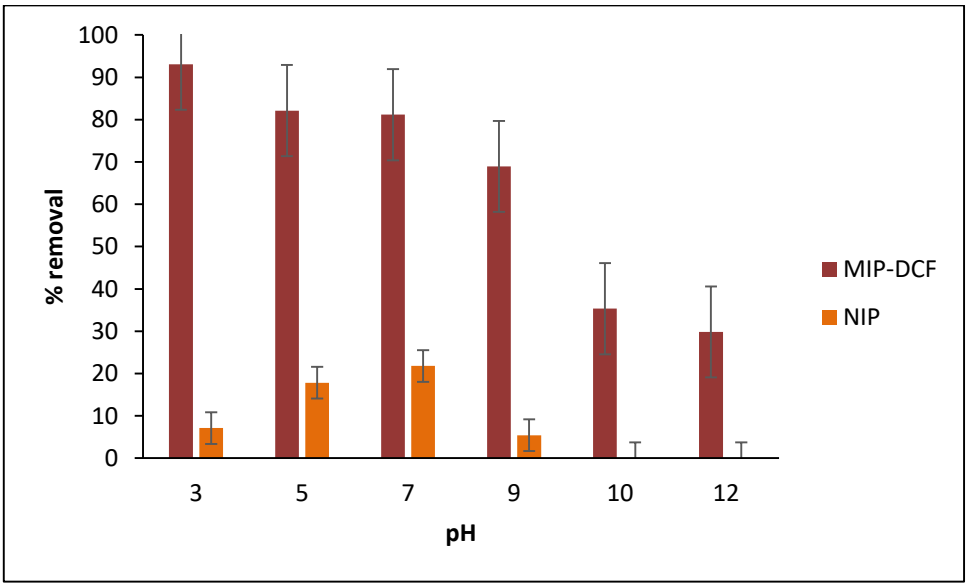


Figure 6 % removal of DCF in different pH solutions (pH 3 – pH 10) using MIP-DCF and NIP, initial concentration: 15 µg/mL of DCF, extraction medium: acetonitrile/water (5% v/v) and agitated for 1 h.

The sorption capacity was approximately 3.0 ± 0.4 mg DCF/ g MIP within neutral pH range (5-9) in 15 µg/mL of DCF solution as initial concentration. With good correlation in regression linear line as shown in Figure 3, it was estimated that at 5 µg/mL the total sorption capacity of DCF would be 1.0 ± 0.1 mg DCF/ g MIP. DCF is a very soluble in neutral-alkaline medium (50 g /L) and is an acidic pharmaceutical (pKa = 4.15) that becomes almost insoluble below pH 4, so below this pH value, diclofenac precipitates.[3] Thus, at pH 3 the DCF removal shown at 2.5 mg DCF/g MIP can be caused by the precipitation of DCF and not by using MIP as the sorbent.

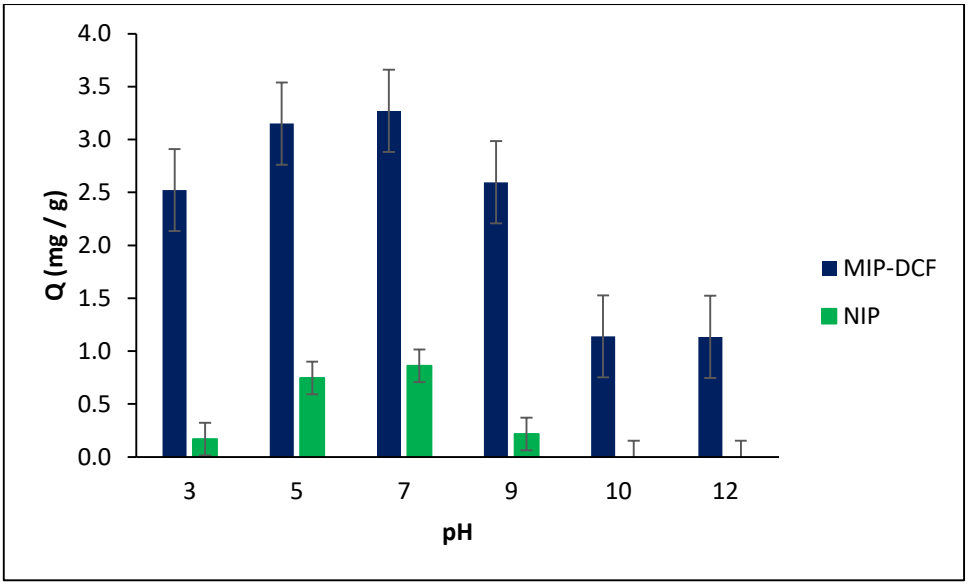


Figure 7 DCF sorbed in different pH solutions (pH 3 – pH 10) using MIP-DCF and NIP, initial concentration: 15 µg/mL of DCF, extraction medium: acetonitrile/water (5% v/v) and agitated for 1 h.

3.3 Selectivity study via simultaneous detection

Detection via spectrophotometer is a simple and direct detection analysis. Hence, in this study, spectrophotometer has been choosen as the instrument for the IDM, DCF and/or IBU detection in two components in a mixture.

To perform the study, simultaneous detection for two components in a mixture consisting of IDM, DCF and/or IBU using MIP-DCF was carried out. In this study, the solutions were tested and being analysed in the presence of interference in order to study the selectiveness properties. Different mixtures consist of two pharmaceuticals in aqueous medium (5% of acetonitrile/water) with initial concentration of 5 µg/mL for each one.

As can be seen from the Figure 8 and Figure 9, zero order absorption spectral shows severe overlapping between each compound. In this study, the two components in a mixture consist of target molecules and one interference will be discussed.

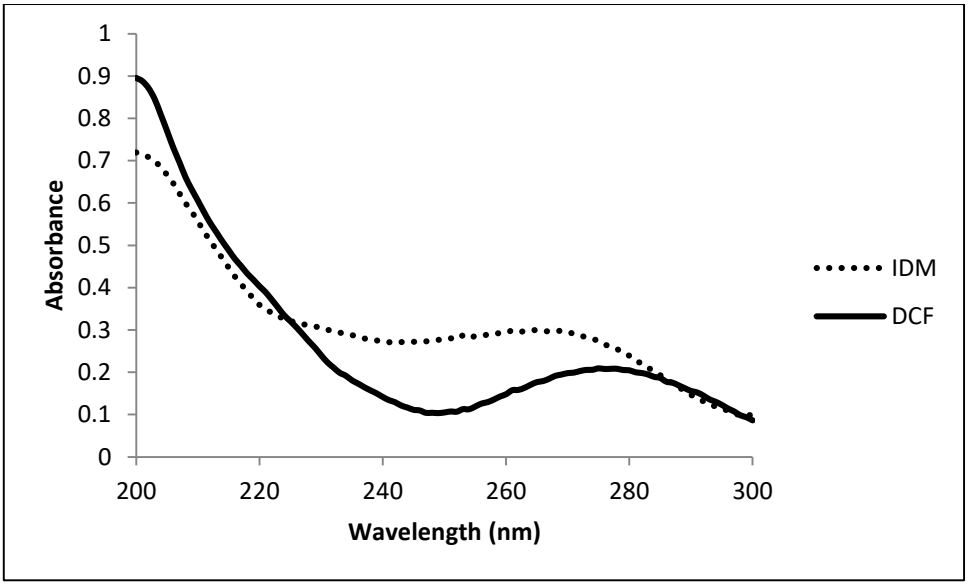


Figure 8 Zero-order absorption spectra of IDM and DCF in mixture 1 with concentration at 5 µg/mL individually prepared using 5% (v/v) ACN/water as blank.

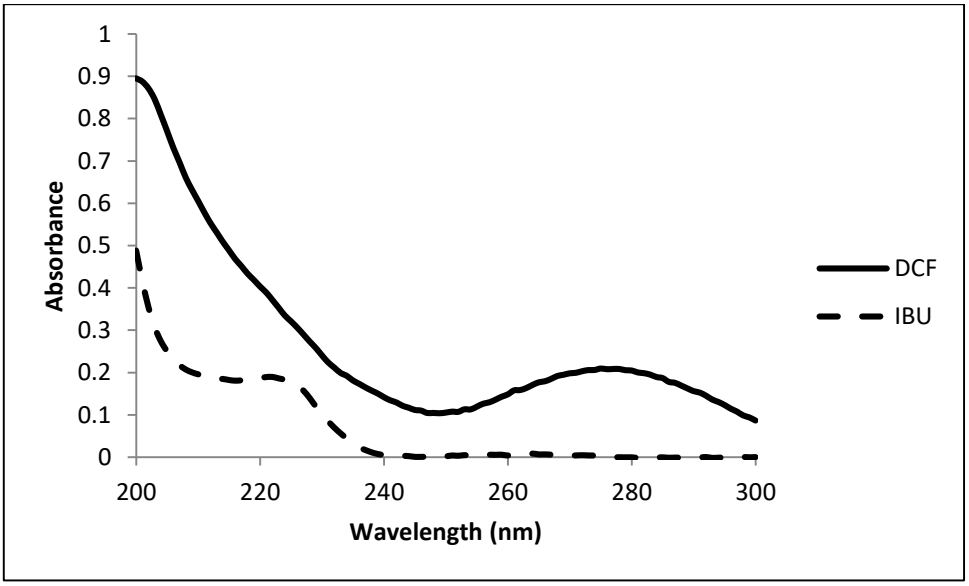


Figure 9 Zero-order absorption spectra of DCF and IBU in mixture 3 with concentration at 5 µg/mL individually prepared using 5% (v/v) ACN/water as blank.

Based on the Beer's Law,

$A = \alpha b C$, ……..…**Equation 2.0**

with

α = absorptivity vector,

b = length of the path cuvette wall = 1 cm

C = concentration at certain wavelength

Consider a mixture of two compounds, X and Y:

$A_m = \alpha_x C_x + \alpha_y C_y$……..……..**Equation 3.0**

A_m = Absorbance for mixture

α = slope

C = concentration certain wavelength

Table 1 shows the linear equations for each different mixtures at different maximum wavelength (λ_{max}) for IDM (260 nm), DCF (280 nm) and IBU (220nm). The slope was determined from the calibration curve for each target molecule at different maximum wavelength, λ_{max}. Next, the calculation on amount sorbed in terms of μmol for each types of MIPs have been performed.

Table 1 Determination of IDM, DCF and/or IBU in the two components mixtures

Template	**Mixture**	**Equations**	
MIP-DCF	Mixture 1 (IDM + DCF)	0.0144x + 0.0123y = 0.0320	At 260 nm
		0.0178x + 0.0088y = 0.0560	At 280 nm
	Mixture 2 (DCF + IBU)	0.0123y + 0.0001z = 0.0300	At 260 nm
		0.0246y + 0.0093z = 0.2600	At 220 nm

*x: IDM, y: DCF and z: IBU amount sorbed in μmol

The selective study has been carried out by MIP-DCF as a sorbent in different mixtures:

- Mixture 1: IDM + DCF
- Mixture 2: DCF + IBU

In mixture 1 with MIP-DCF as a sorbent shows that the removal slightly favored to isolate the DCF molecules (Figure 10). The DCF removal tends to remove three times more than

IDM by using MIP-DCF as a sorbent. It was believed that the functional sites on the target molecule will influence the molecule interaction trapped into the unoccupied template. The other reason for this it is because the size of template molded was sufficient for DCF molecule size to be trapped into the unoccupied templates of MIPs fabricated. This is due to the size of target molecule and functional sites on MIPs that have an ability to interact via hydrogen bonding with the N-H functional group in DCF instead of N-COR functional group that attached on IDM molecule.

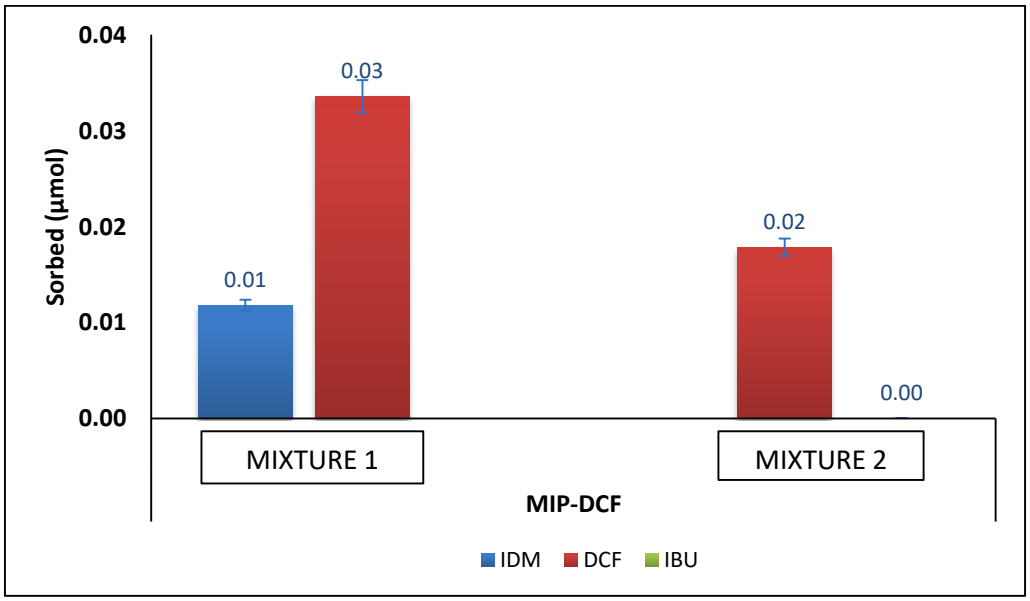


Figure 10 Sorbed (µmol) uptake by MIP-DCF in different mixtures of solution with the initial concentration of 5 mg/L via batch mode, Mixture 1: (IDM ± 0.0006 µmol) + (DCF ± 0.0007 µmol); Mixture 2: (DCF ± 0.0004 µmol) + (IBU ± 0.0012 µmol).

According to the published studies, a few selectivity studies was conducted in individual solutions containing the interference only and not in a mixture containing both, analyte and interference. One example is the study by Amiri and co-workers where the DCF solution was prepared individually and called it as interference study.[28] This was practically unselective procedure towards the analyte according to the definition of selectivity. Selectivity can be defined as the ability of differentiating and quantifying the analyte in the presence of other components from its matrix.[29] Hence, in our study the two components in a mixture were used with the presence of analyte and interference.

Competitive functional sites work as imprinted fingerprint in which it will become selective for N-H functional sites instead of other functional groups. Hence, this can be another benefits of the DCF isolation for selective properties since DCF have the N-H functional group in the molecule. The priority of selective properties will be N-H > N-COR.

As can be seen, there is a carboxylic acid functional group able to interact with thiol groups. Theoretically, the interaction between the carboxylic acid group with thiol groups has been elaborated briefly by Xiao Pei and co-workers.[30] However, at the center of the compounds there are another active functional groups likely to interact via hydrogen bonding with functional sites on MIPs (Figure 11). Nevertheless, there is no functional group at the center of IBU. Hence, the sorption of IBU by MIP-DCF was approximately zero.

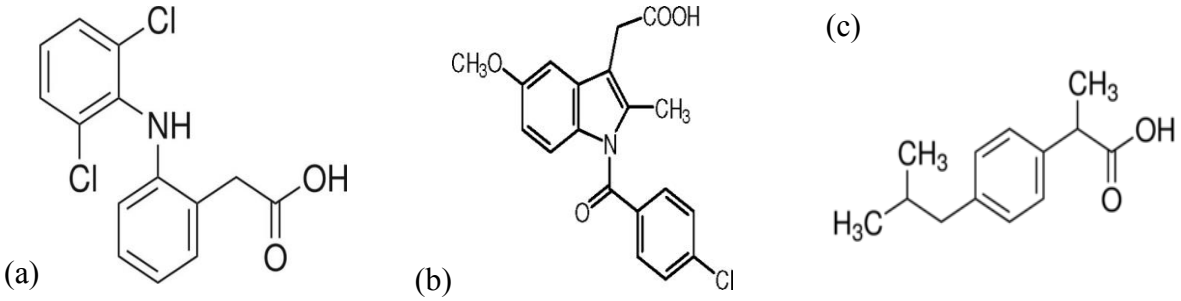


Figure 11 Molecule structure of DCF (a), IDM (b) and IBU (c).

The Scatchard plot for MIP-DCF shown in Figure 12 below is straight line, indicating that the affinities of the binding sites in MIP-DCF is homogeneous among the concentrations tested. The linear regression equation in the Figure 12 below is $y = -16.587x + 0.1346$ (R^2 = 0.95), the unit for B was mmol. The K_D and Q_{max} were calculated to be 0.060 mmol/L and 0.008 mmol/g of dry polymer, respectively, from the slope and the intercept of the Scatchard plot. Similar Scatchard plots were obtained with the case of Meng Dai [25] in which the authors observed one linear line in Scatchard plot analysis in order to study the removal of DCF using MIP synthetised via precipitation polymerization with 2-VP as the monomer.

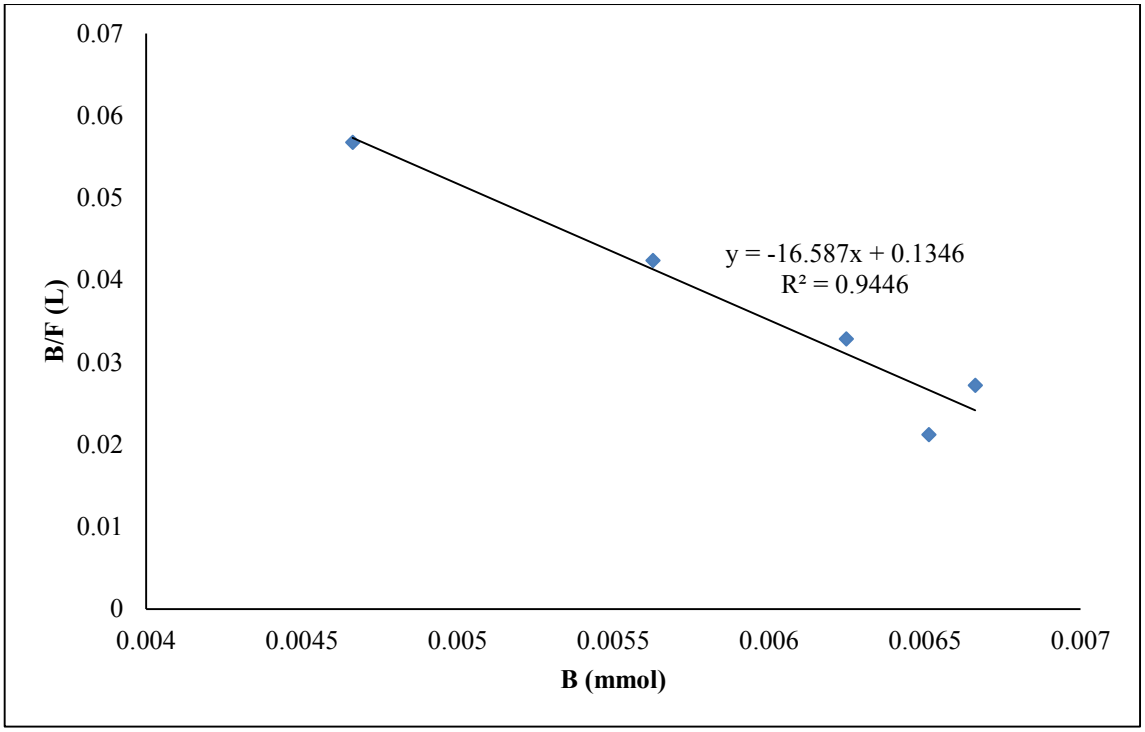


Figure 12 Scatchard plot analysis of the binding of DCF to the imprinted polymer. B is the amount of DCF bound (mmol) to MIP-DCF; F is the concentration of free DCF at equilibrium (mmol/L).

3.4 Pre-polymerization study using ^{1}H NMR spectroscopy

From the results obtained, generally the reaction involved for pre-polymerization was estimated as forward process because the results obtained in mixtures (Mixture 1, Mixture 2 and Mixture 3) shows that the peak in pure DCF or pure AT were decreased and certain significant peaks could not be found in the mixtures spectral. In addition, by applying the Le Chatelier principle, the pre-polymerization occurred with the product favor to be yielded. In this case, the product is the complexes formed.

In order to produce high number of active imprinted sites on the imprinting polymer, it is well known that the ratio between monomer and template is crucial.[10,8,9] According to the previous study conducted by Sun [10] the ratio between monomer and template was found to be optimum at 4 mmol:1 mmol Applying Le Chatelier's principle to the complexes formed prior to polymerization, increasing the binding affinity of the complexes in the pre-polymerization mixture would predict an increase in the pre-polymer complexes. Correspondingly, there is an increase the number of final binding sites in the imprinted polymer, resulting in an

increased binding or selectivity factor per gram of polymer.[31] The structure of the complex formed between DCF and MIP is suggested in Figure 13.

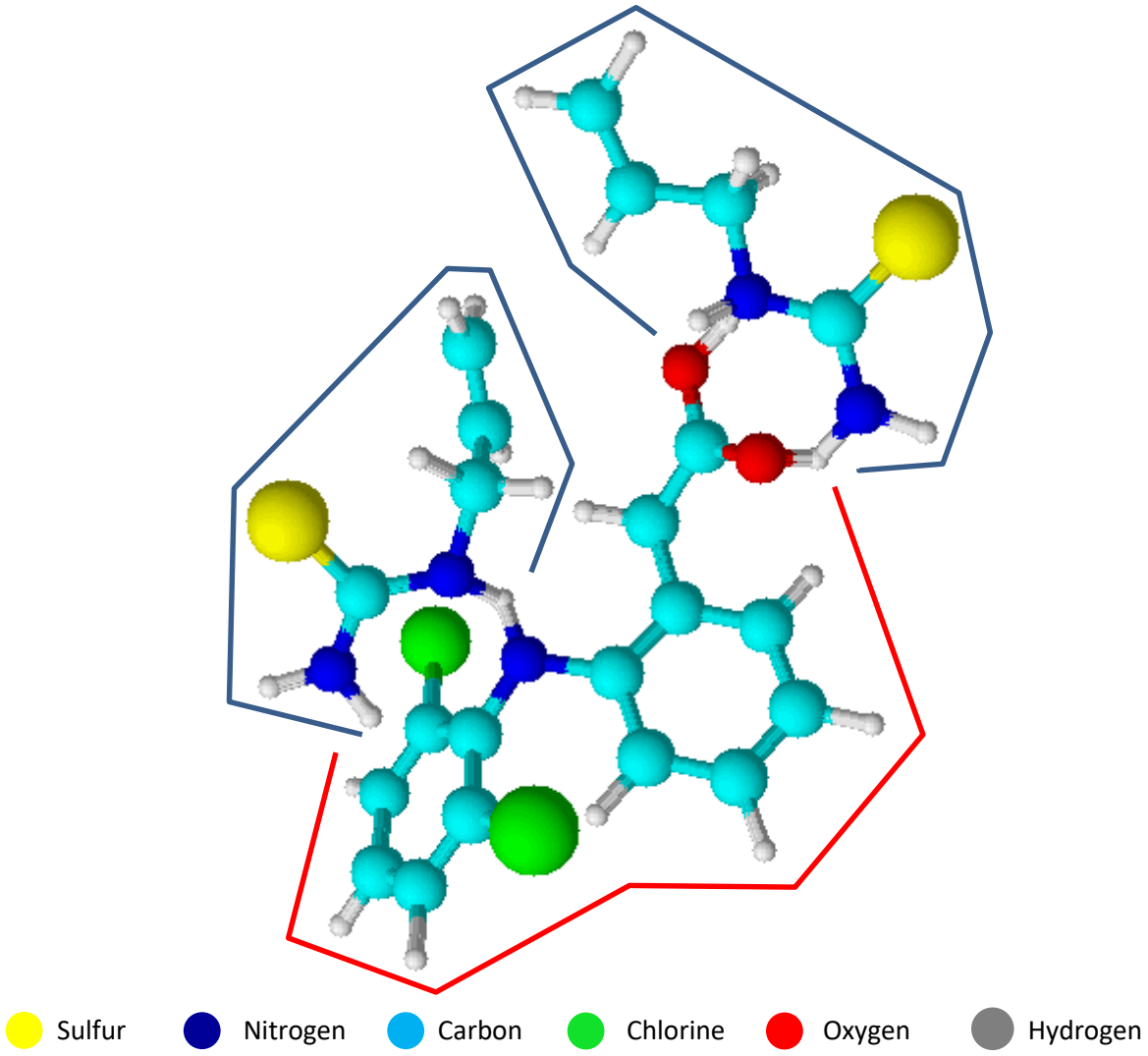


Figure 13 Complexes structure formed between DCF ( ) and AT ( ) with two active functional sites.

From the ^{1}H NMR spectroscopy results, a chemical shift was observed from 6.1 ppm in mixture 1 (low AT concentration) to 6.4 ppm in mixture 3 (high AT concentration) corresponding to the amine group of AT molecule bonded to the carboxylic acid at DCF molecule via hydrogen bonding (Figure 14).

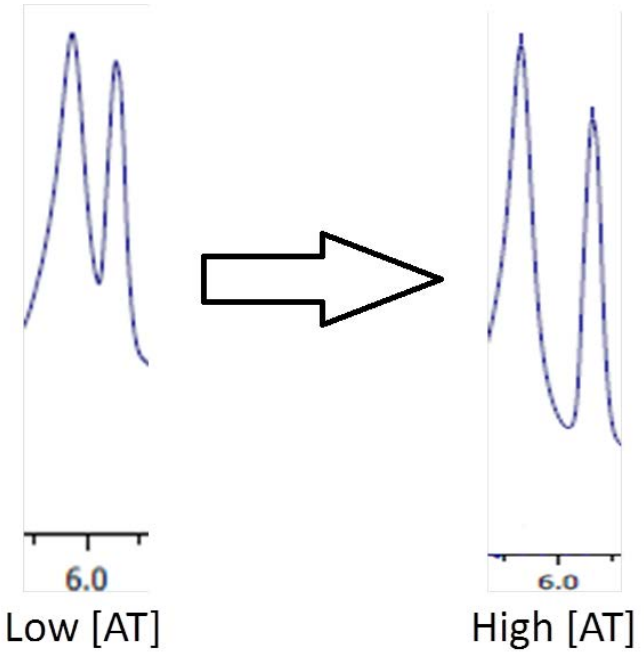


Figure 14 Siginificant peak shows chemical shift of carboxylic acid react with amine group on AT from 6.1 ppm (at low AT concentration) to 6.4 ppm (at high AT concentration).

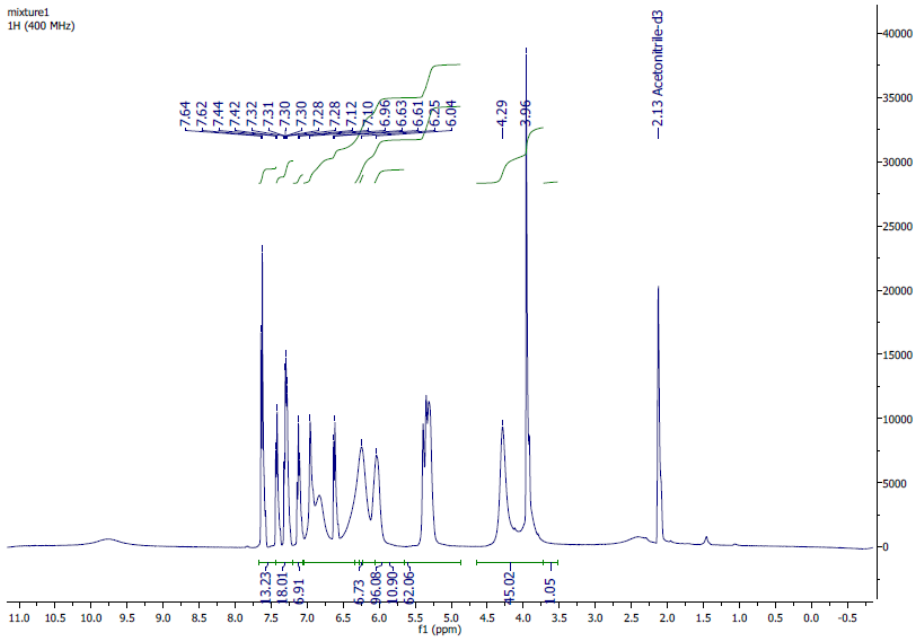
Similar finding was reported by Zhang [30] for the reaction between thiourea and carboxylic acid in open ring compounds. In their work, when the amount of 1,3-bis-(3,5-bis(trifluoromethyl)phenyl) thiourea (TU) (functional monomer) was increased at fixed trifluoroacetic acid (TFA) (template) concentration, the downfield chemical shifted was occurred from 7.83 ppm to 7.94 ppm.[30] The authors stated that TU as anion receptor coordinated with carboxylate by double hydrogen bonding interactions, and was expected to stabilize the anion and lower the pKa of the oxy-acid, thus allowing increase of the electrophilicity of the activated cationic substrate.[30]

However, the results obtained shows that the downfield chemical shifted occurred in the present work was contradicted to the results reported by Sun [10] in which the author claimed an upfield chemical shift, instead of downfield, which occurred from 11.204 ppm to 10.846 ppm.

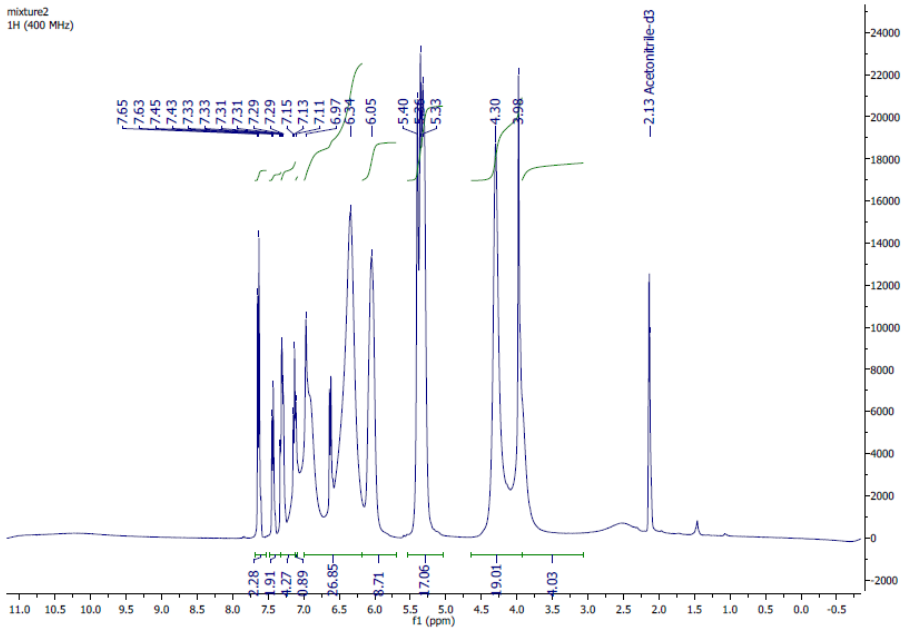
Another downfield chemical shifted observed in our work occurred from 6.6 ppm to 6.9 ppm due to hydrogen bonding between amine group of DCF and nitrogen atom at AT. Similar finding was reported by Sun [10] in which downfield chemical shifted was occurred from 7.70

ppm to 7.82 ppm. All the observation spectrals for the different mixtures prepared are shown in Figure 15.

(a) Mixture 1



(b) Mixture 2



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(c) Mixture 3

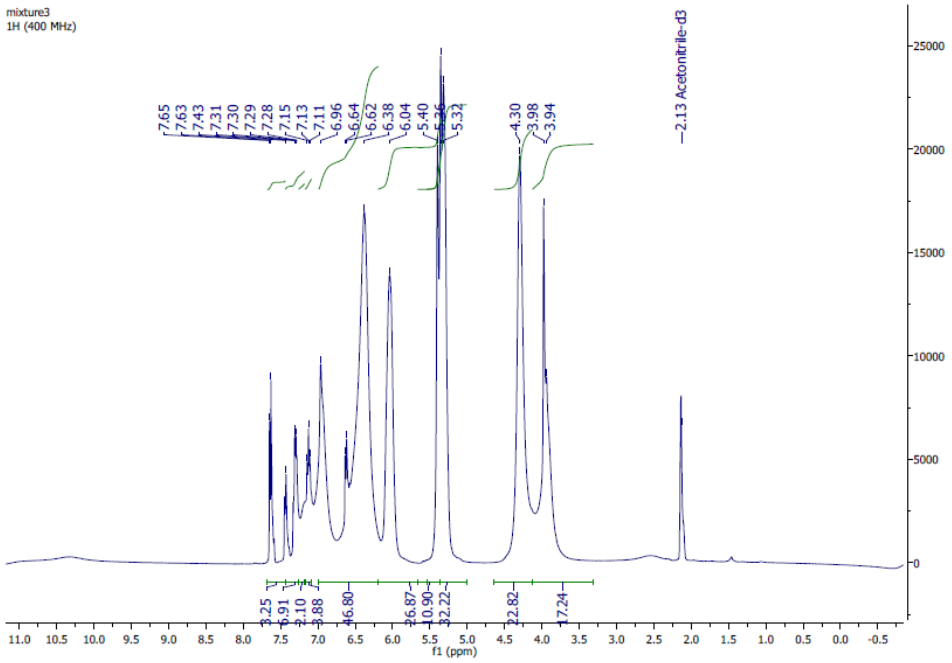
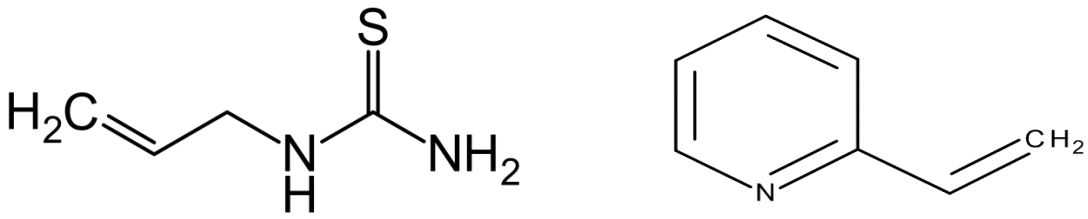


Figure 15 ^{1}H NMR spectral of mixtures consist of (a) 0.1 mol/L AT and 0.05 mol/L DCF (b) 0.3 mol/L AT and 0.05 mol/L DCF (c) 0.5 mol/L AT and 0.05 mol/L DCF in 1 mL of acetonitrile-d_3.

However, Sun [10] used 2-vynilpyridine (2-VP) as the functional monomer for synthesizing the polymers (Figure 16). The clear differences between the two monomers is the only one active functional site on 2-VP molecule whereas there are two active functional sites on AT molecule. Nitrogen atom in 2-VP molecule (pyridine functional site) is less easily protonated compared to the one on the aliphatic amines. Hence, the reaction between amine group with carboxylic acid group is favoured with aliphatic amine compared to pyridine functional sites. To the best of our knowledge, deshielded occurred during downshield chemical shifted whereas shielded occurred during upfield chemical shifted.[32] Thus the pyridine active functional sites on 2-VP molecule was shielded due to the high steric hindrance and less protonated compared to aliphatic amines. Besides, the observation in present work shows that deshielded was occurred and the aliphatic amines were easily protonated. Till to date, study on comparison effects of aliphatic amines and aromatic amines with carboxylic acid is still unknown.



AT 2-VP

Figure 16 Molecule structure of monomers, thiourea (AT) and 2-vynilpiridyne (2-VP).

In this study, the mechanism reaction of MIP-DCF during polymerization has been proposed based on the results observed (Figure 17). In the proposed mechanism, there are two active functional sites on one AT molecule. Thus, it can be estimate that AT as the functional monomer gives higher affinity sites for DCF as the template compared to 2-VP.

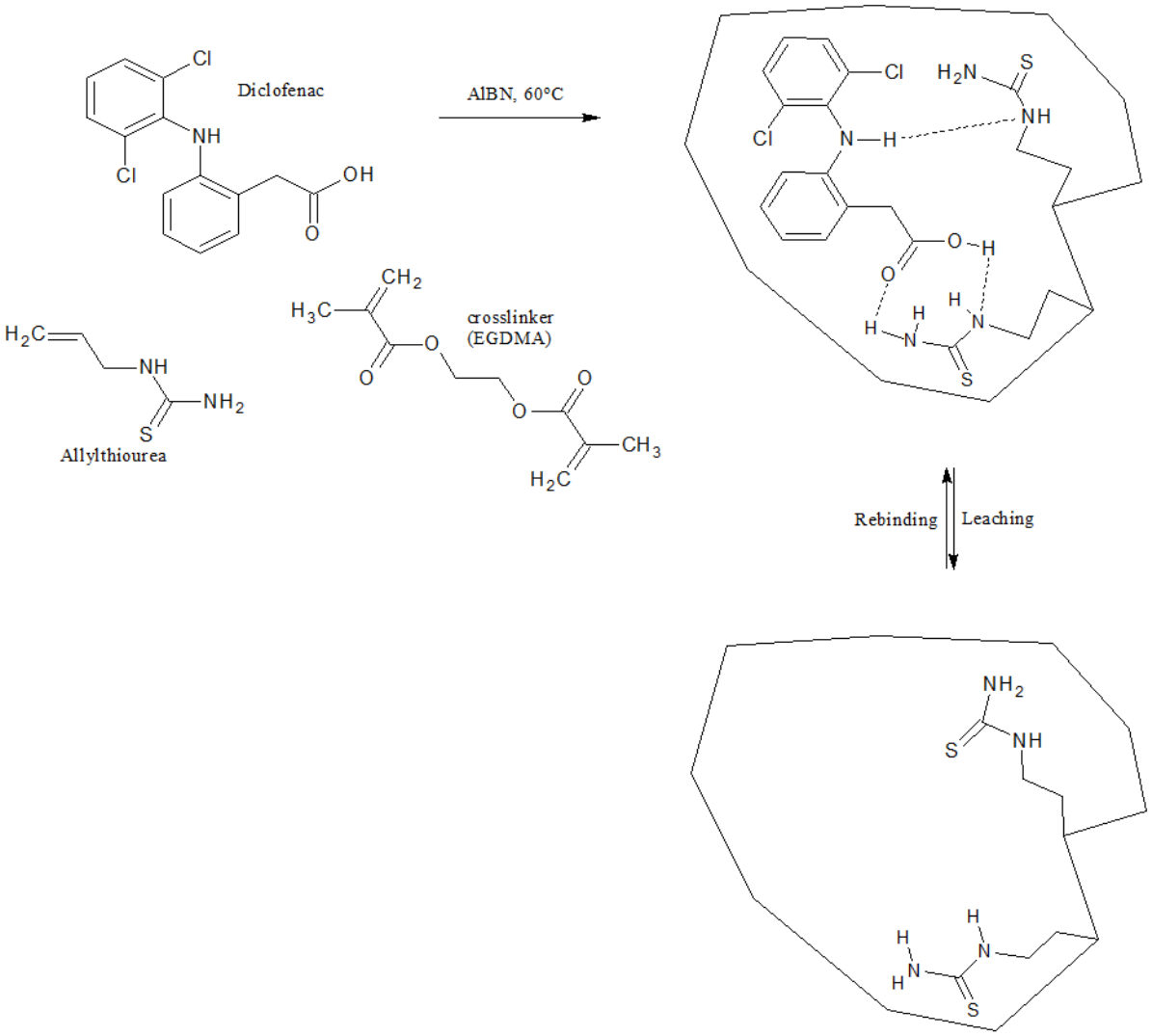
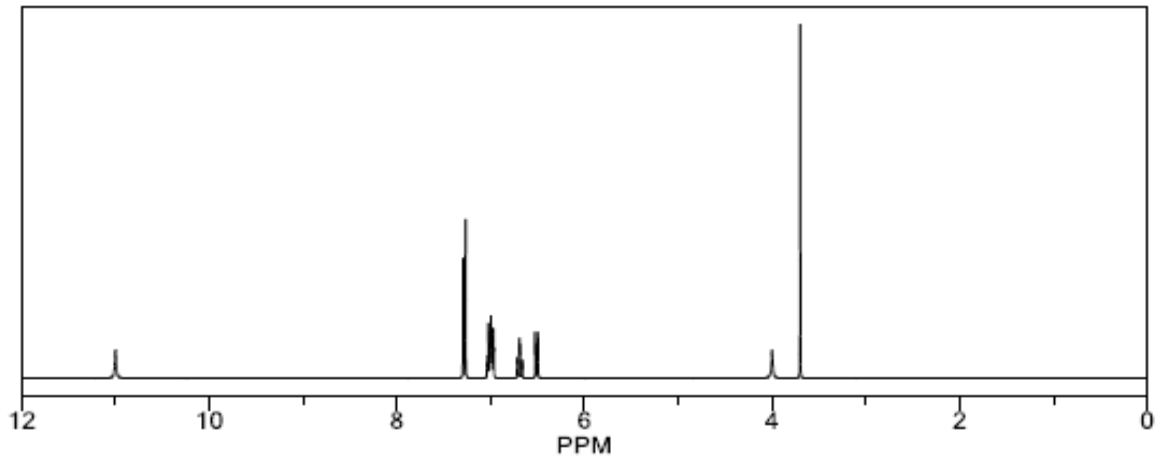


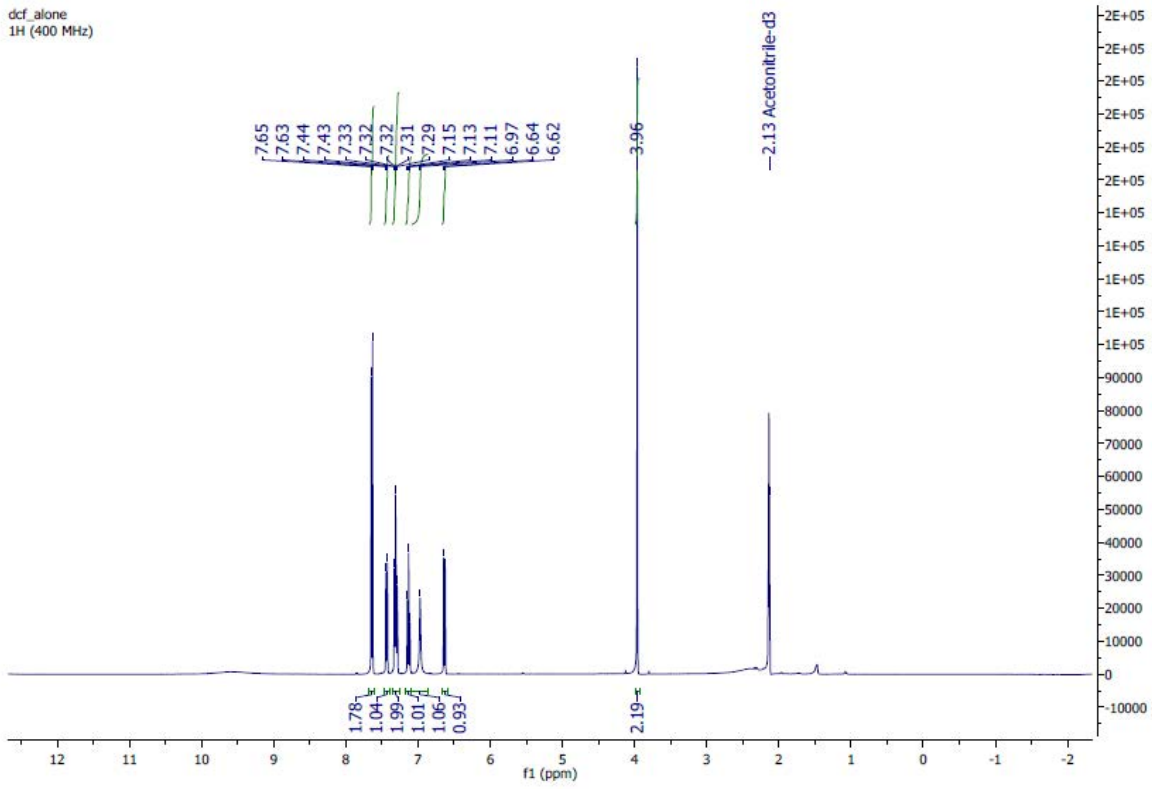
Figure 17 Scheme of proposed mechanism reaction of DCF and AT as the functional monomer in MIP-DCF.

In Figure 18, it is shown the simulation spectra obtained and the experimental result for both, the DCF and the AT. In the experimental result of pure AT, the observed peaks were broad because the thiourea functional sites itself have tendency to react via intermolecular and intramolecular reaction. However, for the experimental of pure DCF in acetonitrile-d_3, the spectral were approximately similar to the simulation spectral.

(a) Simulation of pure DCF.



(b) Experimental spectral of pure DCF.



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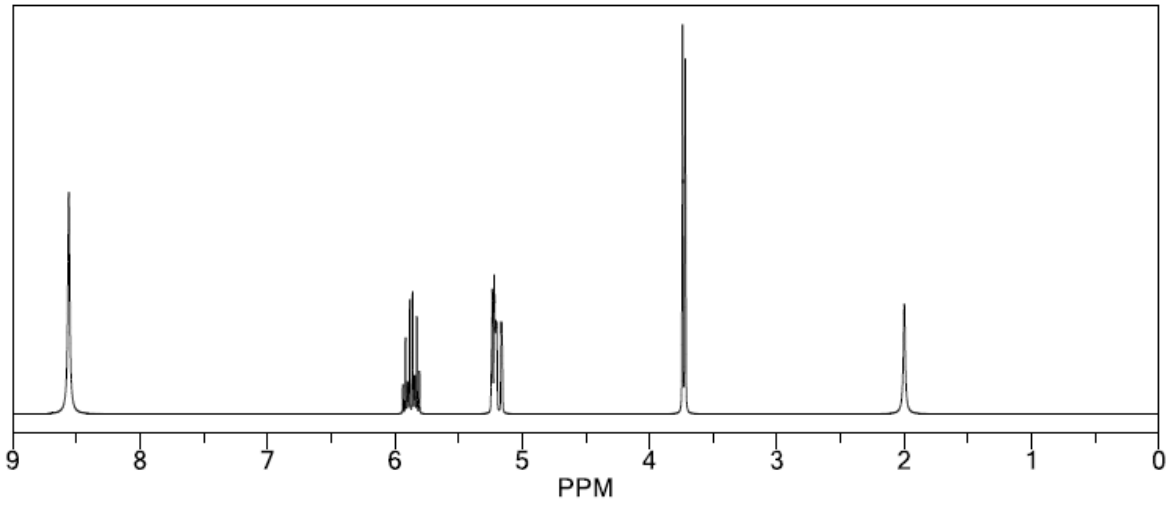
(c) Simulation of pure AT.



(d) Experimental of pure AT.

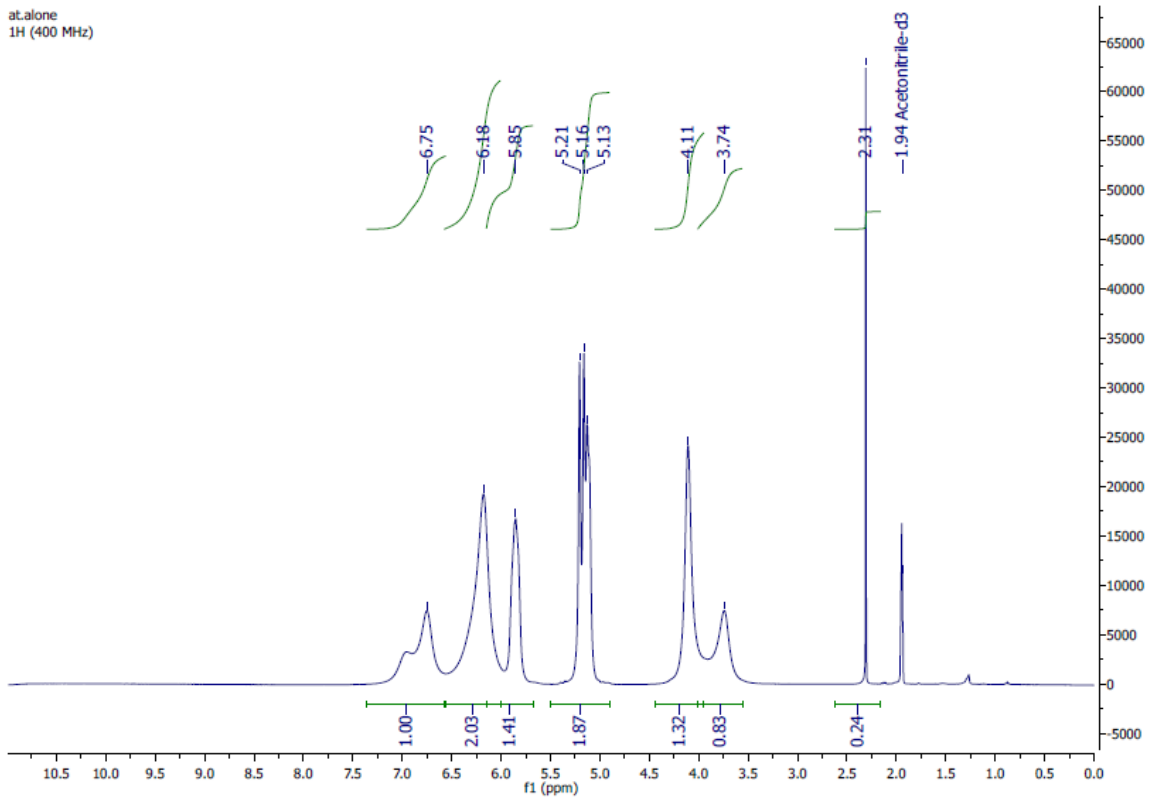


Figure 18 ^{1}H NMR spectral of (a) simulation of pure DCF (b) experimental of pure DCF (c) simulation of pure AT and (d) experimental of pure AT in 1 mL of acetonitrile-d_3

For pre-polymerization study, proton NMR has been used to study the interaction between monomer (AT) and template (DCF). The results show more than one active functional sites

so it can be estimated that the MIPs will have high affinity towards the analyte in sorption process.

4.0 Conclusion

The new molecularly imprinted polymer (MIP) with DCF and IDM as the template using allylthiourea (AT) as the monomer via bulk polymerization was successfully synthetised. One of the significant findings to emerge from this study is that there was more than 90% of efficiency removal within 3 min by MIP-DCF compared to NIP. The present work is much faster and less laborious. pH of the solution during removal processes also has been tested and the best pH value in order to achieved high sorption capacity is at neutral pH (pH 7). The selectivity study shows that the synthetised MIP was possessed towards DCF due to the N-H functional group located at the center of DCF compound which favor react to the active functional sites of the MIP. Hence, DCF was favor to be trapped into the cavities compared to IDM and IBU. The experimental results obtained in pre-polymerization study was consistent to the suggested scheme reaction of MIP synthesis.

Acknowledgements

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