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论文题目　β-环糊精衍生的分子印迹聚合物作为选择性吸附剂用于食品安全分析的应用研究

Applications of β-cyclodextrin-derived molecularly-imprinted polymers as selective extracted sorbents for food safety analysis

作者姓名　素维查（SOVICHEA LAY）
指导教师　沈生荣
学科(专业)　食品科学
研究方向　食品安全
所在学院 生物系统工程与食品科学学院
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Applications of β-cyclodextrin-derived molecularly-imprinted polymers as selective extracted sorbents for food safety analysis

论文作者签名：

指导教师签名：

论文评阅人：

答辩委员会主席：

答辩委员会成员：

答辩日期：

DECLARATION

I hereby declare that this dissertation is my original work carried out under the guidance and supervision by professor Shengrong Shen, college of Biosystems Engineering and Food Science, Zhejiang University, Hangzhou, China and this work has not been submitted for the award of a degree in any higher educational institution.

Date:

SOVICHEA LAY

College of Biosystems Engineering and Food Science, Food Science
Zhejiang University,
Hangzhou 310029,
The peoples' Republic of China

CERTIFICATION

This is to certify that this research work herein entitled *"**Applications of β-cyclodextrin-derived molecularly-imprinted polymers as selective extracted sorbents for food safety analysis**"* was conducted and submitted by Mr. SOVICHEA LAY for the award of Dotoral degree of Food Science at the college of Biosystems Engineering and Food Science, Zhejiang University, under my guidance and supervision from 2012 to 2016 academic session. The research report or any other part has not been previously submitted for any other degree.

Supervisor:

Date:

Prof. Shengrong Shen
Biosystems Engineering and Food Science,
Zhejiang University, P.R China

DEDICATION

This work is dedicated

to my country and people. You are my encouragement and inspiration for this achievement.

And

to my professor whose valuable advices and comments have enabled me to overcome all of the academic challenges during my academics.

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ABBREVIATIONS

HACCP: Hazard analysis critical control point

FAO: Food and Agriculture Organization

WHO: World Health Organization

ISO: International Standards Organization

NACMCF: The USA National Advisory Committee on Microbiological Criteria in Foods

CDC: The Centers for Disease Control and Prevention

HPLC: High Performance Liquid Chromatography

MIPs: Molecularly-Imprinted Polymers

MIT: Molecularly Imprinting Technology

SPE: Solid-Phase Extraction

MISPE: Molecularly Imprinted Solid-Phase extraction

GC: Gas chromatography

MS: Mass spectrometry

B-CD: β-Cyclodextrin

M-MAA: MAA-linked allyl-β-CD imprinted polymers

M-MMA: MMA-linked allyl-β-CD imprinted polymers

M-AN: AN-linked allyl-β-CD imprinted polymers

M-AA: AA-linked allyl-β-CD imprinted polymers

M-βCD: allyl-β-CD imprinted polymers

N-MAA: MAA-linked allyl-β-CD non-imprinted polymers

N-MMA: MMA-linked allyl-β-CD non-imprinted polymers

N-AN: AN-linked allyl-β-CD non-imprinted polymers

N-AA: AA-linked allyl-β-CD non-imprinted polymers

N-βCD: allyl-β-CD non-imprinted polymers

ABSRTACT

Food is the only source of life and it offers invaluable advantages: growth and health promotion, energy, and disease prevention; hence, life will not exist without food. People like enjoying sharing meal-gathering together and like to taste different kinds of traditional cuisines; yet they also demand for consumption of quality and safe food. Principally, food, generally reconsidered as safe (GRAS), should be strictly produced from sanitations, good manufacturing practices, good handling practices, and good agriculture practices to conform with food safety system guideline in order to prevent the contamination or recontamination, especially microbial pathogens and toxic chemicals, because these materials cannot be visually detected. Food contaminations resulting to food poisonings are obviously very serious issues to public health, living welfare, and socio-economic development; therefore, they need to be tightly controlled (prevention, reduction, and elimination) before serving on the tables or markets. To tackle such these difficult problems is a main task and huge challenge for regulatory authorities, public health agencies, and food industries in order to ensure the quality and safe-to-eat food.

Because of visually impossible detection of food hazards, numerous sophistically efficient analytical methods have been successfully developed in order to meet the growing demands for food safety and quality control and supervision. Although food toxicity mostly comes from pathogenic microbes, toxic chemicals; such as pesticide residues, illegal food additives, drug residues; are also a major threat to the life and public health; therefore, there is an increasingly considerable interest in developing new selective and sensitive method for extracting, isolating, and enriching contaminated components from complex food matrices. One of the promising methods that have been innovated so far is a molecularly imprinting technique, which has drawn a great attraction worldwide in the field of chemical separation science. The exploitation of this technique could produce a product of molecularly imprinted polymers (MIPs); which are very robustness, long-term stability, reliability, cost-efficiency, and selectivity; creating MIPs to gain more popularity in chemical separation and analysis.

In this dissertation, we would like to introduce “an analytical method” using MIPs-based materials as a solid phase extraction for selective recognition of given chemical molecules from the food matrices before high performance liquid chromatographic determination. The technique of MIPs synthesis is based on a traditional method, which bulk MIPs need to be ground, sieved, and Soxhlet washed prior to applying them as a

molecularly-imprinted solid-phase extraction (MISPE) for sample clean-up and enrichment. The main functional monomer and cross-linker we use in MIPs polymerization is β (beta)-cyclodextrin, a 7-membered sugar ring molecule and ethylene glycol dimethacrylate (EGDMA), respectively. Our introduced method of MIPs polymerization is simple and inexpensive; moreover, the volume of organic solvent involved in the synthesis of MIPs and the rest of experimental process requires minimally, which is in parallel with environmentally-friendly way. Our findings are as follows.

(1) In chapter two, we prepared a series of di (2-ethylhexyl) phthalate (DEHP) imprinted polymers by using the single use of allyl bromine-β-cyclodextrin (allyl-β-CD) and the combined use of allyl-β-CD and methacrylic acid (MAA), allyl-β-CD and methyl methacrylate (MMA), allyl-β-CD and acrylonitrile (AN), and allyl-β-CD and acrylamide (AA) as the binary functional monomers. The results proved that the binary functional monomers, except for AA monomer, are superior to a single monomer; their average bound substrate from binary monomers was ~110 μmol g^{-1}, whereas a single was ~90 μmol g^{-1} in binding specificity. Finally, M-MAA, M-MMA and M-AN were chosen to run through molecularly imprinted solid-phase extraction (MISPE) to analyze the spiked infant formula of DEHP. For M-AN, the recovery ranged from 93.59-97.98% with relative standard deviations (RSD ≤ 3.21%).

(2) In chapter three, we synthesized three kinds of clenbuterol-imprinted polymers by the combined use of ally-β-cyclodextrin (ally-β-CD) and methacrylic acid (MAA), allyl-β-CD and acrylonitrile (AN), and allyl-β-CD and methyl methacrylate (MMA) as the binary functional monomers. Based upon the results, M-MAA polymers generally proved to be an excellent selective extraction compared to its references: AN-linked allyl-β-CD MIPs (M-AN) and MMA-linked allyl-β-CD MIPs (M-MMA). M-MAA polymers were eventually chosen to run through a molecularly imprinted solid-phase extraction (MI-SPE) micro-column to enrich CLEN residues spiked in pork livers. A high recovery was achieved ranging from 91.03-96.76% with relative standard deviation (RSD ≤ 4.45%).

(3) In chapter four, we exploited binary functional monomers, allyl-β-cyclodextrin (allyl-β-CD) and methacrylic acid (MAA) or allyl-β-CD and acrylonitrile (AN), in a fabrication of molecularly imprinted polymers (MIPs) for selective recognition and large enrichment of pirimicarb pesticide from aqueous media. According to the results, the effect of binding capacity of MAA-linked allyl-β-CD MIPs (M-MAA) demonstrated higher efficiency than that of AN-linked allyl-β-CD MIPs (M-AN) when tested in binding specificity. Finally, M-MAA was chosen to run through molecularly imprinted solid-phase

extraction (MISPE) to analyze the spiked fresh leafy vegetables of pirimicarb. The present proposed technique is a promising tool for the preparation of the receptors which could recognize pirimicarb pesticide in aqueous media.

According to the aforementioned results, we found that our synthesized MIPs could be used for a sample clean-up and pre-concentration of target analytes of interest in food matrices. Our MIPs showed good stability, good selectivity, and high efficient adsorption capacity towards the target molecules; therefore, they could be applied to real food samples as an integral part of analytical method for food quality and safety control and supervision.

There are a variety of applications of MIPs such as off-line or on-line solid phase extraction (SPE), chemical and bio-sensors, catalysis, and drug delivery because MIPs, a synthetically potential artificial receptor-like binding sites with a "memory" for shape and functional group positions of the target molecule, possess a competent ability for selective specificity and recognition for target or unwanted chemical molecules. Among various MIPs applications, the most commonly used is an off-line solid phase extraction application due to its simplicity. Utilizations of MIPs-based materials have been applied to a wide range of chemicals, including food contaminants, pesticides, environmental pollutants, preservatives, and antibiotic drug residues for sample clean-up and pre-concentration, detection, and quantification.

All in all, due to various functionalities of MIPs such as solid phase extraction (SPE), chemical and bio-sensors, catalysis, and drug delivery, molecular imprinting technique has drawn a huge attraction from a wide range of fields, including food safety, chemistry, biology, pharmaceutical engineering, and medicine, etc. MIPs have become a versatile tool of the modern analytical chemistry.

Keywords: Molecularly-imprinted polymer (MIP); Solid phase extraction (SPE); Food safety; Polymerization; High performance liquid chromatography (HPLC)

CHAPTER 1 LITERATURE REVIEW

1.1 Background Information

Food is a basic need of life; none of us can survive without food. Food is an only source of nutrition, energy, and growth for all kinds of living organisms; however, it is also one of the most potential risks if it is not properly handled and processed. People generally need to consume quality and safe food. "Food quality and safety", to our personal point of view, ought to be borne with three attributes: health promotion, growth promotion and disease prevention on consumers. On the other hand, Will and Guenther (2007) defined that food quality and safety are the totally characteristics of food products that bear on their ability to satisfy all legal, customer, and consumer requirement. Due to these factors, a number of food industry, academia, and research institutes have conducted an extensive research to improve more food quality, safety, and nutrition for consumers. However, people are still more growing concerns about food quality and safety after a wide array of serious incidents related to food-borne diseases, resulting from consumption of food-toxic contaminations, endangered lives and even ended up in deaths. In China taken as an example, Chinese central government led by Chinese Premier Li Keqiang has paid a close attention on food manufacturing industry and tightened up strict market supervision and harsh penalties to ensure food safety involving adulterated foods after following a series of food scandals in recent years, especially after a milk scandal in 2008 produced by Sanlu Group, a dairy food manufacturer based in Shijianzhuang of Hebei province, was discovered to have sold milk powder tainted with melamine, an industrial compound used to fabricate plastic that makes the milk appear protein-rich (Qiu Quanlin, 2013), an estimated 300,000 babies in China were sick from the melamine-contaminated milk and six fatalities died from kidney stones and other kidney failure with an estimated 54,000 infants being hospitalized (https://en.wikipedia.org/wiki/2008_Chinese_milk_scandal). Other similar cases of food scandals are related to a di (2-ethylhexyl) phthalate (DEHP), a toxic plastic additive (Wang Yan & Zheng Xin, 2011), a clenbuterol, an illegal food additive chemical and better known as "lean meat powder" that China prohibits its use as an additive in pig feed, and "gutter oil" made from kitchen waste (Zhou & Wang, 2012). "Gutter oil", literally referred to recycled oil dredged from gutters behind restaurants and inedible animal oil or referred to the reprocessing of low quality animal meat, decomposing animal fat, rotten internal organs as raw materials, is believed to have

carcinogen (any substance or agent that promotes cancer), that is extremely dangerous to health if consumed. In a national crackdown in 2011, the Chinese police dealt with 120 cases and 60,000 tons of gutter oil being sold as edible oil (Zhou & Wang, 2012) and have seized over 3,200 tons of a new type of "gutter oil" made from decomposing animal fat and internal organs (Cao Yin & Luo Wangshu, 2012).

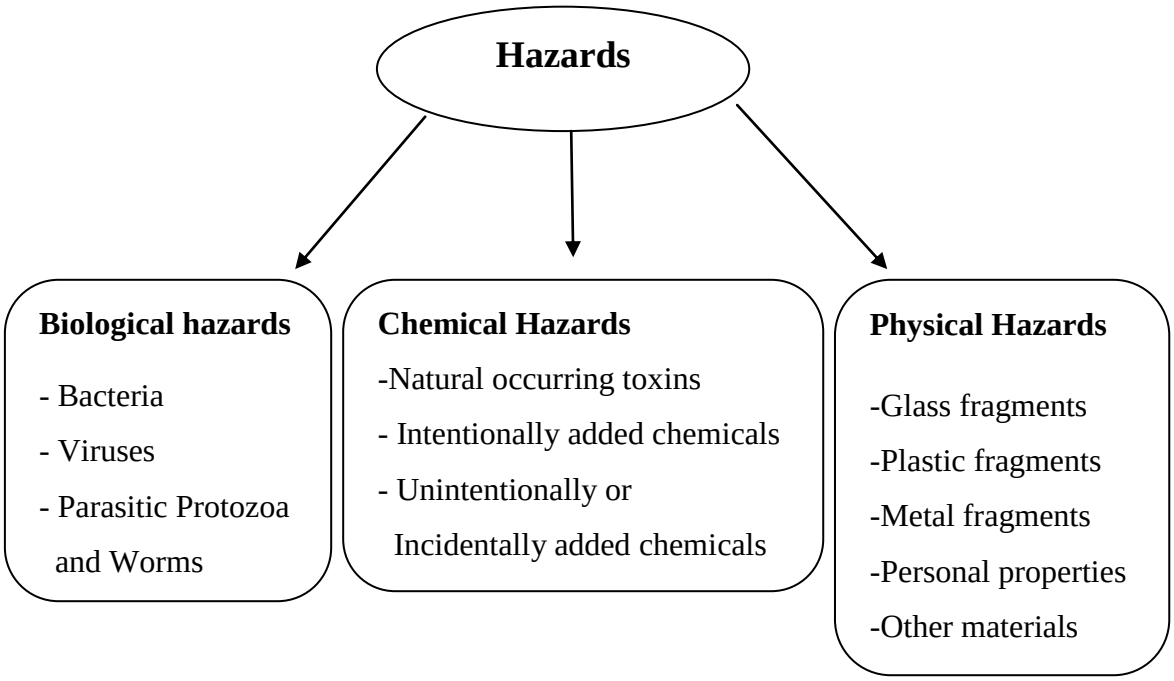
Such a rising number of food scandals have severely damaged consumers' confidence of food safety resulting in their changes of purchasing habits and caused a huge economic loss in food industry. Therefore, innovative analytical detection method of chemical hazards in food is strongly demanded for food quality and safety control and supervision for government and regulation of the policy to safeguard the welfare of people's livelihood.

1.2 Food safety hazard components

Food safety hazard control was a use of the hazard analysis critical control point (HACCP) system to control food safety system in food industries. According to an advisory committee on microbiological criteria, HACCP is a management system in which food safety is addressed through the analysis and control of biological, chemical, and physical hazards from raw material production to procurement, handling, manufacturing, distribution, and consumption of the finished product. HACCP is a common-sense, practical, and achievable food safety approach that industry strives to follow, within the limitations of available technology to produce, transport, procure, and prepare foods that present a minimum level of risk from food-borne hazards. HACCP standard is internationally recognized as the most effective means of controlling food-borne disease, and is jointly developed by FAO/WHO Codex Alimentarius Commission, International Standards Organization (ISO) and the USA National Advisory Committee on Microbiological Criteria in Foods (NACMCF). Especially, HACCP is a logical, effective, scientifically-based and highly structural system of food safety management designed to help plant HACCP teams in producing a program to minimize, manage or control hazards. For instance, numeral countries, including most members of the European Union (EU), Brazil, Canada and the USA have adopted dairy HACCP programmes, which are government-required, for domestic dairy plants, dairy exports or diary imports (Adnan Y. Tamime, 2009).

The term "hazard", defined by HACCP, is a biological, chemical or physical agent that is of sufficient severity or it reasonably likely to cause illness or injury in the absence of its

control. As food safety experts, food processor, public health officials, we should understand the key sources of hazards to guarantee food safety system for consumers. Factually, there are three sources of food safety hazards defined by HACCP: biological, chemical and physical. Sources of contamination; being in the form of pathogenic microorganisms, undesirable pesticides and heavy metal pollutants; could be from air, soil, fecal materials, insects, birds, humans, rainwater, and other animals.



1.2.1 Biological hazards

Table 1.1 shows the biological hazards of pathogenic microorganisms that can potentially cause illness in susceptible individuals if not properly processed.

Food is an easy source of contaminations; therefore, food generally regarded as safe (GRAS) should be absence of food-borne pathogenic microorganisms and toxic chemicals. Mishandling, poor personal hygiene, improper cleaning of storage and preparation areas and unclean utensils could result in contamination of raw and cooked foods, allowing bacteria to grow. Biological contaminants could produce food poisoning that threatens public health safety, food industries, national economy and social well-beings. Foods, tainted with microbiological pathogens such as *Listeria monocytogenes, Salmonella spp., Shigella, Bacillus cereus, Campylobacter jejuni, Clostridium botulinum, Clostridium perfringens,* and *Escherichia coli O157:H7,* have been linked to numerous cases of food-

borne illnesses and deaths. For instance, a case of listeria outbreak traced to Colorado cantaloupes fruits in the United States in 2011 claimed the life as many as 16 victims, the deadliest food outbreak in more than a decade; Listeria is more deadly than more well-known pathogens like salmonella and E.coli. In 1998, twenty-one people died in an outbreak of listeria toxin traced to contaminated hot dogs and possibly deli meat, and another large listeria outbreak in 1985 claimed the life of 52 people linked to Mexican-style soft cheese. The Centers for Disease Control and Prevention (CDC) said that the most susceptible people suffer from listeriosis were the elderly, young (newborn, neonates), pregnant women and other with immune-compromised systems; symptoms of listeriosis include fever and muscle aches, often with other gastrointestinal symptoms. Victims often become incapacitated and unable to speak. Unlike many pathogens, listeia can generate at room temperatures and even low refrigeration temperatures (The Associated Press, 2011). Being ubiquitous in nature, listeria monocytogenes could be also recovered from stool samples of an estimated 1 to 10% of healthy humans (Farber & Peterkin, 1991). McLauchlin et al. (2004) indicated that there were 27 outbreaks of food-borne listeriosis reported worldwide, with about 2,900 cases and about 260 deaths. Between 1998 and 2002, even though listeriosis represented only 0.7% of bacterial food-borne diseases over this 5-year period, it accounted for 54% of all deaths (CDC, 2006). In brief, listeria monocytogenes, a Gram-positive bacterial agent of listeriosis, is of particular concern to food industry since it is a difficult food borne pathogen to control because of its prevalent distribution, tolerance to high levels of salt, and its ability to grow at a relatively low pH and even at refrigeration temperatures. Another example of bacterial food-borne outbreak is salmonella spp, a type of bacteria having a rod-shaped, gram-negative, aerobic bacterium that can cause foodborne illness (salmonellosis) if ingested in large numbers. CDC reported that 732 people in US have fallen sick and 150 people have been hospitalized after eating salmonella-tainted cucumber; the illness usually lasts four to seven days; most persons recover without treatment. According to CDC, salmonella is thought to infect 1.2 million people and 19,000 people were sent to the hospital, and an average of 450 people ends up dying due to the bacteria each year (Jen Christensen, 2015). Salmonella commonly found on meat and poultry. People who have eaten food contaminated with Salmonella often have fever, diarrhea (which may be bloody), nausea, vomiting, and abdominal pain after 12 to 72 hours after infection. The bacterium can enter the bloodstream and cause more severe illness although this rarely happens. Infection with salmonella also may be more serious or fatal in young children, elderly people, and people

with weakened immune systems. Fruits and vegetables that come into contact with salmonella may become contaminated with it, causing illness if eaten. Salmonella lives in the intestinal tracts of some animals, and can live in soil and water for months. Once salmonella has contaminated something, it can be spread from surface to surface.

Another form of pathogenic agents in food contamination comes from viruses. Viruses are ubiquitous and cannot be seen by naked eyes. Viruses can survive in human gut, contaminated water, and frozen food for months. Many viruses were found to have a potent transmission via contaminated foods and subsequently cause sickness in humans. Amongst those viruses, norovirus (NoV) and hepatitis A virus (HAV) are presently recognized as the most vital human foodborne pathogens regarding the number of outbreaks and people affected in the Western world (Marion Koopmans & Erwin Duizer, 2004). Transmission of viruses to foods is often related to poor hygienic practices. For example, people who infected by virus shed the particles via stool. Food handlers with viruses can transmit them to food if they did not sanitize or wash their hands properly. This route can also cause bacterial hazard contamination of foods. Marion Koopman and Erwin Duizer (2004) classified the foodborne pathogens into three main groups according to they produce: first, virus that cause gastroenteritis; second, enterically transmitted hepatitis viruses; and third, viruses that replicate in the human intestine but cause disease after they migrate to other organs, such as the central nervous system or the liver.

Finally, food-borne pathogenic agents come from parasites (protozoa and worm). Naturally, parasites different from virus need a host to survive, living on or within it. There are so many different types of parasites exist worldwide; however, only about 20% can be found in food or water and it is less than 100 are known to infect people through consumption. Two kinds of parasites were noted to infect people: parasitic protozoa and worms. Protozoa are unicellular animals that most of them cannot be seen without an assistance of microscope. The most common parasitic worms include roundworms (nematodes), tapeworms (cestodes), and flukes (trematodes); those worms are varied in size and length from barely visible to conspicuous. Humans and other animals easily get infected by consuming uncooked or undercooked foods, including aquatic vegetation, meat, fish, or shellfish, containing infected parasites (Keiser & Utzinger, 2004; Gulsen et al., 2006).

In short, a huge number of disease outbreaks associated with contaminated food are globally reported every year. An increasingly occurring number of food-borne diseases are more likely to be linked to changes in the food supply, lifestyles, and dietary habits

(Collins, 1997). Sivapalasingam et al. (2004) reported that an increase in the number of outbreaks associated with the intake of the fresh fruits and vegetables in association with an increase in the intake of fresh fruits and vegetables. Since food-poisoning pathogens often contaminated on many foods, knowing the characteristics of such pathogens are essential to an effective control program.

Table 1.1 Food safety hazard components

Biological Hazards	Chemical Hazards	Physical Hazards
I.Bacteria **a. Sporeformers** - *Clostridium botulinum* - *Clostridium perfringens* - *Bacillus cereus* **b. Nonsporeformers** - *Brucella abortis, B. suis* - *Campylobacter spp.* - *Escherichia coli (e.g. E. coli O157:H7)* - *Listeria monocytogenes* - *Salmonella spp. (e.g. S. typhimurium, S. enteriditis)* - *Shigella spp. (e.g., S. dysenteriae)* - *Staphylococcus aureus* - *Streptococcus pyogenes* - *Vibrio spp. (e.g., V. cholerae, V. parahaemolyticus, V. vulnificus)* - *Yersinia enterocolitica*	**I. Naturally occurring toxins** - Fungal toxins (Mycotoxin: aflatoxin, trichothecenes, zearalenone, fumonisins, ochratoxins, patulin, ergot alkaloids) - Scombrotoxin (histamine) - Ciguatoxin - Mushroom toxins (Protoplamic toxins: amatoxins, hydrazine derivatives, orellanine; Neurotoxins: muscarine poisoing, psilocybin poisoning; Gastrointestinal irritants; Disulfiram-like toxins: antabuse syndrome; Carcinogens, hydrazines.) - Shellfish toxins Paralytic shellfish poisoning (PSP) Diarrheic shellfish poisoning (DSP) Neurotoxic shellfish poisoning (NSP) Amnesic shellfish poisoning (ASP)/Domoic Acid **II. Intentionally and unintentionally added chemicals** - Food additives *Direct (allowable limits under Good Manufacturing Practices (GMPs))*	-Glass fragments -Stones -Hairs -Plastic fragments -Metal fragments such as bolts, nuts, bag clips/locks, shavings, wire, machinery, and so on. -Personal properties such as jewelry, earrings, buttons, pens, bandages -Other materials such as nut shells, fruit material (stem, caps, seeds), insects.

b. Nonsporeformers - *Brucella abortis, B. suis* - *Campylobacter spp.* - *Escherichia coli (e.g. E. coli O157:H7)* - *Listeria monocytogenes* - *Salmonella spp. (e.g. S. typhimurium, S. enteriditis)* - *Shigella spp. (e.g., S. dysenteriae)* - *Staphylococcus aureus* - *Streptococcus pyogenes* - *Vibrio spp. (e.g., V. cholerae, V. parahaemolyticus, V. vulnificus)* - *Yersinia enterocolitica* **II. Viruses** – *Hepatitis A and E* – *Norwalk virus group* – *Rotavirus* **III. Parasitic Protozoa and Worms** - *Anasakis simplex* - *Ascaris lumbricoides* - *Cryptosporidium parvum* - *Diphyllobothrium latum* - *Entamoeba histolytica*	+ Preservatives (e.g., nutrite and sulfiting agents) + Nutritional additives (e.g., niacin, vitamins, folic acid) + Color additives (e.g., astaxanthin (AST)) - Agro-chemical pesticides (including insecticides, herbicides, rodenticides, and fungicides) - Illegal food additives (melamine, formalin, phthalates) - Heavy metals (e.g. copper, cadmium, chromium, mercury, lead, arsenic, silver, zinc, cyanide) - Drug residues: + ***Group I :*** Prohibited drugs with no allowable extra-label uses in any food-producing animal species by the US food and drug administration: Chloramphenicol; Clenbuterol; Diethylstilbesterol (Des); Fluoroquinolone (class antibotics); Glycopeptides (all agents, including vancomycin); Medicated feeds; Nitroimidazoles (all agents, including dimetridazole, ipronidazole, metronidazole and other); Nitrofurans (all agents, including furazolidine, nitrofurazone and others) + ***Group II :*** Drugs with restricted extra-label uses in food-producing animal species: Adamantane and Neuraminidase inhibitors in all poultry (these agents are approved for treatment or prevention of influenza.); Cephalosporin (class antibiotics except cephapirin in all classes of cattle, chickens, pigs and turkeys.); Gentian violet (prohibited from use in food or feed of food-producing animal species); Phenylbutazone (in female	

- *Flasciolopsis buski* - *Giardia lamblia* - *Pseudoterranova diceptiens* - *Taenia solium, T. saginata* - *Toxoplasma gondii* - *Trichinella spiralis.*	dairy cattle (20 months of age or older)); Sulfonamide (class antibiotics: in lactating dairy cattle approved used are allowed for Sulfamimethoxine, Sulfabromomethazin and Sulfaethoxypyridazine) -Toxic chemicals: equipment cleaning compounds, sanitizers, lubricants, water treatment additives.	

1.2.2 Chemical hazards

Chemical hazards are listed in details in **Table 1.1**. Chemical contamination can happen at any stage in food production and processing. Those food chemical contaminants and residues can be either from intentional use or from an unintentional use with foods, which can be found under foodborne illness.

Chemicals which are applied according to the recommended regulations do not inflict the public health, animals and environment; however, the most highly concerns of potential risks to humans and environments keep increasing as chemicals are easy to be illegally accessible and exploited by food manufacturing producers in order to increase their profit returns. As previously described in preceding section 1.1, toxicity related to illegal use of chemicals in food products were described such as clenbuterol-tainted meats, melamine-tainted milks, DEHP-tainted foods, pesticide residues, antibiotics, etc. For example, Gianfranco et al. (2000) reported about a case of the clenbuterol epidemic poisoning of tainted beef meat in Italy resulting in sickness of 15 people following the consumption of tainted meat. The author described that there are diverse clinical symptoms in patients such as distal tremors, palpitations, headache, tachipnoea-dyspnoea, and also moderate hyperglycaemia, hypokalemia and leucocytosis. Outbreaks of clenbuterol-tainted food toxins due to consumption of bovine liver were reported elsewhere (Martinez-Navarro, 1990; Pulce et al., 1991).

Pesticides, one of the chemical hazards, are among the most public health threats and hazardous environmental pollutants owing to their high toxicity and bioaccumulation. Pesticides have been widely used in a protection of agricultural production to improve quality and yield. Pesticides, including insecticides, herbicides, rodenticides, and fungicides are used in crops as a means of controlling pests; however, when used them not based on their recommendation, they produce toxins on people and environment such as waters (González-López et al., 2005), soils (Arias-Estévez, et al., 2005), and leafy vegetables (González-Rodríguez et al., 2008). Maciej Tankiewicz et al. (2010) reported that there are 53,346.7 tonnes of crop-protection chemical products being sold in Poland in 2008 and the member countries of the European Union (EU) utilize an estimated 300,000 tonnes of pesticides to crops every year. Exposure to pesticides (foodstuffs, air, and water) is very harmful to human health and all living organisms. Generally, human health problems depend on the type of the used pesticides and also the extent of exposure. The immediate symptom of poisonings from pesticides include mild headaches, flu, skin rashes,

blurred vision and other neurological disorders, but rarely, paralysis, blindness, and even death. Long-term persistence of exposure could induce cancer, infertility, miscarriage, male sterility, birth defects, and effects on nervous system (Moses, 1995).

Another chemical hazard is result from aflatoxins, which are naturally occurring toxins. Aflatoxins are amongst the most potently toxic mycotoxins. Aflatoxins are odorless, tasteless and colorless which help them hard to be detected. These toxins constantly remain a high potential jeopardy to human health, farm animals and poultry; thereby, the reliable procedure should be developed to prevent this hazard. The major threat of among aflatoxins is B1 type; it is typically more abundant and induced liver disease (aflatoxicosis) and also induced an increased protein requirement in livestock and poultry that consume it. Aflatoxins are metabolized in the liver of all living organisms. High concentrations of aflatoxin consumption can lead to acute liver disease or even death within 3 days. Its low levels of aflatoxin exposure, however, require continuous consumption for several weeks to months in order for signs of liver dysfunction symptoms (Bingham et al., 2003). The aflatoxin B_1 is the most well-known producing carcinogenic natural toxin to mankind; moreover, aflatoxin B_1, the most commonly found in foods, was placed as group I carcinogens by the International Agency for Research on Cancer (IARC, 1993a). Aflatoxins, basically, have six main chemical forms, including aflatoxins B_1, B_2, G_1, G_2, M_1, and M_2 (Murphy et al., 2006; Weidenborner, 2001). The aflatoxins are mycotoxins produced primarily by toxigenic strains of aspergillus flavus and aspergillus parasiticus, so-called saprophytes and opportunistic pathogens, which commonly occur in the tropics and sub-tropics on a wide range of agricultural commodities and food matrices. Of these two species, aspergillus flavus produces aflatoxin B, meanwhile aspergillus parasiticus produces both aflatoxin B and aflatoxin G. Susceptibly agricultural materials to aflatoxin contamination include maize and other cereal grains such as rice, wheat, tree nuts, groundnuts, sorghum, millet, cottonseed, sunflower seeds and a variety of spices (Gordon S. Shephard, 2009) intended for human or animal consumption. Therefore, aflatoxins can get into the food supply chain easily when processed inattentively. It was found in both pet and human foods, as well as livestock feeds for agricultural animals and even in cosmetics. Fratamico et al. (2008) reported that aflatoxin transformation products are sometimes found in eggs, dairy products and meat when animals are fed contaminated grains. Aflatoxins B_1 and B_2 under shortwave UV was blue fluorescence, whereas aflatoxins G_1 and G_2 was green. Besides, aflatoxins M_1, and M_2 are the products of conversion of B form through metabolic processes in animals and excreted in milk. In the animal models tested,

aflatoxins were found to induce acute necrosis, cirrohosis, and carcinoma of the livers as well as acute poisoning with 50% lethal dose (LD_{50}) values ranging from 0.5 to 10 mg/kg body weight. The United States Food and Drug Administration's action level and legislation in the EU for aflatoxin in foods is 20 ppb (2 μg/kg), and for aflatoxin M_1 in milk, it is 0.5 ppb (0.05 μg/kg) because of the high consumption of milk by infants and children (Vijay K. Juneja & John N. Sofos, 2010). For instance, there were reported that the food poisoning outbreaks of aflatoxins (aflatoxicosis) occurring in Kenya between 2004 and 2005 resulted in over 150 deaths; maize samples contaminated up to 46,400 ppb of total aflatoxins, and over 55% of maize samples exceed regulatory limits in Kenya (Lewis et al., 2005). In brief, not only animals but also humans are facing highly more at risk of aflatoxins-contaminated compounds on agro-product industry. The growing rate of cancer disease is probably one of the main causes of aflatoxin-contaminated consumption. That is why, there are a lot of attempts trying to prevent the aflatoxin contents in the food chains, and the government also takes a preventive measure action to prohibit the aflatoxin-contaminated products from the commercially trafficking market.

Another form of chemical hazard is heavy metals. Heavy metal toxicity also poses serious health problems to people. Heavy metals; including zinc, arsenic, lead, cadmium, mercury, aluminum, silver, nickel, manganese, selenium, thallium; enter our body through food, water, skin, and air; those heavy metals can induce potential health risks if they present in high amounts in the body. Consumption of plants grown on heavy metal-contaminated soil can result in a potential risk to human and animal health (Gupta & Gupta, 1998; McBride, 2007; Monika & Katarzyna, 2004). Of the heavy metals, lead and cadmium are regarded potent carcinogenic agents and other related disease-causing materials, especially cardiovascular, kidney, neurological disorder, blood, and bone diseases (Jarup, 2003). Human and animal exposure of heavy metals is through soil-plant-human pathway. Heavy metal-contaminated soil is a root cause of human exposure to metals through food chain. The major source of soil and water pollutions can be from mining (Ping Zhuang et al., 2009), wastewater irrigation, solid waste disposal, sewage sludge application, and industrial discharge (Chen et al., 2005; Singh et al., 2004; S. Khan et al., 2008). For instance, Asia's largest realgar mine in China was forced to shut down after it polluted arsenic contaminated soil and water leaving local villagers with sickness and dying. Out of a total population of 3,000, over 1,200 residents have been diagnosed with arsenic poisoning. From 1971 to January, 2013, more than 600 workers at the mine factory died from arsenic toxicity, more than 400 of who suffered from cancer (Chinadaily,

2014). Environmental pollutants caused by heavy metals pose a serious problem for people. To solve this problem, it is required to have a strict regulation and supervision to control the heavy metal toxicants and is required an advanced, intellectual knowledge on how to remove all of these materials from soil and water.

1.2.3 Physical hazards

Physical hazards must be evaluated in food safety hazard because they are materials that could be likely to inflict injury or choking. Physical hazards, including fragment of stone, glass, plastic, or metals, particularly from packaging materials or processing facilities could incidentally enter a food chain if careless (**Table 1.1**). Hard or sharp foreign objects in foodstuffs may trigger traumatic injury including laceration and perforation of tissues of the mouth, tongue, throat, stomach and intestine as well as damage to the teeth and gums. However, natural fragments (i.e., hard or sharp) coming from a food itself are unlikely to cause injuries because of awareness of the parts by consumers that those fragments are a natural and intrinsic component of that particular product. As a consumer mistakenly consumes the foreign object, it is probably to cause choking, injury or other adverse health effects. Physical hazards are the most widely reported consumer complaints because the injury occurs immediately or shortly after consuming, and the source of hazard is often not difficult to identify.

1.3 Research Direction

As stated in the preceding section 1.2, there are three distinctive hazard components that HACCP categorizes with regarding to the food safety manner. In this present work, we decide to choose “chemical hazards” as our main research direction for food safety analysis and develop an analytical method to separate those hazardous chemicals from a food matrix by exploiting imprinting technique to establish the molecularly-imprinted polymers (MIPs) coupled with a high performance liquid chromatography (HPLC). To determine the concentration of unwanted chemical materials in foods is indispensible and challenging tasks for government, public health personnel, and food operator because food must be strictly supervised and regulated in order to guarantee the public health safety and people living’s well-beings.

1.4 Research Objectives

The specific research objectives are listed below:

1. To develop an analytical method based on molecularly-imprinted polymers (MIPs) for extraction and enrichment of hazardous chemical materials in food matrices.
2. To find a better way of MIPs polymerization by taken traditional bulk polymerization protocol base.
3. To evaluate and characterize the performance of synthesized MIPs according to sophisticated instruments and experimental processes.
4. To evaluate the effect of different functional monomers in the creation of MIP particles.
5. To review the current application of β (beta)-cyclodextrin as functional monomers in the fields of imprinting technique.

1.5 The introduction of molecularly-imprinted polymers

Over the recent years, the technique of molecular imprinting has rapidly gained an interest amongst the academic community and the industry. As a result, significant progress has been made in developing polymerization methods that generate enough MIP formats with rather good binding attributes expecting an enhancement in the performance or to suit the desirable final application, such as beads, membranes or nanoparticles. Molecularly imprinting technology (MIT) is one of the most promising methodologies to synthesize artificial receptors as ideal materials with a predetermined selectivity and specificity for a target analyte; the material of molecularly imprinted polymer (MIPs), obtained by using an imprinting technology, has a molecular recognition ability enabling to memorize natural recognition entities, such as antibodies and biological receptors, from a complicated environmental sample like biological fluids (Guiseppe et al., 2011).

MIPs are popularly considered a versatile tool in a field of separation science because they are able to separate and recognize both biological and chemical molecules; including amino acids (Morelli et al., 2010; Scorrano et al., 2011; Kempe & Mosbach, 1995; Vidyasankar et al., 1997), proteins (Bossi et al., 2007; Rachkov & Minoura, 2001; Shi et al., 1999), nucleotide derivatives (Longo & Vasapollo, 2008), pesticides (Obana et al., 2003; Schenck et al., 2002; Kaijie Tang et al., 2008; Israel S´anchez-Barrag´an et al., 2007; Qingzhi Zhu et al., 2002), steroidals (Zhong et al., 2001; Asanuma et al., 2000), pollutants

(Dabrowska et al., 2003; Pichon & Chapuis-Hugon 2008; Tamayo et al., 2005), drugs (Puoci et al., 2007; Masci et al., 2001; Ansell & Mosbach, 1998), antibiotics (Edward & Stanley, 2003; Christine et al., 2000), carbohydrates (Wullf, 1995; Mayes et al., 1994) and food contaminants (Baggiani et al., 2007); with predesigned imprinted cavity networks. To date, MIPs have been widely adopted in various areas including solid-phase extraction (SPE) (Brüggemann et al., 2004; Ariffin et al., 2007; Turiel et al., 2007; Hassan et al., 2000), online column-packed chromatography stationary phase (Jiaping et al., 2002; Mena et al., 2002; Qin et al., 2008; Kempe & Mosbach, 1995; Xie et al., 2001; Huang et al., 2003); chemo- and biosensors (Piletsky et al., 2006; Sadaf et al., 2011; Shaoqin et al., 2013, Subramanian et al., 2012; Li & Li, 2007); catalysis (Li & Li, 2007; Alexander et al., 1999; Wulff, 2002); drug delivery (Puoci et al., 2008); biological antibodies and receptors mimics (Bossi et al., 2007; Longo & Vasapollo, 2008b; Ge & Turner, 2009; Andersson et al., 1995; Shi et al., 1999).

Even though the first use of MIPs was reported in 1972 (Wulff et al., 1972), the widespread application of MIPs started booming in many fields until 1983, shortly after Vlatakis et al. (1983) published a paper demonstrating its potential effects and usefulness in selectivity and affinity of a target molecule.

Fundamentally, to synthesize MIPs is required three basic components; the template molecule (analyte), the functional monomer, cross-linking agent; to form a complete building process of MIPs production. Once the synthetic MIP is obtained, the template molecule was washed out under certain conditions resulting in selective imprinted binding sites leaving on an imprinted polymer matrix (commonly known in the scientific community as a molecularly imprinted polymer (MIP)) which is able to complementary with size, shape, and functionality of the template molecule rebinding. **Figure 1.1** shows the schematic illustration of MIP polymerization. The chief advantages of MIPs are their selectivity and affinity for a designed target molecule and they possess a physical robustness, strength, and resistance to atmospheric temperature, pressure, acids, bases, metal ions, and organic solvents. More importantly, they are inexpensive to be fabricated, and are capable of long, stable extended shelf life for years at room temperature (Giuseppe et al., 2011).

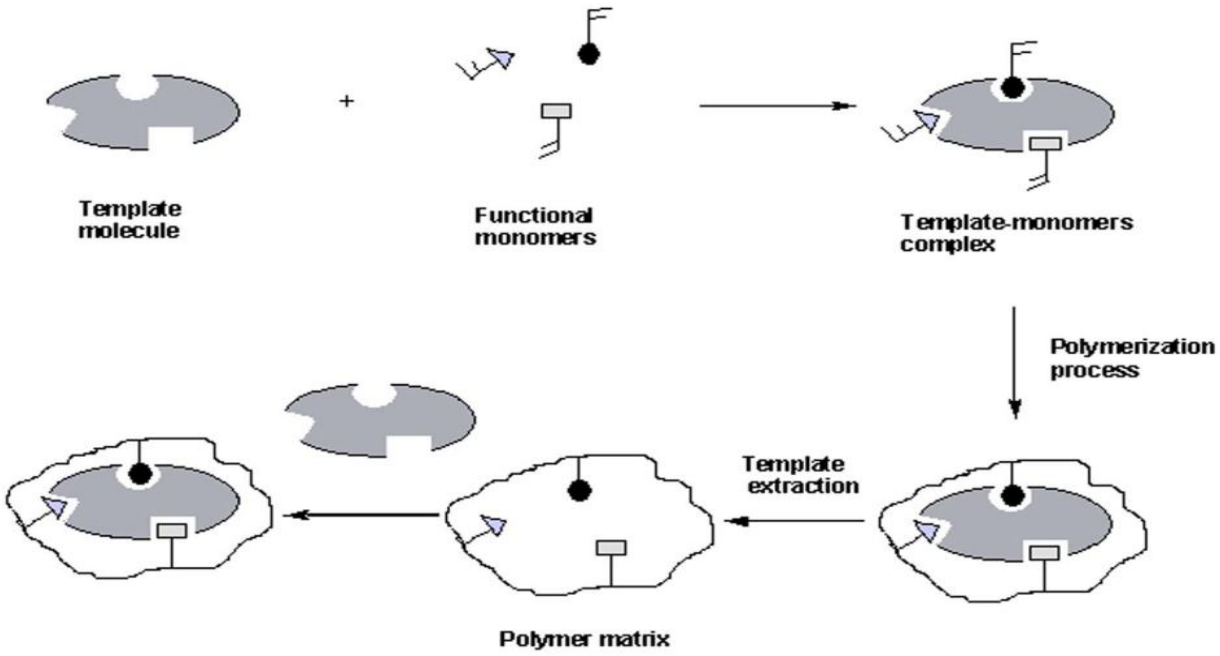


Figure 1.1 The schematic illustration of MIP polymerization (Adapted from Giuseppe et al., 2011).

1.6 Synthesis of MIPs

In pre-polymerization, careful selection of functional group is crucial to attain the highest quality of MIP material. Choosing the right functional ones will determine, on one hand, the stability of the complex formed before and during the polymerization process and on the other hand, the subsequent ability of the MIP product to interact selectively with the target analyte (Beltran et al., 2010); therefore, the careful choice of monomer functional group is necessary choice to provide complementary interaction with the template and substrates (Hongyuan & Kyung, 2006). The interaction of template onto the polymeric matrix can be established to organize the functional monomers around the template through a variety of mechanisms such as hydrogen boding (H-bonding), ionic bonding (π-π bonding), dipole–dipole or electrostatic interaction, hydrophobic effect and metal ion-coordination (Xu et al., 2007). A published review paper of molecularly-imprinted polymers as useful sorbents for selective extraction conducted by Beltran et al. (2010) showed that methacrylic acid (MAA) and 4-vinylpyridine (4-VP) are the most widely use as functional monomers in imprinting technique because both functional compounds can fabricate a strong hydrogen bond around a template molecule and they can be used for extracting either acidic or basic compounds, as it can be seen in **Tables 1.2-2.4**, which

summarize some of their recent MISPE application. **Figure 1.2** illustrates common functional monomers used in non-covalent molecularly imprinted polymers.

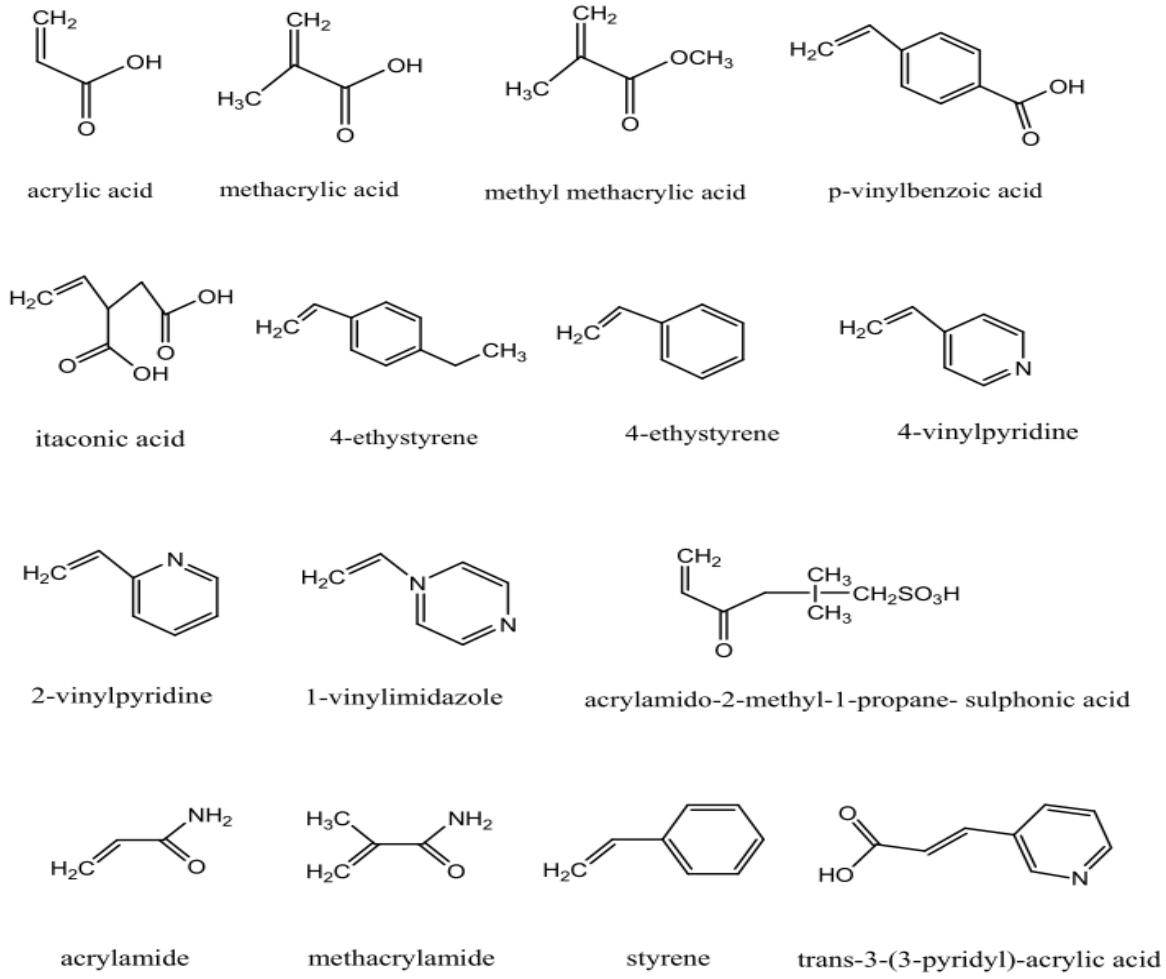
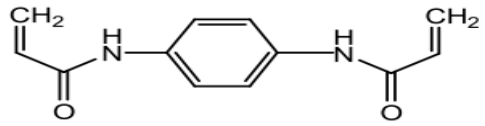
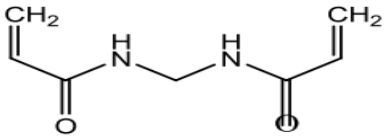


Figure 1.2 Common functional monomers used in non-covalent molecularly imprinted polymers (Adapted from Hongyuan & Kyung, 2006)

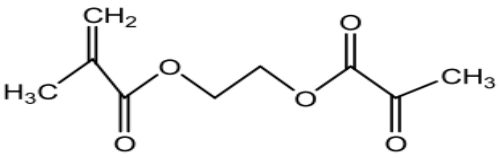
Another core component involved in the synthesis of a MIP is the cross-linker, whose function is to maintain mechanical stability to the polymer matrix, stabilize the molecular recognition site, and control the porosity of the polymer. Though there are many commercially available cross-linking agents to choose from, the most commonly used is ethylene-glycol dimethacrylate (EGDMA), as can be seen in **Tables 1.2-1.4**. From a polymerization point of view, the amount of cross-linking content is always preferably employed excessively to both the template and functional monomers in order to be able to generate materials with adequate mechanical stability. **Figure 1.3** shows chemical structures of common cross-linkers used in non-covalent molecular imprinting technique.



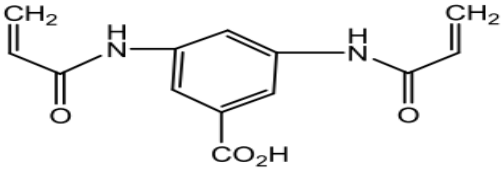
N,N'-1,4-phenylenediacrylamine



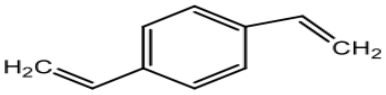
N,N'-methylenediacrylamide



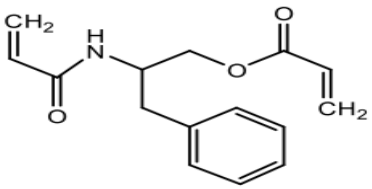
ethylene glycol dimethacrylate



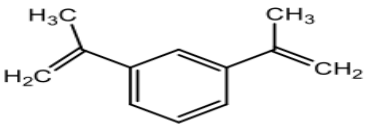
3,5-bis(acryloylamido)benzoic acid



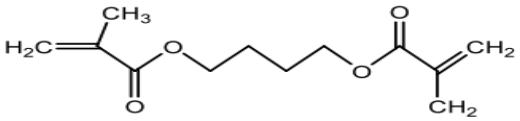
divinylbenzene



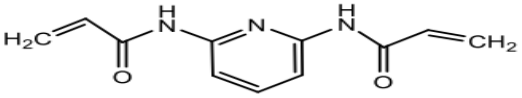
N,O-bisacryloyl-phenylalaninol



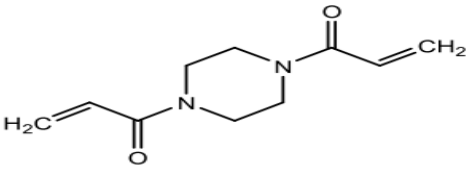
1,3-diisopropenyl benzene



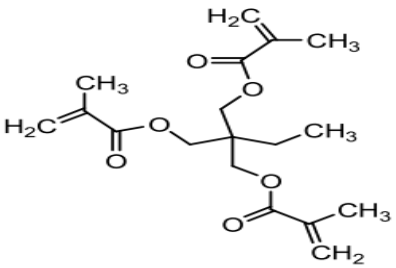
tetramethylene dimethacrylate



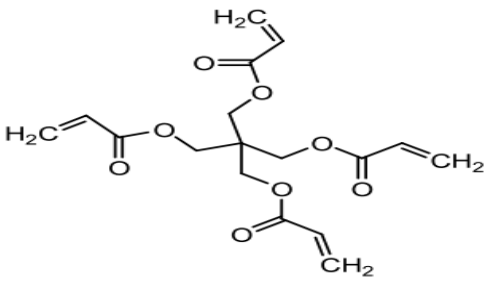
2,6-bisacryloylamidopyridine



1,4-diacryloyl piperazine



trimethylpropane trimethacrylate



pentaerythritol tetraacrylate

Figure 1.3 Chemical structures of common cross-linkers used in non-covalent molecular imprinting technique (Adapted from Hongyuan & Kyung, 2006)

Factually, there are several methods of MIPs synthesis that have been invented to obtain a product of molecularly imprinted polymer particles. The below information will describe each point of the procedure.

1.6.1 Covalent, non-covalent and semi-covalent approaches

One way to attain an imprinted polymer particle is based on from either covalent, non-covalent (self-assembly), or semi-covalent approaches. The classification of MIPs is according to an interaction between the functional monomer and the template molecule during polymerization, and a purpose of MIP would be used.

Covalent approach involves covalently linking the template to the monomer. After copolymerization with cross-linking agent, the imprint molecule is chemically cleaved between the template and the functional monomer through a required acid hydrolysis procedure from the highly cross-linked polymer. Due to the slow kinetics involved in constructing a covalent bond during extraction of the analyte from the sample and the cleavage of this covalent bond during elution of the analyte from the MIP, the covalent approach of MIP fabrication is not popular.

The most frequently used method of MIP synthesis is a non-covalent approach because of its simplicity, an easy cleavage of template from polymeric networks, and a greatly available variety of functional monomers. The specialized binding site of MIPs made of a non-covalent approach is formed by the self-assembly between the template's and monomer's interactions such as H-bonding, π-π bonding, electrostatic interaction, hydrophobic effect, followed by a highly cross-linked co-polymerization (Svenson et al., 2004; Ekberg& Mosbach, 1989). The non-covalent imprinting approach is more likely to hold more dominance of future molecular imprinting because a vast number of compounds, even biological ones, are capable of non-covalent bonding with functional monomers (Michael et al., 1995; Sellergren & Kenneth, 1993).

Finally, another synthetic method for obtaining MIPs is the semi-covalent approach. In this case, the interaction between the template molecule and the functional monomer before the polymerization is covalent, whereas the interaction of the target analyte and the MIP once the polymer is in use is through non-covalent interactions (Beltran et al., 2010). Owing to the varying interaction established between the template and the functional monomer before the polymerization process in the non-covalent or semi-covlaent approach, a different ratio of template to functional monomer is required to ensure the best imprinting

on the finished polymer. The ratio generally applied to the semi-covalent approach to be rather low 1:1 or 1:2, whereas ratios typically range from 1:4 to 1:8 for the non-covalent approach, depending on the complexicity of the template and affinity of the functional monomer to the template (Beltran et al., 2010). For example, Cacho et al. (2006) studied an interesting comparison between the non-covalent and the semi-covalent approach from two different studies. The authors compared a semi-covalent MIP with one that they had previously obtained using the non-covalent approach (Cacho et al., 2003) to determine their respective efficiency in the selective extraction of triazines from real samples. For this comparison, the authors pointed out that the semi-covalent MIP enabled a better cleaned-up sample than the non-covalent MIP. Another separate example of semi-covalent and non-covalent MIP was reported by Ester et al. (2002). The authors synthesized two types of molecularly imprinted polymers (MIPs) for the selective extraction of 4-nitrophenol (4-NP) from water samples. One polymer was synthesised via a non-covalent approach and the other via a semi-covalent approach. The selectivity of the polymers for 4-NP was evaluated when these polymers were applied in on-line solid-phase extraction (MISPE) coupled to reversed-phase HPLC. The MISPE conditions for both MIPs were optimised and a clean-up step was included to eliminate non-specific interactions. Differences between the two MIPs were observed with the non-covalent MIP being the more selective of the two, whereas the recoveries were slightly higher for the semi-covalent MIP.

All in all, although covalent imprinting is generally believed to yield better defined and more homogenous binding sites than the non-covalent approach since the template-functional monomer interactions are far more stable and defined during the imprinting process than the template-functional monomer complex in the non-covalent approach, the general applicability of the pre-organized approach is limited due to the difficult design of suitable binding sites for the target analyte in which covalent bond formation and cleavage are readily reversible under mild conditions. In turn, non-covalent imprinting approach is much more flexible regarding the binding sites that can be exploited towards a range of templates. Importantly, the non-covalent approach is far simpler than covalent imprinting because the complexation step of polymerization is achieved simply by mixing the template with the functional monomers in a suitable solvent. No chemical derivatisation of the template is needed and template removal basically involves simply washing the imprinting polymers repeatedly with a suitable solvent or solvent mixture. A main drawback of non-covalent method is an unavoidable heterogeneity of the binding sites obtained arising from the multitude of complexes formed between the template and the

functional monomers which are factually preserved to some extent during the polymerization. The non-covalent bonding is principally not strong; therefore, an excess of functional monomer relative to the template is usually demanded to favor template-functional monomer complex formation and to maintain its integrity during polymerization. Consequently, a fraction of the functional monomers are randomly incoropated in the polymer matrix resulting in the formation of non-selective binding sites (Ester et al., 2002). **Figure 1.4** shows schematic representation of covalent and non-covalent molecular imprinting approach.

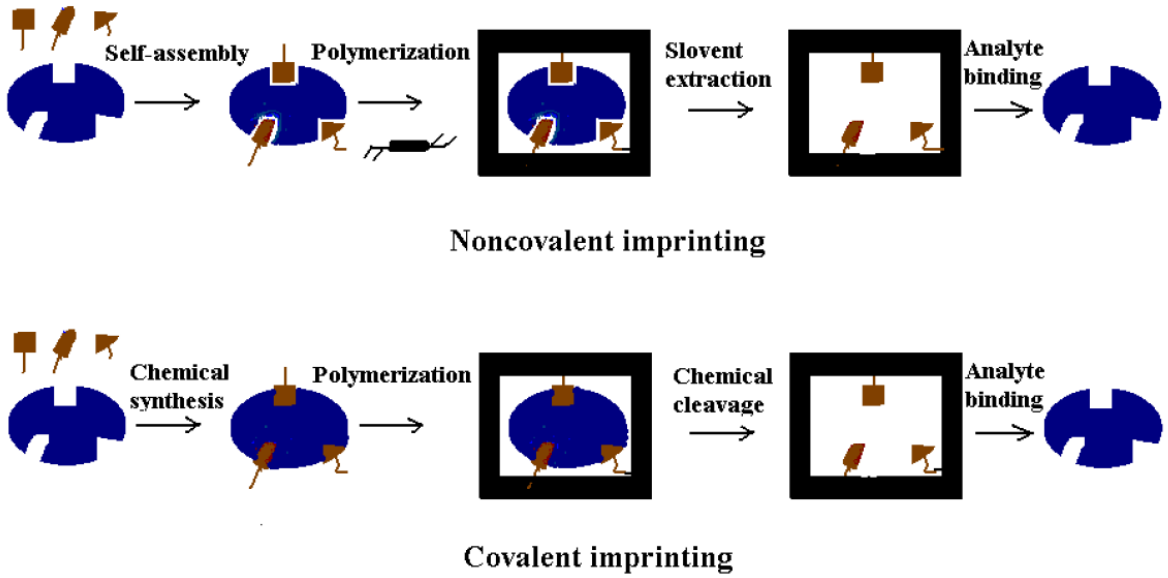


Figure 1.4 Schematic representations of covalent and non-covalent molecular imprinting procedures (Adapted from Hongyuan & Kyung, 2006).

1.6.2 Synthesis protocols

There are several protocols of MIP preparations that have been developed depending on the target molecular type (template) and the final application of MIPs. Fundamentally, MIPs are synthesized in various formats such as nano/micro spherical particles, nanowires and thin film or membranes. They are fabricated with different polymerization techniques; including bulk, precipitation, multi-step swelling, suspension, emulsion, dispersion, gelation polymerization; some of which we discussed below.

A. Bulk polymerization

The first polymerization method invented for MIPs was based on "bulk" or solution polymerization. Preparation method of MIP through bulk polymerization is also known as a traditional method for MIP polymerization. All the components (template, monomer, cross-linker, and initiator) are mixed together in a low volume of a suitable organic solvent, also known as porogen, subsequently leaving to polymerize for some periods before obtaining a resultant monolithic polymer. The resultant bulk polymer obtained by a conventional method must be crushed, ground, and sieved to obtain a certain size of polymeric particle before it could be used in the extraction method such as molecularly imprinted solid-phase extraction (MISPE), online column-packed chromatography stationary phase, immobilized thin layer chromatography (TLC) etc. Sellergren & Shea (1995) reported that the suitable particle size used in chromatographic studies is usually less than 25 μm. The major constraints of bulk polymerization method are irregular shapes and sizes between 20 and 50μm, substantial loss of imprinted sites, laborious and time consuming due to grinding and sieving.

Despite all these drawbacks, this is still the most widely-adopted protocol because this technique is the simplest method and versatility along with requiring low volume of organic solvent and also it does not require a sophisticated instrument and skills of operators.

B. Precipitation polymerization

Precipitation polymerization is the most widely-used technique for obtaining spherical particles of a size suitable for MISPE application amongst the techniques innovated to overcome the drawbacks of conventional protocol (**Tables 1.2-2.4**) (Beltran et al., 2010). The underlying principle of this approach is that polymer particles precipitate from the solution as the polymeric chains growing in solution reach a certain critical mass. The major constraint of this technique is that obtained particle size is typically not larger than 10 μm (Cacho et al., 2009), and are sometimes even in the sub-micrometer range, which is a limitation for MISPE procedure. Furthermore, precipitation polymerization is not as strong as bulk polymerization in term of imprintability, i.e., the possibility of imprinting any given template molecule (Beltran et al., 2010). Therefore, to overcome with such these constraints, the polymerization conditions must be thoroughly chosen in order to yield

good polymeric quality products, i.e., ratios of functional monomers and porogen or functional monomers and template molecule should be applied in the reaction process. **Figure 1.5** shows different particles of synthesized MIPs between traditional polymerization and precipitation polymerization

Several reports where MIPs obtained using the precipitation polymerization method were studied as sorbents in MISPE application (Hu et al., 2007; Turiel et al., 2005), capillary electrochromatography (Boer et al., 2002; Sp´egel et al., 2001), and chromatography-grade molecularly imprinted beads (Li et al., 2003). Compared to a traditional polymerization protocol, the precipitation polymerization was found to be outperformed the traditional one, not only a yield of polymeric products (particle size uniformity), but also sorbent capacities when applied in MISPE protocols (Beltran et al., 2007; Beltran et al., 2009).

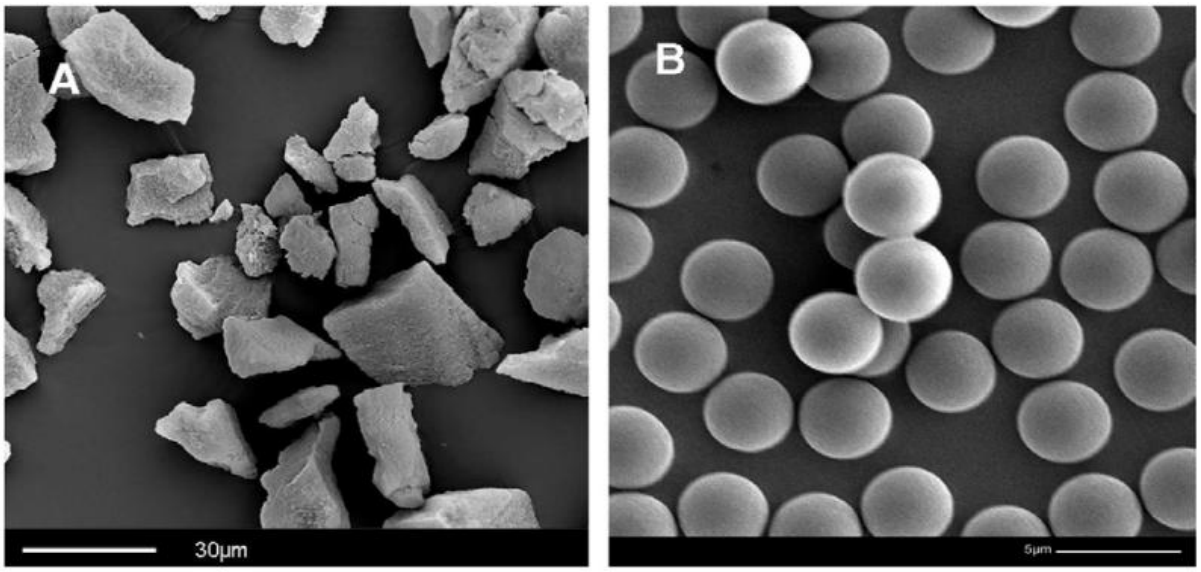


Figure 1.5 Schematic illustration of grinding MIP particles made from traditional polymerization (A), and spherical MIP particles made from precipitation polymerization (B) (Adapted from Beltran et al., 2010).

Table 1.2 Molecularly-imprinted solid-phase extraction (MISPE) protocols involving extraction from foodstuff samples (Adapted from Beltran et al., 2010)

Template	Funct. monomer	X-Linker	Analyte	Polymerization	Matrix	Analytical method	On/off-line
Enrofloxacine	MAA, 4-VPy, DEAEM	EGDMA	Norfloxacin, Enrofloxacin, Ciprofloxacin	Suspension	Milk	LC-UV	Off-line
Ofloxacin	2-hydroxyehtyl methacrylate	EGDMA	Enrofloxacine, Ciprofloxacine	Traditional	Milk	LC-UV	Off-line
Chloramphenicol	2-(diethylamino)ethyl methacrylate	EGDMA	Chloramphenicol	Suspension	Milk, Shrimp	LC-UV	Off-line
Tetracycline	MAA	EGDMA	Tetracycline	Traditional	Fish	Fl-Chem	On-line
Fumonisin B_1	2-diethylaminoethylmethacrylate	TRIM	Fumonisin B analogues	Traditional	Bell pepper, Rice, Corn	LC-MS-MS	Off-line
Zeralone mimic	1-allylpiperazine	TRIM	Zeralenone	Traditional	Wheat, Rice, Corn, Barley	LC-Fluor.	Off-line
Propazine methacrylate		EGDMA	Simazine, Atrazine, Propazine	Precipitation	Soil, Potato, Corn	LC-UV	Off-line
Isoproturon, Linuron	MAA, TFMAA	EGDMA	Fenuron, Linuron, Metoxuron, Clortoluron, Isoproturon, Metobromuron	Silica particles	Potato, Pea, Corn		On-line
Tebuconazole	MAA, 4-VPy	TRIM	Tebuconazole	Precipitation	Cabbage, Pannage, Shrimp, Orange juice, Water	LC-UV	Off-line
o-phthalic acid	4-VPy	EGDMA	Domoic acid	Multi-step swelling	Blue mussels	LC-UV; LC-MS	On/Off-line

Table 1.3 Molecularly-imprinted solid-phase extraction (MISPE) protocols involving extraction from biological samples (Adapted from Beltran et al., 2010)

Template	Funct. monomer	X-linker	Analyte	Polymerization	Matrix	Analytical method	On/off-line
Alfuzosin	MAA	EGDMA	Alfuzosin	Traditional	Plasma	LC-UV	On-line
Alfuzosin	MAA	EGDMA	Alfuzosin	Traditional	Plasma, Soil	LC-UV	Off-line
Cefathiamidine	4-VPy	EGDMA	Cefathiamidine	Traditional	Plasma, Serum	LC-UV	Off-line
Diazepam	MAA	EGDMA	Benzodiazepines	Traditional	Hair	LC-MS-MS	Off-line
Lamotrigine	MAA	EGDMA	Lamotrigine	Traditional	Serum	LC-UV	Off-line
Ketamine	MAA	EGDMA	Ketamine, Norketamine	Traditional	Hair	LC-MS-MS	Off-line
Enrofloxacine	MAA	EGDMA	Enrofloxacine, Ciprofloxacine	Traditional	Urine, liver	LC-UV	Off-line
Commercial MIP			Atrazine + 3 metabolites	Traditional	Urine	UV	In-line
Oxfloxacine	MAA	TRIM	9 quinolones	Traditional	Urine	LC-UV	Off-line
Clomiphene (analogue)	MAA	EGDMA	Tamoxifen	Traditional	Urine	LC-UV	Off-line
Carbamazepine	MAA	EGDMA DVB	Carbamazepine Oxcarbazepin	Traditional Precipitation	Urine	LC-UV	Off-line
Amoxicillin Cephalexin	MAA	EGDMA	Amoxicillin Cephalexin	Traditional	Urine	LC-UV	Off-line
Dopamine	MAA, Acrylamide	EGDMA,MBAA	Dopamine	Traditional	Urine	LC-Fluorescence	Off-line
Trimethoprim	MAA	EGDMA	Trimethoprim	Traditional, Suspension	Urine	LC-MS	Off-line

Table 1.4 Molecularly-imprinted solid-phase extraction (MISPE) protocols involving extraction from environmental samples (Adapted from Beltran et al., 2010)

Template	Funct. monomer	X-Linker	Analyte	Polymerization	Matrix	Analytical method	On/off-line
Enrofloxacine	MAA, 4-VPy, DEAEM	EGDMA	Norfloxacin, Enrofloxacin, Ciprofloxacin	Suspension	Milk	LC-UV	Off-line
Ofloxacin	2-hydroxyehtyl methacrylate	EGDMA	Enrofloxacine, Ciprofloxacine	Traditional	Milk	LC-UV	Off-line
Chloramphenicol	2-(diethylamino)ethyl methacrylate	EGDMA	Chloramphenicol	Suspension	Milk, Shrimp	LC-UV	Off-line
Tetracycline	MAA	EGDMA	Tetracycline	Traditional	Fish	Fl-Chem	On-line
Fumonisin B_1	2-diethylaminoethylmethacrylate	TRIM	Fumonisin B analogues	Traditional	Bell pepper, Rice, Corn	LC-MS-MS	Off-line
Zeralone mimic	1-allylpiperazine	TRIM	Zeralenone	Traditional	Wheat, Rice, Corn, Barley	LC-Fluor.	Off-line
Propazine methacrylate		EGDMA	Simazine, Atrazine, Propazine	Precipitation	Soil, Potato, Corn	LC-UV	Off-line
Isoproturon, Linuron	MAA, TFMAA	EGDMA	Fenuron, Linuron, Metoxuron, Clortoluron, Isoproturon, Metobromuron	Silica particles	Potato, Pea, Corn		On-line
Tebuconazole	MAA, 4-VPy	TRIM	Tebuconazole	Precipitation	Cabbage, Pannage, Shrimp, Orange juice, Water	LC-UV	Off-line
o-phthalic acid	4-VPy	EGDMA	Domoic acid	Multi-step swelling	Blue mussels	LC-UV; LC-MS	On/Off-line

C. Multi-step swelling polymerization

Multi-step swelling polymerization is another alternative approach to obtain spherical particles. In this technique, preformed uniformly-sized seed particles are suspended in water, followed by several additions of well-defined organic solvents, and ultimately the initially suspended particles begin swelling to a final size in a range of 5-10 μm. When the particles have swollen to the desired size, all the involved components in the MIP synthesis have to be added to the solution by creating incorporation into the particles during the swelling state, and polymerization is then induced. Compared to precipitation polymerization protocols, this multi-step swelling polymerization approach is more robust because none of the components associated with the polymerization influences the process of acquiring spherical particle. Due to its greater complexity, this approach has not been as commonly used as either bulk or precipitation polymerization (**Tables 1.2-1.4**) (Beltran et al., 2010).

There are several studied of MIP fabrication using multi-step swelling polymerization methods reported elsewhere (Hoshina et al., 2009; Sambe et al., 2007; Kubo et al., 2006; Haginaka & Kagawa, 2002; Hosoya et al., 1996; Haginaka & Sakai, 2000; Nakamura et al., 2005).

D. Suspension polymerization

Suspension polymerization, which is also not involved in grinding and sieving process, is a rather simple method for obtaining a spherical polymeric particle. In this technique, all the involved compounds required in the polymerization process are dissolved together in an appropriate volume of organic solvent and this solution is further added to a higher volume of an immiscible solvent, and the mixture solution is continually stirred rigorously with a mechanically magnetic machine to form droplets (typically in the micrometer range 10-100 μm) before the polymerization process is induced. A basic common method between suspension polymerization and multi-step swelling polymerization is that any given molecule (template) can be exploited to deliver imprinted materials and the most widely-used dispersing solvent in both cases is water although the presence of water during the polymerization process may interrupt proper interactions between template and functional monomer (Beltran et al., 2010). To avoid

interruption or interference, there are a few attempting reports using of liquid perfluorocarbon solvent instead of water (Mayes & Mosbach, 1996; Zhang et al., 2003).

Several studies using an aqueous suspension polymerization protocol in forming of non-covalently imprinted microsphere were reported elsewhere (Qu et al., 2008; Shi et al., 2007, Hu et al., 2005; Lai et al, 2002; Lai et al., 2001; Matsui et al., 1997; Flores et al., 2000). An interesting study of a comparison between a suspension method MIP and a traditional method MIP conducted by Hu et al. (2005) was shown that the traditional method MIP in term of adsorption capacity and selectivity was higher than the suspension method MIP due to the larger pore size and the number of active sites within a traditional method MIP. Lai et al. (2002) reported using of a suspension polymerization to prepare molecularly imprinted microspheres (MIMs) for use as the HPLC stationary phase for separating and recognizing the target molecule trimethoprim (TMP), using both electrostatic interaction and hydrophobic interaction between monomer/MIMs and the substrate. In 2001, the author also reported the separation and determination of 4-aminopyridine and 2-aminopyridine by high-performance liquid chromatography with molecularly imprinted microspheres, prepared by aqueous suspension polymerization, as stationary phase (Lai et al., 2001).

E. Surface imprinting polymerization (Grafting/emulsion)

Surface grafting is another polymerization technique aimed at delivering spherical particles; however, this is also used to form composite materials (Tamayo & Martı´n-Esteban, 2005). Silica particles was a starting material, which all components involved in the polymerization process are grafted within the silica particles before the polymerization process starts. When the polymer is established, the silica is etched way to reveal a final product of spherical particles (Yilmaz et al., 2007). This technique requires more knowledgeable about synthetic skill and the use of corrosive solvents.

Surface grafting of MIP layers onto preformed beads have gained much attraction to obtain chromatography-grade imprinted materials. Sellergren et al. (2002) and Ruckert (2002) successfully used thin imprinted layers as coatings on chromatography-grade porous silica using several synthetic skills to retain the radical polymerization on the surface of the beads. Also, Tamayo & Martı´n-Esteban (2005) exploited this technique to synthesize MIPs and applied

them for extraction herbicides from water and vegetables. Numerous reports were published elsewhere regarding surface imprinting polymerization (Sreenivasan, 2006; Say et al., 2005; Araki et al., 2005; Piacham et al., 2005).

F. Monolithic imprinted polymerization (In-situ polymerization)

Monolithic molecularly imprinted technique is a combination of monolithic column and molecularly imprinted method. Monolithic MIPs were prepared by a simple, one-step, in-situ, free-radical polymerization "molding" process directly within a chromatographic column without the tedious procedure of grinding, sieving, and column packing. This type of synthesized MIP is expected to enhance separation and enable direct analysis with high-speed and high performance after in-situ polymerization (Hongyuan & Kyung, 2006).

Matsui et al. (1993, 1995) was the first study of using the in-situ polymerization technique for preparation of molecularly imprinted monoliths. In this case, all involved components (template, monomer functional group, cross-linker, and initiator) were dissolved in porogenic solvents (cyclohexanol and 1-dodecanol). The mixture solution was degassed prior to pouring into a stainless steel chromatographic column. After polymerization, the template and porogenic solvents were removed by exhaustive washing solvents containing methanol and acetic acid.

This synthetic technology has attracted huge interest due to their ease of preparation, high reproducibility, high selectivity and sensitivity, and rapid mass transport; moreover, the preparation is more cost-effective because amount of template molecules required is relatively much lower and the MIP product has greater porosity, good permeability and high surface area which is suited for both small and large molecules (Hongyuan & Kyung, 2006). Over the recent years, the uses of monolithic media for superior chromatographic separation in HPLC and capillary electro-chromatography have received considerable attention (Liu et al., 2000; Svec et al., 2000; Yan et al., 2005; Wulff, 2002; Huang et al., 2004; Quaglia et al., 2004; Yan et al., 2003; Zhang et al., 2005; Li et al., 2005; Kim & Georges, 2005).

1.7 Template removal

After polymerization, the final product of MIP is obtained. The next challenging task is a template removal from imprinted cavities where serving an underlying sites for rebinding and extracting process; therefore, an efficient template removal process must be demonstrated in order to reach a full potential in analytical and biotechnological applications. The process of template removal is the least cost-efficient and most time consuming process in the production of MIP (Ellwanger et al., 2001).

Currently, there are several various methods of extraction being used for template removal. These have been divided into 3 groups: solvent extraction, physically assisted extraction, and subcritical or supercritical solvent extraction.

1.7.1 Solvent extraction

a) Soxhlet Extraction: this technique has known as a standard extraction protocol with organic solvents since its innovation over a century ago. This method consists of placing the MIP particles into a cartridge inside the extraction chamber before the solvent is heated and condenses inside the cartridge leaving to contact the MIP particles and then extracting template. The major advantage to this technique is the repeated washing of MIP particles with anew extracting solvent, and cause solubilization because hot solvent was used. Besides, there is required no filtration upon completion to collect the MIP particles. The instrument is affordable, versatile and could be applied to mostly any polymer matrix. The major disadvantages are a long time process of extraction along with required large volume of organic solvent, and the possible degradation for temperature-sensitive polymers. Additionally, the static nature of Soxhlet extraction method does not facilitate solvent flow through MIP (Luque de Castro & Priego-Capote et al., 2010).

Because our work is used Soxhlet extraction method to remove the template from polymer matrix, we try to design this technique by ourselves. **Figure 1.6** shows the design of Soxhlet instrument in our laboratory. Based on this technique, we can facilitate solvent flow through MIPs, which can increase the ability of extraction process.

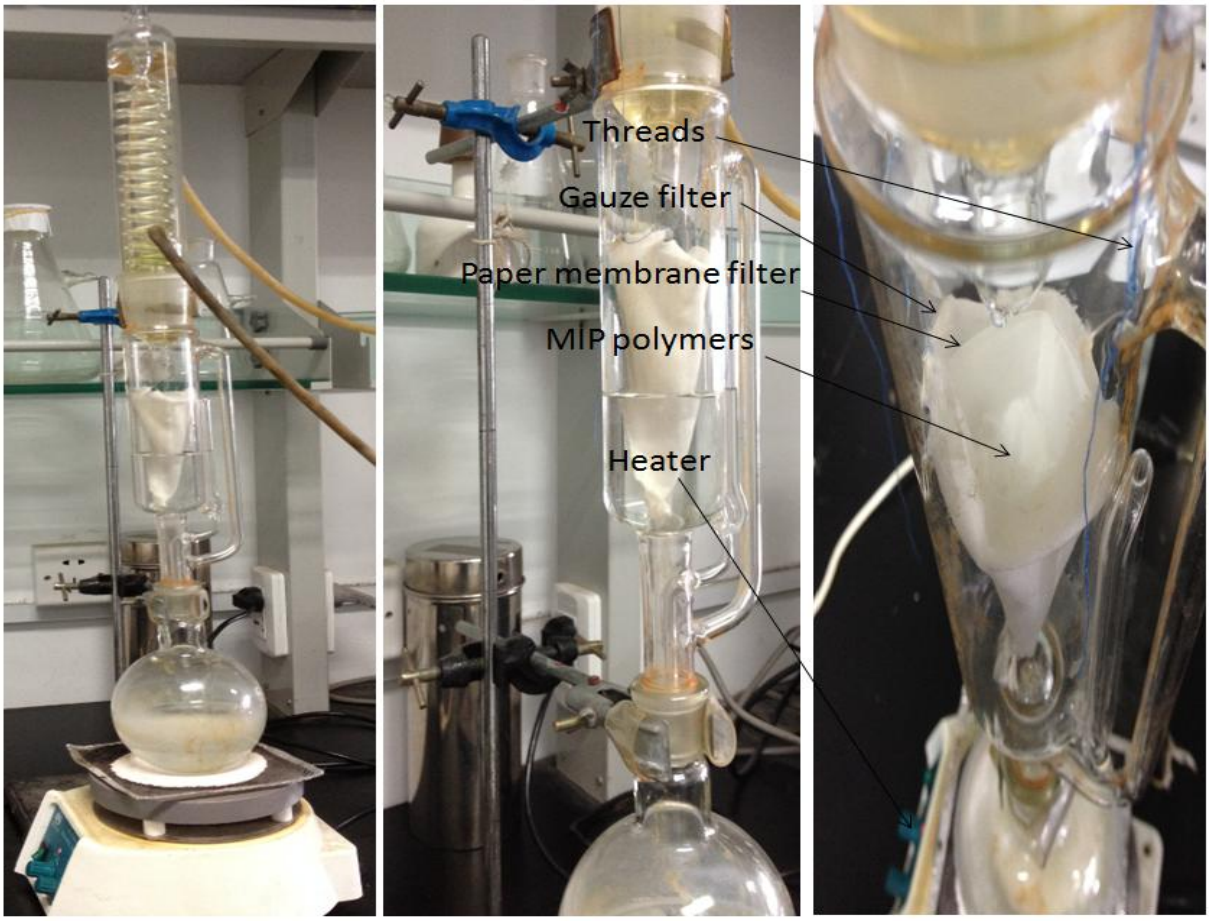


Figure 1.6 The close-up schematic illustration of Soxhlet apparatus designation by our group.

b) Incubation: this technique focuses on the immersion of molecularly imprinted polymer particles into extracted solvents which could induce swelling of the polymeric network and simultaneously dissociate the template from the polymer. Normally, this procedure is executed under mild condition; thereby, an imprinted site is not affected. It is much likely to the Soxhlet extraction technique that is very time-consuming (Hillberg et al., 2009).

1.7.2 Physically-assisted extraction

a) Ultrasound-assisted extraction (UAE): this technique uses an Ultrasound-based system which is a cyclic sound pressure with a frequency greater than 20 kHz. It runs through the process known as cavitation which generates small bubbles in aqueous medium that could

trigger the mechanical erosion of solid particles. The local increase in temperature and pressure induced by Ultrasound favor solubility, diffusivity, penetration and transport of solvent and template molecules (Cintas & Luche, 1999; Luque-Garcia & Luque de Castro, 2003).

b) Microwave-assisted extraction (MAE): this technique uses a microwave-based system which literally interacts with the molecules causing Ionic conduction and dipole rotation. The utilization of microwares for extraction causes the template to remove from the imprinted cavity rapidly, but one must be very cautious to avoid excessively high temperatures if the polymers are heat sensitive. This method could produce the best results as it is used with strong organic acids; nonetheless, this also could pose another problem because it is likely to cause particle MIP degradation (Ellwanger et al., 2001). This method considerably decreases the time required to extract the template, reduces the solvent costs, and is regarded to be a clean technique (Tobiszewski et al., 2009).

C) Mechanical extraction: the micro-contact molecular imprinting technology causes mechanical removal of the target molecules (large biomolecules, proteins etc.) from polymer. This method combined with the biosensor applications is a promising technique for solution of diverse biotechnological, environmental and medical matters (Ertürk et al., 2014).

1.7.3 Subcritical or Supercritical solvent extraction

a) Subcritical water (PHWE): this technique uses the water-based solvent, which is the most environmentally friendly and cheapest method. The temperature and pressure must be controlled in a range of 100 and 374^{o}C, and 10 and 60 bar, respectively. This technique is according to the high reduction in polarity that liquid water undergoes when heated to high temperatures. This permits water to solubilize a wide spectrum of polar, ionic, and non-polar constituents. Under these conditions, the decreased surface tension and viscosity also favor diffusivity. In addition to this, the high thermal energy could assist break intermolecular forces such as dipole-dipole interactions, vander Waals forces, and hydrogen bonding between the template and the polymer matrix (Mendiola et al., 2007; Teo et al., 2010; Ong et al., 2006).

b) Supercritical CO_2 (SFE): Carbon dioxide is a unique solvent because it bears the ability to alter its solvency power simply by adjusting the thermal energy and pressure of the CO_2 during the extraction. When the temperature and pressure are above 88F and 1083 psi, carbon

dioxide is taken as “supercritical”. If the temperature is decreased below 88F, carbon dioxide transforms to a liquid and considered as “subcritical”. Both supercritical CO_2 and subcritical CO_2 behave like a solvent, which could extract oil and other related compounds from polymeric matrix or plant materials. As the pressure is dropped (typically to below 600 psi), it changes to a gas and loses its ability to hold oils in solution and separates the extracted oil from the current gaseous CO_2. This technique is becoming a key commercial and industrial solvent because of its role in chemical extraction along with its low toxicity and environmental impact. The relatively low thermal energy of the process and the stability of CO_2 also permit a large variety of compounds to be extracted with mild damage or denaturing; the solubility of many extracted constituents in CO_2 additionally differs with pressure, allowing selective extractions. With regards to plant oil extraction, subcritical CO_2 has lower solvency power, hence it tends to extract mainly lighter oils and leave behind most waxes, paraffin and resins. Basically, subcritical CO_2 is relatively cold so that it is very effective at extracting temperature sensitive volatile oils. Subcritical CO_2 is of great importance at pulling and retaining light oils from the plant material. However, the extraction process can be time-consuming and yields are usually lower due to the fact that there will still be residual waxes and resins that are left behind in the plant material. On the contrary to subcritical CO_2, supercritical CO_2 is a much stronger solvent than subcritical CO_2. In addition of functionality of extracting the lighter oils, supercritical CO_2 could also extract the heavier molecular weight materials such as waxes, parafins, lipids, and resins, from the plant providing for higher efficiency in yields and a more complete extraction. The stronger solvency power makes the extraction times faster, too (http://www.apekssupercritical.com/why-supercritical-co2-for-botanical-applications/).

1.8 Applications of molecularly-imprinted polymers

There are various applications of MIPs that have been applied in molecular imprinting technology so far. The below information will present the main advantages of MIPs in the field of separation science.

1.8.1 Molecularly-imprinted solid-phase extraction (MISPE)

The major advantage of MIPs in MISPE protocols is high improvement in extraction selectivity and efficacy because the adsorbent offers stronger retention for selective extraction of the target molecule than that of the rest of compounds also present in the sample. The MIP quite often can also extract closely-related structures of the target analyte (often from the same family) through an effect known as cross-selectivity, enabling cleaner extraction of the analytes of interest (Beltran et al., 2010).

The MIPs can either be applied to off-line MISPE or on-line MISPE coupled to liquid chromatography. The most-studied method is the off-line protocol due to the fact that the eluting solvent is the mobile phase of the chromatographic system (Beltran et al., 2010). Before we can exploit the high selectivity of MIPs, pre-treatment method is required, especially for biological samples. In this aspect, the most commonly used technique is to deproteinize the sample using methanol or acetonitrile to avoid blocking the cartridge prior to using the MISPE procedure. After the enrichment of analyte of interest is collected through MISPE, the analyte was dried under the streaming of nitrogen gas (N_2) before HPLC, HPLC coupled with mass spectrometry (MS), HPLC/MS/MS, gas chromatography (GC), or GC/MS determination. Applying MISPE before chromatographic detection could minimize the interference compounds from the foreign materials while simultaneously enhance the sensitivity of analytes of interest through pre-concentration and enrichment based on MISPE protocol. **Figure 1.7** shows the schematic diagrams of MISPE protocol's designation by our group.

On the other hand, other highly environmental complex matrices required the analytes of interest to be extracted with an organic solvent beforehand are food samples. There are two prime advantages of performing extraction on the sample: Firstly, it offers the extracted fraction in the most appropriate solvent for running the MISPE procedure, which can be accomplished by employing either the most suitable extraction solvent for the MISPE (Xiong et al., 2006) or through evaporating the solvent to dryness and reconstituting the sample in a well-defined solvent to carry out the MISPE protocol (Urraca et al., 2006). Secondly, the final volume of the extracted fraction is quite often low, which is an advantage for MISPE due to the well-known, generally low ability of MIPs (Beltran et al., 2010).

MISPE has been widely used not only in biological samples (like plasma and urine) to pre-concentrate the analytes of interest such as antibiotics, illicit food additives, and so on due to they contain the most important information for observing the health of an individual, but also in environmental samples (like water and soil) such as pesticides. For example, the most commonly-studied herbicide compounds from environmental samples or other kinds of matrices are atrazines because of their high potent toxicity and widespread use. Therefore, these compounds must be tightly controlled, and this is most effectively accomplished by MIPs because of their high selectivity; anyways, commercially-available MIPs are already on market for the selective extraction of triazinic compounds (Beltran et al., 2010). For more information related to solid-phase extraction, several reviews of literatures were published elsewhere (Giuseppe et al., 2011; Beltran et al., 2010; Yolanda Picó et al., 2007; Hassan et al., 2000).

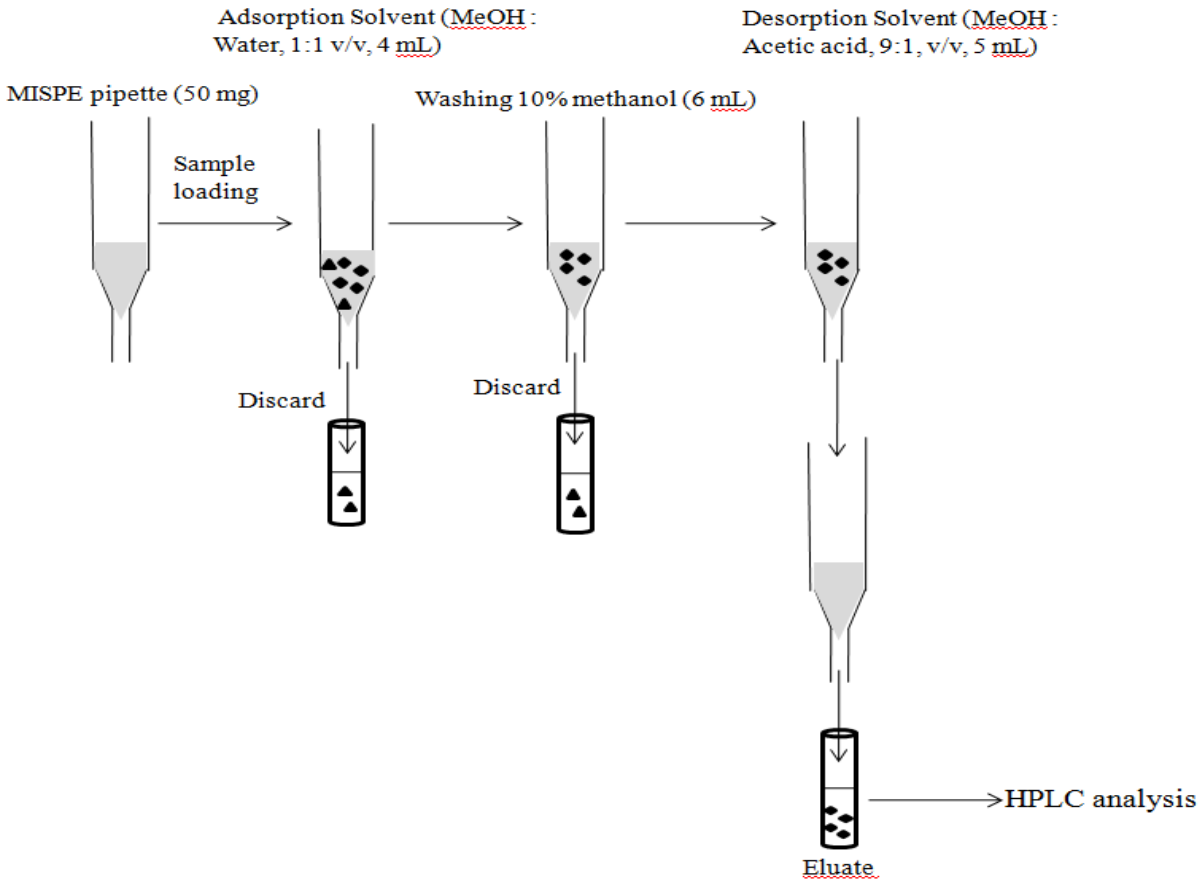


Figure 1.7 The schematic diagrams of MISEP protocol designation by our group.

1.8.2 Molecularly-imprinted polymers as chemical sensors and biosensors

Over the last decade, chemo-sensors and biosensors are gaining the increasing attraction of interest in the areas of modern analytical chemistry. This is a new demand of detective method that is emerging, particularly in food hazard analysis, environmental analysis, and clinical diagnostics. Due to an outstanding functionality of MIPs, a synthetic artificial receptor-like material being capable of binding an analyte of interest with comparable affinity and selectivity to natural antibodies or enzymes, MIPs can be integrated with different types of transducer, a new kind of functionality. Transducer is a device, usually electrical or electronic, that converts one kind of energy to another. Most transducers are sensors or actuators. Moreover, the chief attributes of MIPs making them a good candidate in chemical analysis are chemical inertness, long-term stability, and insolubility in water and most organic solvents. In recent years, a wide spectrum of transducers has been innovated with MIP-based sensors. The first and foremost kind of widely used transducer is optical transducer, such as fluorescence assays, surface plasmon resonance (SPR), and chemical luminescence. Second type of electrochemical transducer is also quite often used in MIP-based biosensors because these sensors are simple to utilize, rapid, sensitive, and inexpensive. The last one is mass-based transducer, such as the quartz crystal microbalance (QCM) (Liu et al., 2013). **Figure 1.8** exhibits schematic diagrams of fluorescent chemosensor MIPs for selective recognition of cholesterol by molecular imprinting technique (Cheng et al., 2014).

Typically, there are two processes involving MIP-based sensors for optical or fluorescent sensors. First, to exploit assemble MIPs as a chemo-sensor or biosensor, MIPs-based recognition sites needed to be fabricated as layers or thin films by deposition or grafting onto surface of transducer platform (Hedborg et al., 1993; Syu et al., 2006). The polymerizable functional monomers binding to the template molecules are copolymerized onto the surface of transducers via the traditional bulk polymerization technique or electropolymerization. After self-assembly (polymerization), the template (target molecule) is removed by vigorous washing steps to leave specific recognition cavities in the polymer networks (Liu et al., 2013). Controlling thickness of the film or layer fabrication coating onto the transducer is imperative because it determines a response of sensors. Optical transducer (Jakusch et al., 1999), acoustic transducer (Dickert et al., 1998), and electrochemical sensors (Blanco-Lopez et al., 2004) were constructed according

to this technique. Second, transformation of analyte binding into a measurable signal; therefore, the efficiency of MIP-based sensors is largely reliance on the sensitivity and selectivity of the MIP materials used to target analytes (Liu et al., 2013).

Although bulk polymerization MIP materials display great selectivity, they have some constraints such as low binding capacity, poor site accessibility, and slow binding kinetics due to the fact that most imprinted sites are embedded in the interior of a highly rigid polymer matrix (Xie et al., 2008) or are broken down during grinding process. As a result, to establish more effective recognition sites and enhance the accessibility of binding sites, synthetic nano-technological particles as the supporting materials for preparation of core-shell structural MIPs were exploited (Li et al., 2012).

For more information related to chemical sensors and biosensors, several comprehensive reviews of literatures describing the development of chemo-sensors and biosensors were published elsewhere (Liu et al., 2013; Sergey & Anthony, 2002; Subramanian et al., 2012; Jiang et al., 2007; Rao et al., 2007).

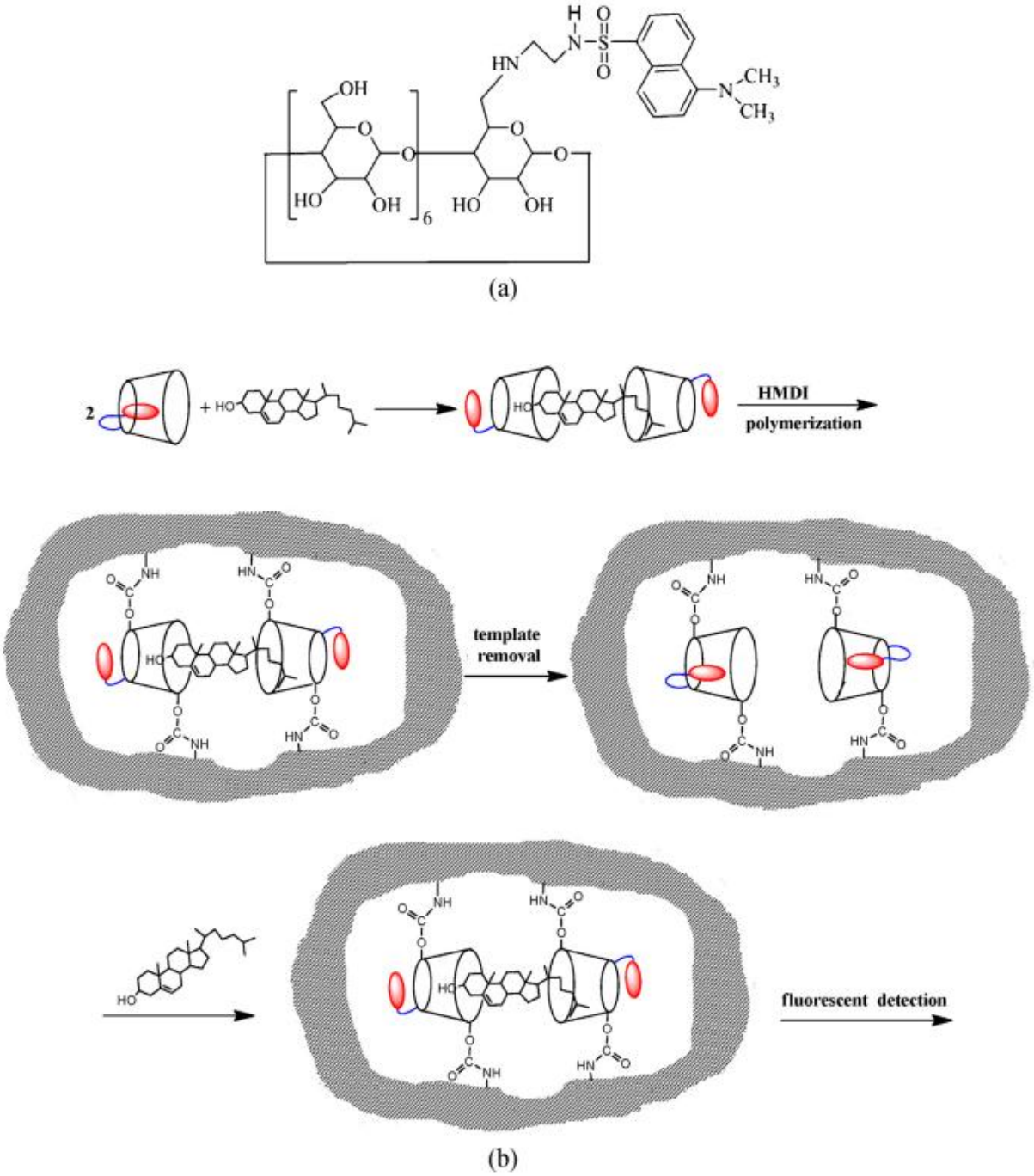


Figure 1.8 The schematic diagrams of fluorescent chemosensor MIPs for selective recognition of cholesterol by molecular imprinting technique (Cheng et al., 2014).

1.8.3 Molecularly-imprinted polymers as catalytic materials

To extent the advantage of a possible use of MIPs for catalytic application has been attempted with considerable efforts (Li & Li, 2007; Wulff, 2002). A high selectivity, strength,

and resistance of MIPs make them a proper tool to be used at elevated temperature and pressure along with the presence of variety of organic solvents and also under acidic or basic reaction condition. Owing to these attributes, MIPs have been utilized in place of bio-molecules such as enzymes and natural catalytic antibody (Shokat et al., 1989) that are highly susceptible to certain temperature and pressure.

The exploitation of MIP for catalytic application is very necessary because MIP catalysts are able to mimic the selectivity and specificity of the binding domains of antibodies and enzymes, a generally used catalyst in diverse reactions. Catalytically active imprinted particles were obtained using analogues of substrates, transition states, or products as templates in imprinting technology (Ramstrom & Mosbach, 1993). The correct placement of functional groups in the binding sites in line with a type of catalytic process must be ensure in order to obtain polymer matrix, which is similar to the shape of template use.

The strategy of using substrate analogues as template involves the use of compounds that mimic the reaction mixture between the substrate and the matrix. The catalytic groups will be introduced in correct position of imprinted cavities of polymers, and they will subsequently behave catalytically in presence of the real substrate. Alternatively, another approach in the polymerization process was the use of basic Transitional State Analogue (TSA) and methacrylic acid (MAA) as basic template and acidic monomer functional groups (Wulff, 1996). **Figure 1.9** illustrates the schematic catalysis diagrams of MIPs development system.

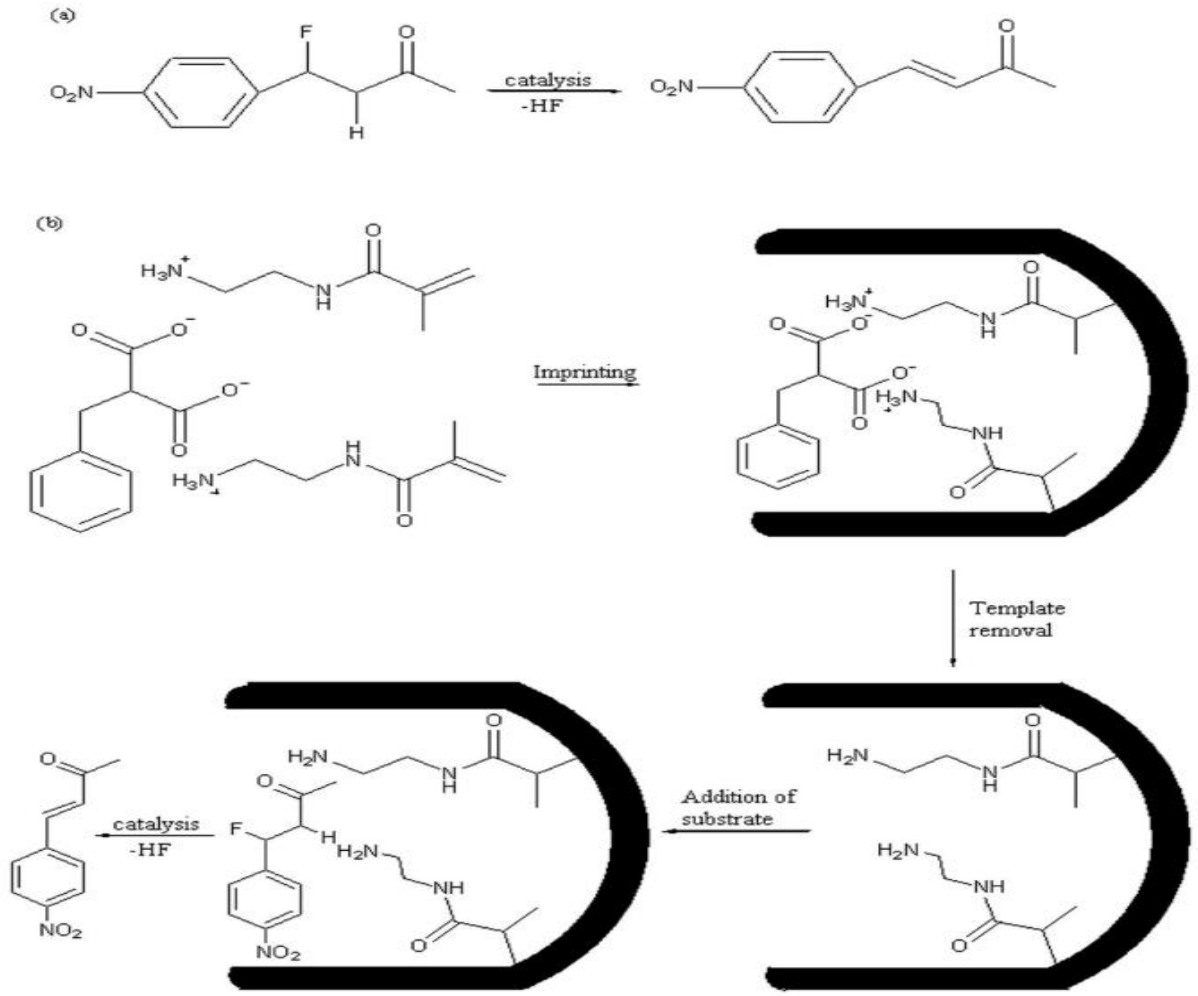


Figure 1.9 The schematic catalysis diagrams of catalytic MIPs development system (Adapted from Beach & Shea, 1994).

1.8.4 Molecularly-imprinted polymers as drug delivery

A revolution of imprinting nanotechnology has made MIPs enlarge a further area of functionality as "drug delivery", which bears an ability to transport the therapeutic drug to a particular tissue in the body. This is an integral part of clinically therapeutic treatment techniques. In recent years, numerous publications have been released about the use of MIPs in drug delivery application (Alvarez-Lorenzo & Concheiro, 2004; Hilt & Byrne, 2004) providing their physiochemical properties to protect the drug from degradation by enzymes during systemic transportation in the body. An efficient drug delivery system ought to make sure that the carried drug is released at the right site, in the right dose and for right period of time (Chien

& Lin, 2002). This invented drug vehicle mechanism employed drug template-imprinted polymers as a carrier tool could be capable of prolonging the release profile of specific therapeutic agents with better performance as compared to more traditional drug delivery system (Sellergren & Allender, 2005). Regarding the clinically therapeutic treatment via drug delivery technology, cancer is one of most widely studied diseases, known as cancer nanotechnology. Cancer nanotechnology, an emerging interdisciplinary field involving in chemistry, biology, pharmaceutical engineering, and medicine, has drawn a lot of attraction from researchers around the globe. Nanosystems with a single style and integrated technology are a promising tool for the detection, prediction, prevention, and the targeted therapy of cancer. Nanoparticles can be functionalized, and they are likely to interact with biosystems in a more efficient way to regulate biological processes in disease development. One of the most notable advantages is that targeting ligands (drug template) can be fabricated in the imprinted nanoparticles, increasing specificity for target cancer cells without causing substantial damage on normal tissues (Nel et al., 2009; Misra et al., 2010; Lee et al., 2014; Hoppens et al., 2014). This mechanism is a great challenge in cancer nanotechnology for selectively delivering nanomaterials to target tumor tissues, meanwhile reducing side effects on normal tissues (Vigderman & Zubarev, 2013; Kim et al., 2014). The present cytotoxic chemotherapy for liver cancer indicates low specificity and effectiveness; only 2-5% of therapeurtic drugs; including cytotoxic drugs (estramustine, oxaliplatin, and doxorubicin), antiangiogenic drugs such as sorafenib, bevacizumab, sunitinib, and thalidomide, and trans-arterial chemoembolization; are absorbed by tumors, yet more than 90% of drugs accumulated in normal tissues (Cheong et al., 2006; Guan et al., 2012); therefore, molecularly imprinted polymer nanoparticles come to be an alternative method of cancer treatment due to its strong drug delivery mechanism.

Regarding to MIPs as drug delivery, we have drawn a discussion about the role of imprinting technique nanotechnology for tea-bioacitive compound delivery as follows.

Tea is one of the most popular beverages in the world. People like consuming it habitually for enjoyment and healthy. Generally, in the people's mindset, tea could keep them healthy and avoid diseases due to the potent effects of its bioactive components. On the contrary, in the experimental evidence, there is as yet no conclusive scientific evidence on its intrinsic value because of the conflicting results of the research to date. Therefore, more research is needed

mainly focusing on not only the conventional way of tea drinking, but also advanced methods, especially the nanotechnological approach, as our future outlook.

A recent review article on "Tea and Human Health" conducted by Chen and Lin (2015), published in Journal of Zhejiang University-SCIENCE B (Bio-medicine & Biotechnology), was one of the published review papers associated with tea drinking benefits to human health (McKay and Blumberg, 2002; Cabrera et al., 2006; Wolfram, 2007). This paper does not represent a significant advance over previous ones. We think that it is not appropriate that the authors started the article with a very long historical discussion of the evolution of tea rather than discussing the most recent developments; this makes it less interesting. In addition, there are still no conclusive evidence to prove that the bio-active component of tea is really beneficial to human health because the experimental evidence is still conflicting, e.g., Yang et al. (2009) demonstrated that there is no significant difference between the treatment group and control group on the preventive effect of tea. Moreover, there are many reports finding that the relationship between drinking tea habit and cancer is not clear in terms of reduced cancer risks (Bonner et al., 2005; Sun et al., 2006; Li et al., 2008; Zhou et al., 2008). The article also lacked presentation of the modern techniques in clinically experimental medicine against diseases. For example, epigallocatechin gallate (EGCG), the most abundant and active catechin in green tea, was exploited to treat the liver cancer cells of humans and animals through cancer nanotechnology, a treatment which is far beyond what the traditional tea-drinking habits would provide of the EGCG bioactive component as normally obtained in the body through oral intake. The results demonstrated that the EGCG-functionalized nanoparticles exhibited potent anti-cancer effects on a liver cancer-specific manner (Zhou et al, 2015). The conclusion could be drawn that this technique may serve as the underlying basis of new development strategies on the treatment of liver cancer and other malignancies. Last but not least, the article should have included other biologically active components such as fluoride since the fact is that numerous review papers have already been produced discussing similar compounds, including polyphenols, catechine, flavonoids, and theaflavins. Fluoride is another public health concern that poses a great threat to human health when there is overexposure (WebMD News Archive, 2005). It is a natural ability of the tea plant that it can absorb fluoride from surrounding soil. It is estimated that up to 98% of the accumulated fluoride content in the tea plant is deposited in its leaves, particularly the old ones (Lu et al., 2004). Excessive consumption of fluoride could induce

skeletal or dental fluorosis. A newly published article related to skeletal fluorosis due to excessive tea drinking was reported, "A 47-year-old woman had a symptom of bone pain after habitually consumed a pitcher of tea from 100 to 150 tea bags daily for the past 17 years; brewed tea has one of the highest fluoride contents among beverages in the United States. She reported a 5-year history of pain in the lower back, arms, legs, and hips. Because of brittleness, all her teeth were extracted. After scanning radiography, she was eventually diagnosed as skeletal fluorosis," (Kakumanu & Rao, 2013).

In conclusion, though bioactive compounds in tea plants are generally considered to have a beneficial effect on human health, there is as yet no conclusive scientific evidence on its intrinsic value because of the conflicting results of the research to date. Therefore, more research is needed mainly focusing on not only the traditional way of tea drinking, but also advanced methods, especially the nanotechnological approach. We believe that nanotechnology, using a bioactive tea's component as an agent, could provide stronger supplementary evidence in establishing its real effect on human health. **Figure 1.10** displays the schematic illustration of preparation and characterization of an imprinted polymer nanoparticle for drug delivery system of liver cancer.

Apart from drug delivery system, MIPs can be also used to bind several substances in gastrointestinal tract and blocking their absorption in the body, a part of the pharmacological therapy. In this case, MIPs as chemical traps to remove undesirable substances from the body, such as glucose, cholesterol (Davidson & Hayes, 2002; Sellergren, 1998; Spizzirri & Peppas, 2005), bile acid (Huval et al., 2004) and melittin (Hoshino et al., 2010) were innovated.

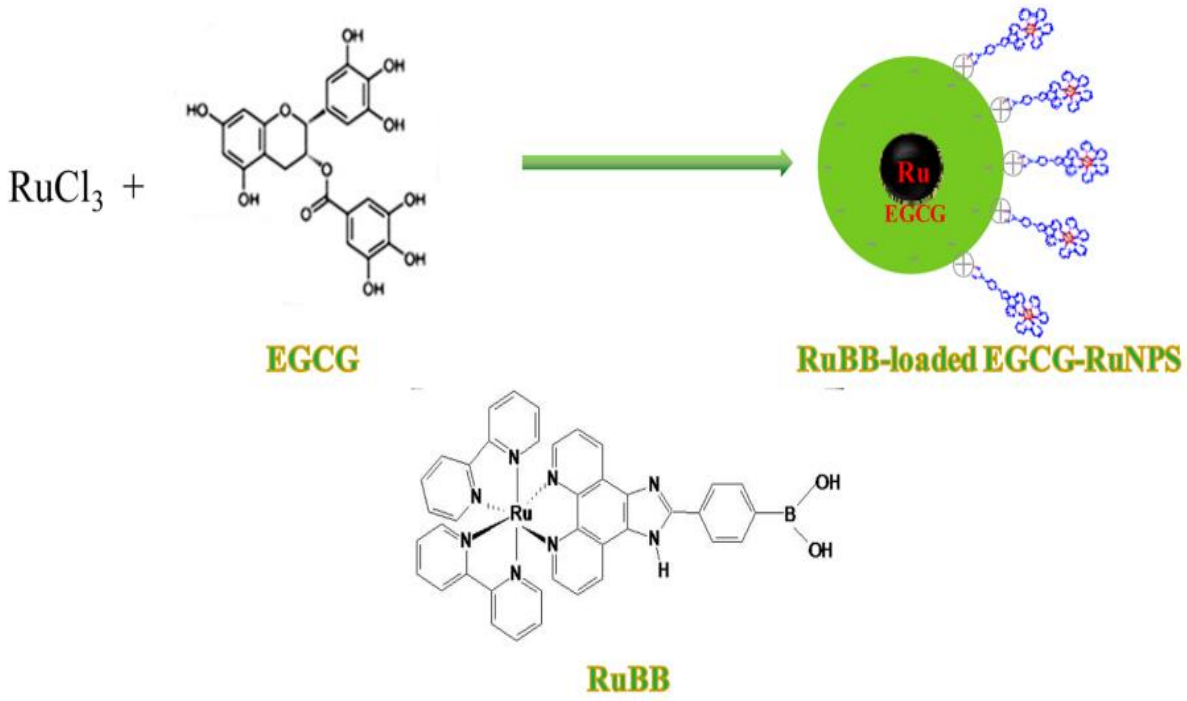


Figure 1.10 The schematic illustration of preparation and characterization of RuBB-loaded EGCG-RuNPs (EGCG: epigallocatechin gallate; RuNPs: functionalized ruthenium nanoparticles; RuBB: $[Ru(bpy)_2(4\text{-}B)](ClO_4)_2 \cdot 2H_2O$) (Adapted from Zhou et al., 2015).

1.9 Conclusion

As aforementioned at the above sections, we can conclude that due to robustness, stability, and flexibility, varying functionalities of MIPs, the molecularly imprinting technique has drawn a huge growing interest in the field of science separation. A wide range of synthetic MIPs protocols, including bulk, precipitation, multi-step swelling, suspension, emulsion, dispersion, gelation polymerization, have been successfully developed to meet the increasing demands for analytical molecular methods and bio-clinical medicines. Despite the drawbacks of bulk polymerization, the traditional technique is still the most commonly-used protocol for MIPs synthesis. Of the three approaches of MIP synthesis, the non-covalent method is the most popular for obtaining MIPs.

CHAPTER 2 EXPLOITING ALLYL-β-CYCLODEXTRIN AS FUNCTIONAL MONOMERS IN MOLECULAR IMPRINTING FOR SELECTIVE RECOGNITION OF PHTHALATES

2.1 Introduction

Growing public health concerns over food safety hazards have prompted national and international food regulatory authorities to formulate food safety system frameworks in order to minimize or eliminate the risk of food toxicants from a range of sources. Some food manufacturers have used illicit food additives in pursuit of increased profits; this act has been shown to have very serious health consequences for consumers and has, in some cases, resulted in death (Tyan et al., 2009). Phthalic acid diesters (phthalates) are commonly used as a plastic additive to increase the flexibility, transparency, durability and longevity of polyvinyl chloride (PVC) plastics such as food packaging products, medical devices, and toys. These plasticizers are easily abused by addition to foodstuffs as a substitute for conventional and more expensive emulsifiers, such as palm oil. They are highly lipophilic (fat soluble) and not chemically bound to the PVC matrices, hence they easily leach out, migrate, or evaporate into the atmosphere and body from the plastic-derived materials when heated, used, or disposed of (Staples, Peterson, & Parkerton, 1997a). Di (2-ethylhexyl) phthalate (DEHP) and dibutyl phthalate (DBP), which are the most widely employed additives, are suspected agents of endocrine disruption by means of carcinogenesis (Naarala, & Korpi, 2009; Benson, 2009; Blandeau, 1999), and hormonal and reproductive malfunction (Poon et al., 1997; Foster, Mylchreest, Gaido, & Sar, 2001; Duty, et al., 2005), according to toxicological findings in animal models. Of these phthalates, DEHP is the most commonly used plasticizer; more than 2 million tons of DEHP alone was annually produced worldwide (Lorz et al., 2002). In addition, over 800,000 tons of the three primary plasticizers; DEHP, diisononyl phthalate (DINP), and diisodecyl phthalate (DIDP); was manufactured in Western Europe in 2003 (Koch, Preuss, & Angerer, 2005). As the result of their potent adverse health effects and wide usage, the European Union has called for an emergency restriction on the utilization of six phthalates; dibutyl phthalate (DBP), butyl benzyl phthalate (BBP), di-n-octyl phthalate (DOP), DEHP, DINP, and DIDP; in the production of items intended for children in the age group of 0—3 years (Rastogi, & Worsoe, 2001). According to

the US Food and Drug Administration (USFDA), the acceptable tolerance limits for DEHP was 3.5 μg mL^{-1} for adults and 0.3 μg mL^{-1} for neonates and infants (Chaudhary, et al., 2010).

It was reported during the food safety scandal of DEHP-tainted products, the Chinese mainland suspended imports of 948 items from Taiwan, including beverages, food products, and food additives manufactured by 280 companies which were suspected of using DEHP, according to a notice issued by the Food Safety Commission in 2011. Moreover, the Center for Food Safety in Hong Kong suggested that the reference dose (RFD) of DEHP is 1.5 mg kg^{-1}; and that any item exceeding the limit standard should be reclaimed and destroyed (Wang, & Xin, 2001).

Molecularly imprinted polymers (MIPs) have been popularly exploited for chemical separation and analysis for various applications such as sensors, enantiomeric separation, biomedical and analytical applications amongst others, because they possess potentially artificial receptors for selective adsorption of target molecules (Khan, Khan, & Park, 2008). The attractiveness of MIPs can be attributed to their stability, durability, reliability and effective cost of production. The main compositions engaged in the creation of MIPs are the template molecule, the functional group, and the cross-linking agent. In the last decade, β-cyclodextrin (β-CD) and its derivatives were typically chosen as either a single (Tsai & Syu, 2005; Ning, Byun, & Bittman, 2001; Xu, et al., 2008) or binary functional monomer (Kang, et al., 2012; Xu, Kuang, Feng, & Zhang, 2010; Xu, et al., 2007; Chen, Chen, & Chung, 2007); the binary functional monomer-synthesized MIPs have since been demonstrated to obtain greater affinities towards the target analytes (Xu, Kuang, Feng, & Zhang, 2010).

In this study, the MIP was prepared using DEHP as the template, allyl bromine-β-cyclodextrin (allyl-β-CD) as a single functional monomer; and the combined use of allyl-β-CD and methacrylic acid (MAA), allyl-β-CD and methyl methacrylate (MMA), allyl-β-CD and acrylonitrile (AN), and allyl-β-CD and acrylamide (AA) as the binary functional monomers; ethylen glycol dimethacrylate (EGDMA) as a cross-linker. An array of assays was performed to evaluate the selective binding efficacy of prepared MIPs, and finally, MIPs were applied to test the spiked milk with DEHP constituent to determine the recovery rates through the MISPE-HPLC method.

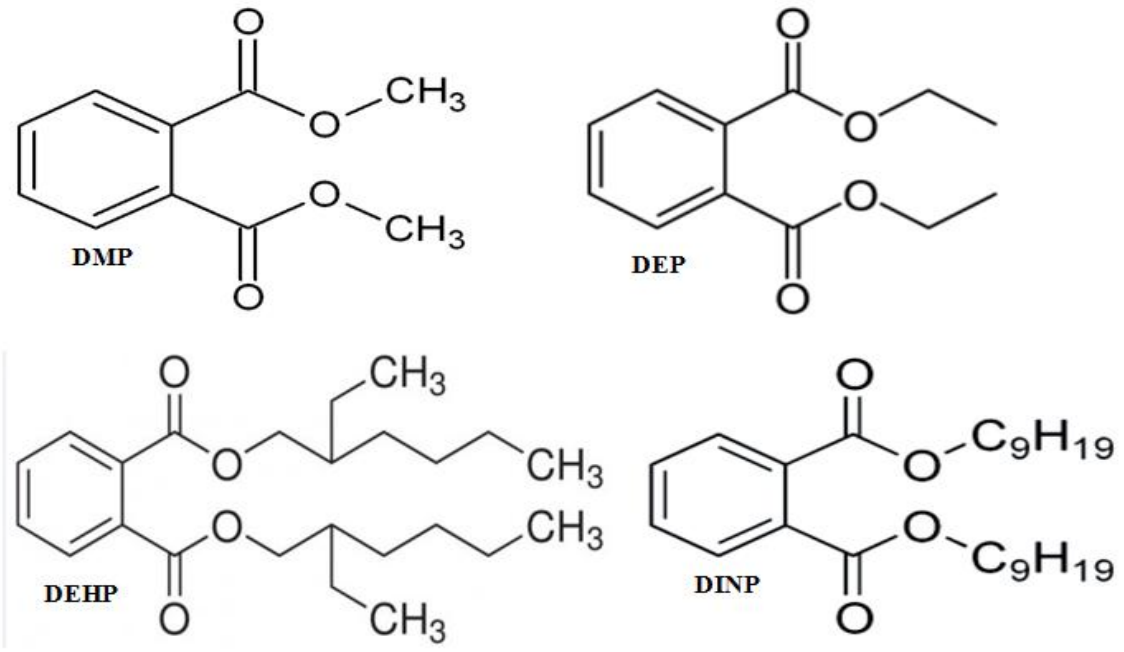


Fig. 2.1. Chemical structures of DEHP and its analogues.

2.2 Experimental Section

2.2.1 Chemicals

The analytical working standard di (2-ethylhexyl) phthalate (DEHP, 99% pure), diisononyl phthalate (DINP, 99%), dimethyl phthalate (DMP, 99%), diethyl phthalate (DEP), β-cyclodextrin (β-CD, 96%), allyl bromide (98%), methacrylic acid (MAA, 99%), methyl methacrylate (MMA, 99%), ethylen glycol dimethacrylate (EGDMA, 98%), azo-N,N-bisisobutyronitrile (AIBN, 99%), N,N-dimethylformamide (DMF, 99.5%), and isopropyl-2-ol (99.5%) were procured from Alladin Chemistry (Shanghai, China). Acrylonitrile (AN, 99%), acrylamide (AA, 99%), and HPLC grade methanol were purchased from Gracia Chemical Technology (Chengdu, China), Biosharp (Anhui, China), and Tianjin Sayfo Technology (Tianjin, China), respectively. Ultra-pure water, used for molecularly imprinted polymers (MIPs) synthesis and HPLC mobile phase, was produced from a laboratory water purification system (Ultra pure UF model, Shanghai, China).

β-CD and AIBN reagent were re-crystallized from water and methanol, respectively. The selected re-distillation of MAA, MMA, and AN functional monomers and already prepared

DEHP stock solution (1mmol per milliliter in HPLC grade methanol) were stored in the refrigerator at 4 ^{o}C prior to their use for MIP bulk polymerization. All the reagents used in this work were of high-purity grade.

2.2.2 Instruments

The method of HPLC operation was largely unmodified from the previous studies (Chaudhary, et al., 2010). The chromatographic analyses were performed on an Agilent 1200 HPLC system, equipped with a UV-visible variable-wavelength detector (225 nm monitored), binary pump (G1312B), degasser (G1322A), autosampler SL, and temperature-controlled compartment (Santa Clara, CA, USA). The reversed-phase column was SB C18 4.6 x 100 mm (1.8 micrometer). The mobile phase composition of methanol, isopropyl-2-ol and water was blended in a ratio of 250:100:50, respectively. The flow rate was run at 1 mL min^{-1} and the controlled temperature was 30 ^{o}C. The injection volume of 10 μL and running time of 6 min were observed to result in a sufficiently good response and separation of DEHP esters.

Fourier Transforms Infrared (FT-IR) Spectra (Nicolet, USA) and Scanning Electron Micrograph (SEM) (Hitachi, Japan) were employed to characterize the synthetic properties of MIP formations and its morphological attributes.

2.2.3 The preparation of allyl-β-CD interaction

The reaction of allyl bromide and β-CD was prepared as follows: 20 g of sodium hydroxide (NaOH) was first dissolved with 20 mL of water in a 100 mL glass beaker. The solution was continually stirred until it was well-mixed before 20 g of β-CD was poured into it. This solution was mechanically stirred in the water bath at 60^{o}C for 2 hours. Afterwards, 4 mmol allyl bromide was put in the solution and continually stirred under nitrogen gas for 2 hours in the inert atmosphere at 60^{o}C. The solution was finally transferred back to the water bath for 30 min prior to desiccating in the oven at 60^{o}C. After 3 days, the rigidly dried yield of reaction mixture obtained was crushed and ground with a mortar and pestle into fine powder and washed 5 times with absolute ethanol (5 min each time), in order to remove the residues from the resulting product.

2.2.4 The synthetic polymerization of MIPs

The procedure of synthesized MIPs was modified from the previous report (Kang, et al., 2012). 2.80 g of allyl-β-CD synthetic stock compound was first dissolved in 40 mL DMF for 30 min prior to adding 2 mmol DEHP stock to the solution. The reactive solution was processed for 2 hours at a controlled temperature of 60^{o}C under continuing magnetic stirring with nitrogen gas purging. A 12 mmol functional monomer (MAA, MMA, AN, or AA) was then supplied to the solution, allowing 1 hour for reaction. Afterwards, 60 mmol EGDMA was supplemented for another 1 hour, followed by adding 40 mg AIBN as an initiator; the resultant solid-state reaction mixture was obtained after about 30 min and was then incubated in a water bath at 60^{o}C for 24 hours. The bulk solid, removed from the thermal bath, was continually desiccated in an oven for two days before being crushed and ground into free-flowing powder through a 400 mesh steel sieve. Fine particles were collected and washed with hot water to minimize possible contaminants. They were subsequently Soxhlet extracted with methanol and acetic acid (9:1, v/v) until no DEHP was detected by UV-vis spectrophotometry. The MIPs were finally washed with methanol to get rid of acetic acid residue prior to testing its binding characteristic capabilities.

Non-imprinted polymers (NIPs) were simultaneously prepared under the same conditions except that the DEHP template was removed.

2.2.5 Binding assays

2.2.5.1 The influence of solvent on binding factor

Varying methanol and water ratios of 10:0, 9:1, 8:2, 7:3, 6:4, 5:5 (v/v) were utilized as a source of solvent for binding assays of smart MIPs and NIPs. This was chosen as the analyte is in the ester groups which is non-polar and water immiscible; therefore, water concentrations were chosen that were equal and/or less than methanol. To make this adsorption solvent, we mixed methanol with DEHP first, thoroughly swirled this solution for a few minutes prior to decanting water into solution. To test this assay, MAA-linked allyl-β-CD MIP (M-MAA) was selected as a model amongst the prepared MIPs to evaluate the intensity of its binding performance for DEHP in different testing solvents because numerous studies have reported on

high effects of MAA monomers in MIPs-building (Kang, et al., 2012, Beltran, Borrull, Cormack, & Marce, 2010; He, Lv, Zhu, & Lu, 2010; Shi,Wu, Qu, Li, & Zhang, 2007). 20 mg particles of polymers were inoculated in 3 mL of 1mM DEHP solvents in 10 mL conical flasks by shaking at 130 rpm at the controlled temperature of 30^{o}C.

After the defined time of inoculation, the solvent was removed by filtering through 0.22 µm for HPLC analysis. The percentage of bound substrate to the polymer matrixes was calculated as the following equation:

% Bound = [(initial amount – free amount after adsorption)/ initial amount] x 100

2.2.5.2. The binding specificity

All the synthetically imprinted and non-imprinted polymers were tested for their rebinding capacity with a target binding molecule of DEHP in solvent media. The amount of template bound to polymers (Q, µmol g^{-1}) was determined by equilibrium binding experiments and calculated according to the eq. (Lv, et al., 2007):

$$Q = \frac{V(Ci - Ca)}{m}$$

Where V (mL), the volume of the solution; Ci (µmol mL^{-1}), the initial concentration; Ca (µmol mL^{-1}), the free concentration after adsorption; m (g), the dried mass of polymers.

Distribution coefficient (Kd, mL g^{-1}) of DEHP was utilized to understand its binding affinity between polymers and solution. The higher the amount of Kd, the greater the role the polymers have played. The equation of distribution coefficient is followed by the previous study (Zhu, Haupt, & Knopp, 2002):

$$Kd = \frac{Q}{Ca}$$

To understand the significant variation of the amount of uptake between MIPs and NIPs, the specific binding capacity ΔQ (μmol g^{-1}) was applied according to:

$$\Delta Q = Q\ (MIP) - \ Q\ (NIP)$$

The molecular imprinting factor (IF) was employed to validate the imprinting result. IF was calculated as follows:

$$\mathrm{IF} = \frac{Kd\ (MIPs)}{Kd\ (NIPs)}$$

2.2.5.3. Binding dynamics and competitiveness

Various contents of template ranging from 0.1 - 1 mM were studied. The equilibrium binding experiment procedure was referred to section 2.5.2. Briefly, 20 mg particles of polymers were inoculated in 3 mL of soluble DEHP solvents in 10 mL flasks by shaking at 130 rpm at the temperature of 30^{o}C.

A series of structural analogues of DEHP phthalate esters, including DMP, DEP, DINP, were tested under the same conditions to characterize the polymers' competitive binding affinity and efficiency, as well as functional recognitions of its structurally-related counterpart. The performed concentration of solution was 1 mM in all circumstances. **Fig. 2.1** shows the chemical structure of DEHP and its analogues.

2.2.5.4 Preparation of Molecularly Imprinted Solid-Phase Extraction (MISPE) Procedure

A. Binding adsorption. An aliquot (50 mg) of MIPs and NIPs was packed into a pipet with a piece of cotton as a stopcock, followed by a thin layer of quartz crystal sand on the top of polymers. The polymers were pre-conditioned with 3 mL methanol and 3 mL water prior to loading the sample. The standard solution of DEHP was prepared in methanol with the concentration of 100 μmol mL^{-1}. After conditioning, 1 μmol and 3 μmol DEHP stock was first

singly loaded in the micro-column, then 1 mL methanol was added, followed by 1 mL water. A total of 4 mL adsorption solvent was used per sample. It was later washed out with 6 mL water containing 10% methanol in order to displace any non-adsorbed components of the DEHP solution in the column. 5 mL elution of methanol containing acetic acid in a ratio of 9:1 (v/v) was run to remove the template material. The eluate was continually collected in a test tube, and dried out under nitrogen gas purging. It was then re-dissolved with 1 mL methanol for HPLC analysis. The elution solvent was eluted through the column with a flowing rate of 0.5 mL min^{-1}.

B. Competitive binding. The purpose of this assay was to understand the effect of synthetic MIPs on memory binding, competing for its template's versus analogue's chemical structure by separation of free and bound to polymers when they are mixed together. Thereby, DMP, DEP, and DINP structural-related analogues, will be selected to compete with DEHP template. DEHP and its analogues were mixed in the same stock of methanol to a final concentration of 40 μmol mL^{-1}in a ratio of 1:1:1:1. 0.4 μmol of an aliquot was loaded in the column for further analysis. The running procedure was followed as before.

C. Real sample analysis. It is believed that milk power was contaminated by esters during the processing operation through milking machines. Thereby, a commercial brand of baby-powder formula was purchased from a supermarket and used as our sample. Milk fat separation was carried out primarily based on the previous study (Li et al., 2011), but with some modifications. In brief, 1 g of infant formula was accurately weighed into a 50 mL polypropylene centrifuge tube and 10 mL methanol was then added to the sample. The mixture was blended by a vortex agitator for 15 min then 0.5 g of sodium chloride (NaCl) was added to the solution and it was homogenized once again prior to sonicating for 10 min. This solution was separated by centrifuge at 3500 rpm for 10 min; the collected supernatant was decanted into a test tube and either directly loaded or stored in the refrigerator at $4^{o}C$ before analytical application. Here, we used 6 mL elution for desorption. The recovery experiment (R%) was evaluated by spiking blank baby milk powder with various concentrations of DEHP standard solutions (100 ng, 200 ng, 300 ng, and 400 ng per milliliter of methanol), and the blank control sample of raw milk was carried out by the same sample preparation procedure except that DEHP standard solution was removed. The recovery calculated as follows:

$$\% \text{ R} = \frac{C\ mean}{C\ spike} \text{ x } 100$$

Where C_{mean} is the mean of the fortified milk concentration, and C_{spike} is the spiked concentration.

2.3 Results and Discussion

2.3.1 Characterization of the synthesized MIPs

The MAA-linked allyl-β-CD MIPs (M-MAA) was taken as a demonstrated model for characterization by FT-IR spectroscopy. A very sharp peak at 1727.15 cm^{-1} was a carbonyl group (C=O) from MAA and EGDMA which is consistent to the previous report (Kang, et al., 2012). And 1157.16 cm^{-1} was C-O stretch, a band in the region of 1454.91 cm^{-1} was carbon-carbon double bond existing on the allyl-β-CD molecules. The absorption peak at 1637.75 cm^{-1} and 1560.49 cm^{-1} were only observed in the spectrum of MIP with template molecule indicated the aromatic ring and the O-H stretching of carboxylic acid from DEHP, respectively (**Fig. 2.2A**). These confirmed that ester group of DEHP and functional monomers were co-existing in the polymeric networks. Furthermore, a very broad band between 3600-3000 cm^{-1} was O-H stretching and 2957.94 cm^{-1} was C-H stretch which is compatible to the β-CD constituent functional structure. For non-imprinted polymers **(Fig. 2.2B)**, it was observed that the location and shape of the major bands were similar to those of imprinted polymers, which is also in agreement with the previous study (Xu et al., 2008).

The morphological study of polymers was done by SEM. It was evident that imprinted and non-imprinted polymers were remarkably distinctive. The surface of both imprinted M-AN (A1) looked highly rough, friable and porous, whereas the surface of non-imprined N-AN (A2) was slightly rough and compact. The arrow heads showed the surface area of imprinted and non-imprinted polymers (**Fig. 2.2C**). The irregularly high rough, porous imprinted outer layer is most likely formed by the template removal during Soxhlet apparatus operation creating the particular sites of rebinding cavities.

Looking deeper insight in the binding formation process between template and polymer, we can depict that in the pre-polymerization process for M-MAA synthesis, when allyl-β-CD molecules were mixed with DEHP template in DMF, the possibility of aromatic rings of DEHP can be either trapped inside the hydrophophic core of ally-β-CD, or protruded outside of the stereo-shape cavities owing to its chemical nature. Whenever the aromatic rings were outside, the carbonyl group (-C=O-) of DEHP will form stronger ionic interactions with MAA functional monomer as MAA was added. Hence, the allyl-β-CD and MAA were complementary, and the position and mutual conformation of allyl-β-CD and MAA were firmly fixed through the crossing-linking agent. This imprinting process is illustrated in **Fig. 2.2D**.

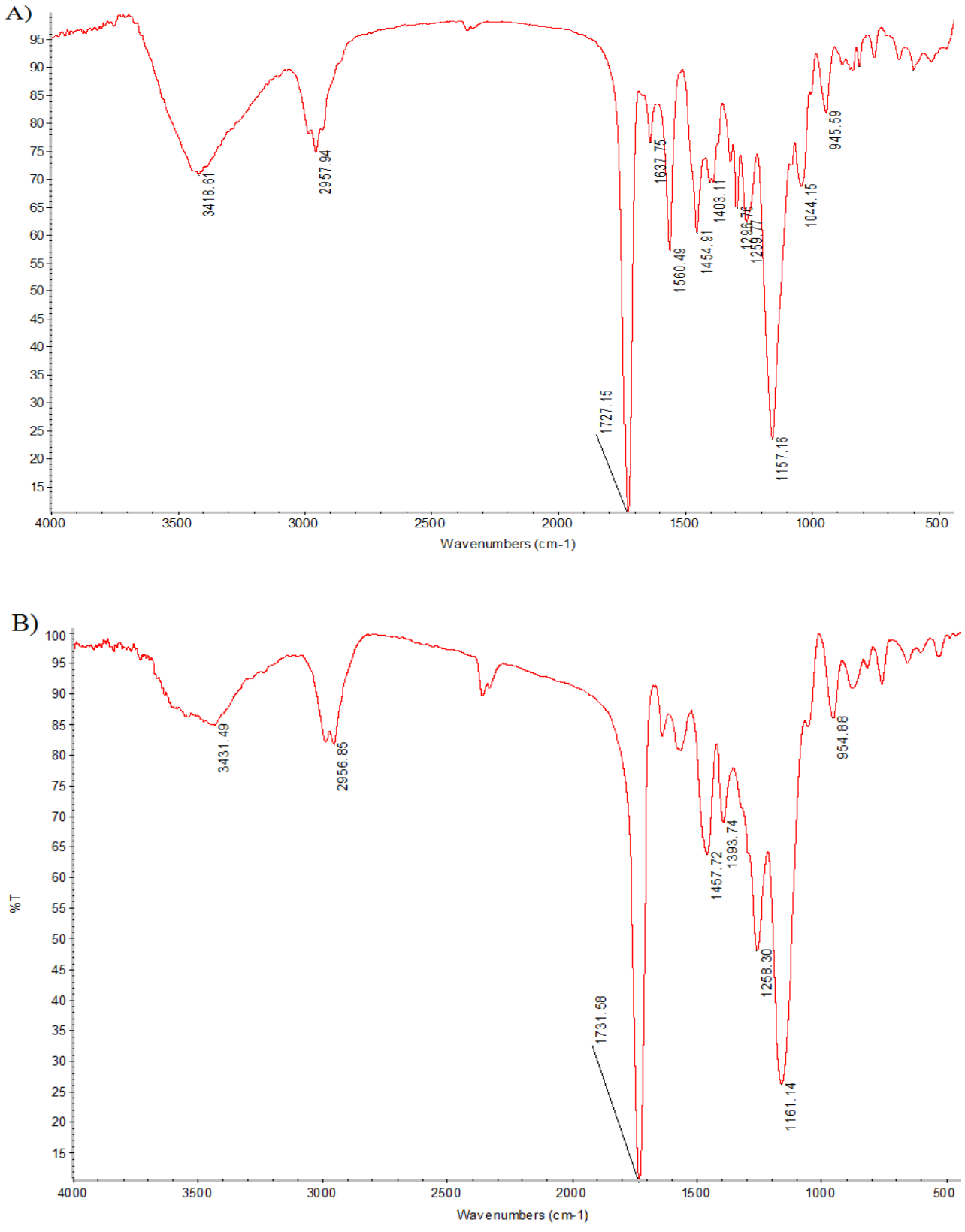


Fig. 2.2. FT-IR spectrum of (A) M-MAA and (B) N-MAA

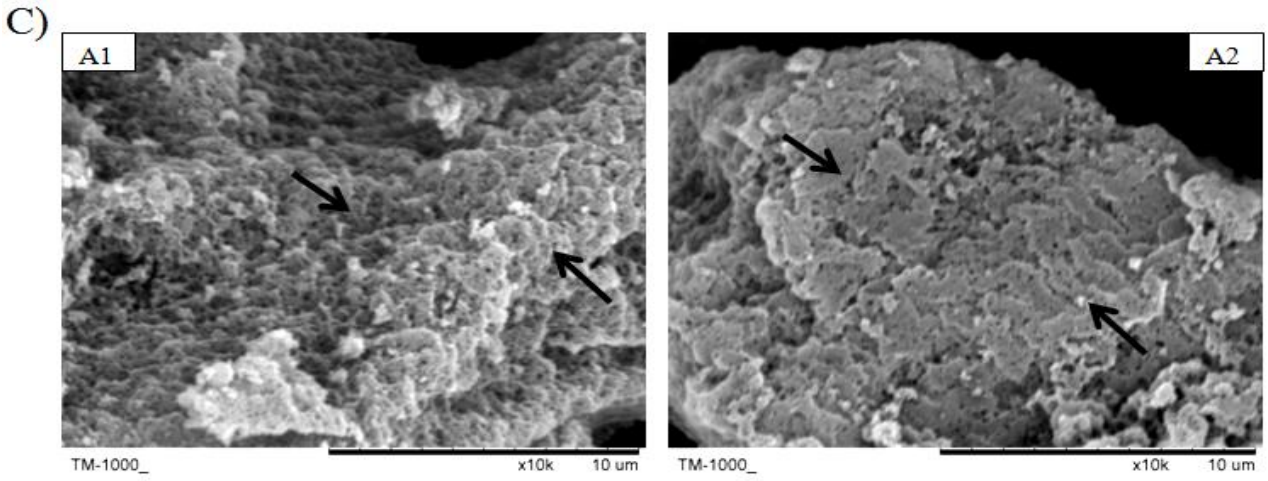


Fig. 2.2. (B) N-MAA; (C) SEM micrographs of selected MIPs: [A1] M-AN, AN-linked allyl-β-CD MIPs and [A2] N-AN, AN-linked allyl-β-CD NIPs.

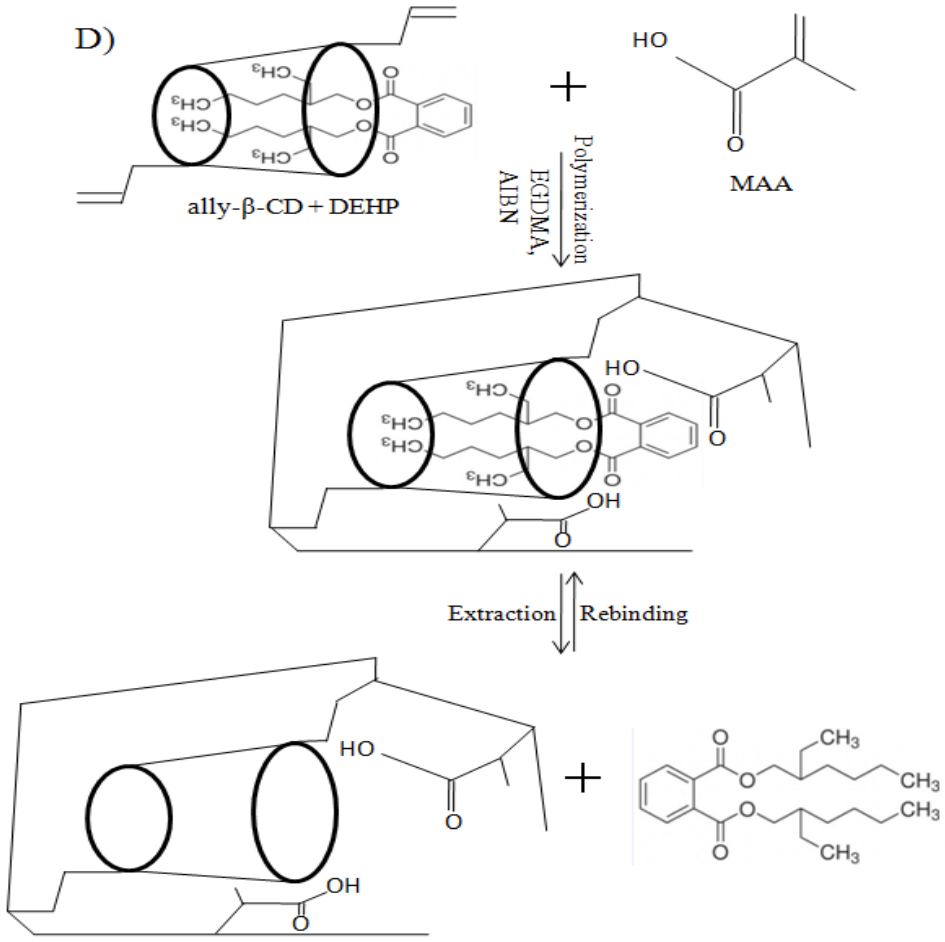


Fig. 2.2. (D) Schematic representation for the preparation of DEHP- imprinted polymer made of MAA-linked allyl-β-CD (M-MAA).

2.3.2 Binding assays

3.3.2.1 The influence of solvent on binding factor

Different volumes of water were taken as an integral part of solvent combination with methanol to evaluate the influence of water on the binding of DEHP on the synthesized polymers. As previously mentioned in **section 2.2.5.1**, MAA monomers were demonstrated to have greater yield in binding affinity by numerous reports; therefore, M-MAA and N-MAA were taken as a model in this investigation.

As seen in the **Fig. 2.3**, differing ratios of methanol and water concentration has influenced the binding efficiency of polymers. Water promoted binding capacity. As the water content was gradually increased, more template molecules could be driven into the imprinted cavities of polymers. In this binding aspect, there were two factors that made attractive bonds between MIPs and templates. The first was imprinting process, which is precisely complementary in size, shape and functional group to the template molecule. The second was unspecific interaction, which is by (O^- - H^+) hydrogen bonding from template and functional groups and hydrophobic force induced by water, where NIPs and template were formed only by unspecific. Therefore, in all circumstances, the attractive forces of MIPs were superior to NIPs. As a result, the highest yield was obtained when the water and methanol ratios in the adsorption solvent were reached equilibrium level (1:1, v/v). This result was also consistent with previous studies that impressed water content as part of a solvent mixture in β-CD-formed MIPs (Xu et al., 2008; Xu, Kuang, Feng, & Zhang, 2010; Xu, Kuang, Liu, & Deng, 2007). Hence, the M-F solvent was considered as an optimized adsorption medium for our further research. In addition, although there were assigned amounts of varying time (hours) of inoculation, no any sign of sizeable changes of responses in imprinted polymers were observed. Thus, one hour's suspension of polymers in media was observed to produce the optimal results.

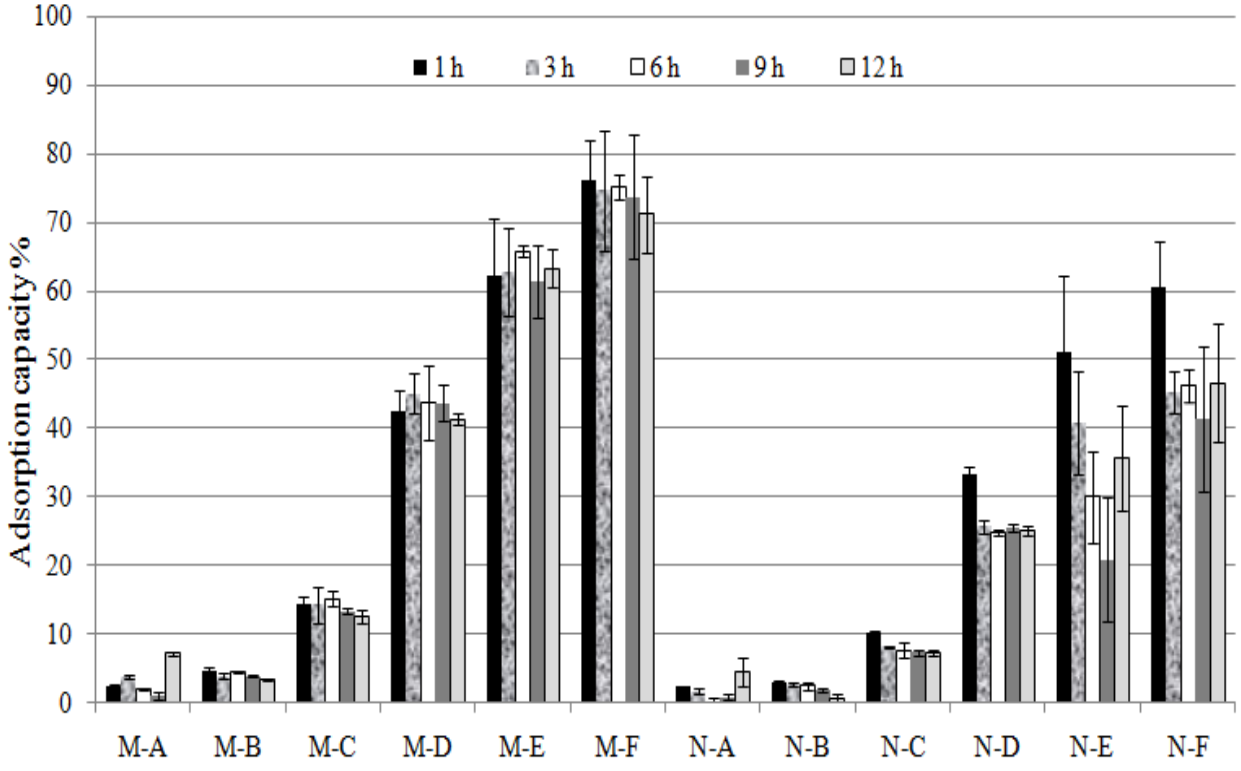


Fig. 2.3. The influence of solvent on binding factor. M-A, M-B, M-C, M-D, M-E, and M-F were methanol and water solvent, corresponding to a ratio of 10:0, 9:1, 8:2, 7:3, 6:4, and 5:5 (v/v), respectively. Triplicate independent results were made.

2.3.2.2 The binding specificity

A number of different types of the synthesized polymers were tested in the equilibrium binding experiments (refer to **section 2.2.5.2**). The variable amounts of substrates bound to the MIPs and NIPs as illustrated in **Table 2.1**. According to the results, the MIPs had a much stronger quality of affinity than the NIPs in all regards, including Q, Kd, ΔQ, and IF; indicating that the synthesized NIPs do not possess imprinting cavities inside the polymeric matrixes. Amongst the various MIPs, AA-linked allyl-β-CD MIPs (M-AA) was found to attain the poorest value (≤47 μmol g^{-1}), revealing that an AA functional monomer did not create strong hydrogen bonding and hydrophobic effect in polymeric networks compared to others that were tested. An AA monomer was also described elsewhere having a poor functionality (Xu, Kuang, Feng, & Zhang, 2010; He, Lv, Zhu, & Lu, 2010). In spite of allyl-β-CD MIPs (M-βCD) showing results (≤94 μmol g^{-1}) that were two-fold higher than M-AA, M-βCD was still significantly lower

compared to M-MAA, M-MMA, and M-AN. Therefore, the adsorption capability was improved when MAA, MMA, and AN monomers were supplemented to allyl-β-CD monomers linking them into the binary groups. The average amount of bound substrate (Q) in M-MAA, M-MMA, and M-AN were ~110 μmol g^{-1}, whereas M-βCD and M-AA were ~90 μmol g^{-1} and ~40 μmol g^{1}, respectively. While different amount of binding time (hours) was given, the values demonstrated relative similarities, implying that the polymeric particles became saturated in about one hour. In light of these results, we decided to choose one hour as an optimal time and drop out M-AA and M-βCD for our further experiment.

Table 2.1 Recognition of DEHP amount uptake on the polymers prepared with different functional monomers*

Eq.	Time(h)	M-MAA	N-MAA	M-MMA	N-MMA	M-AN	N-AN	M-AA	N-AA	M-βCD	N-βCD
Q	1	106.88±1.06	69.66±3.76	110.64±1.68	58.68±6.61	111.34±3.06	85.29±2.78	47.00±3.00	41.10±4.10	92.23±3.19	55.99±6.76
	3	110.82±0.56	70.58±1.63	114.12±0.36	61.25±1.19	111.43±0.12	91.31±2.12	46.60±3.80	34.15±2.73	94.15±2.29	52.11±2.00
	6	105.16±2.54	64.95±0.96	110.55±1.67	49.78±2.06	110.12±0.15	81.74±2.52	39.78±6.95	22.35±6.34	91.98±3.74	43.82±6.35
Kd	1	371.81	130.07	421.68	96.40	432.08	197.71	68.45	56.61	239.49	89.35
	3	424.36	133.31	477.24	103.53	433.47	233.40	67.61	44.23	252.93	79.85
	6	351.88	114.56	420.35	74.52	414.23	179.64	54.15	26.27	237.85	61.91
ΔQ	1	37.22		51.96		26.06		5.90		36.24	
	3	40.24		52.87		20.12		12.45		42.05	
	6	40.21		60.76		28.38		17.43		48.17	
IF	1	1.53		1.89		1.31		1.14		1.65	
	3	1.57		1.86		1.22		1.36		1.81	
	6	1.62		2.22		1.35		1.78		2.10	

* The result was triplicate independent variable values. Initial concentration: 1mM; solvent: methanol/water (1:1, v/v); volume: 3 mL (M: imprinted polymers, N: non-imprinted polymers).

2.3.2.3 Binding dynamics and competitiveness

As stated in the preceding **section 2.3.2.2**, three distinctive imprinted polymer properties; M-MAA, M-MMA, M-AN; were selected for this experiment. A series of different contents of DEHP were tested with the MIPs and NIPs in order to determine its binding capacity for a target molecule. We selected five variable degrees ranging from 0.1 to 1 mM (refer to section 2.5.3). The polymers were suspended in the media for one hour prior to defining its amounts of adsorbed substrates to the polymeric sorbent. Both of the MIPs and NIPs displayed very good responses; with higher initial concentration of template, we saw greater responses of binding acquired onto polymers (**Fig. 2.4A**). According to this figure, the responsiveness of all MIPs polymeric adsorbents yielded comparatively similar effects and was clearly superior to that of NIPs.

As already described in **section 2.2.5.3**, DEHP and its analogues (DMP, DEP, DINP) were performed in the competitive binding of the polymers with 1mM standard solution. The formation of molecular structures had played a fundamental role in the functioning recognition of the MIPs. The different molecular structures triggered the variable correspondences; the bigger structure induced the better responses (**Fig. 2.4B**). The result may be explained by the fact that both of the DMP and DEP structures were comparatively very small relative to the template; therefore, their structures and the imprinted cavities of MIPs were inappropriately complementary, leading to poor binding responses. However, the rebinding of DINP and DEHP molecule onto polymers generated high outcomes because their molecular formula was very closely-related, so that the polymers could easily recognize and specifically bind into its pre-formed cavities more effectively. Undoubtedly, the selective bindings of the tested compounds in MIPs were all higher than that in NIPs, proving that MIPs were more effective and efficient adsorbents.

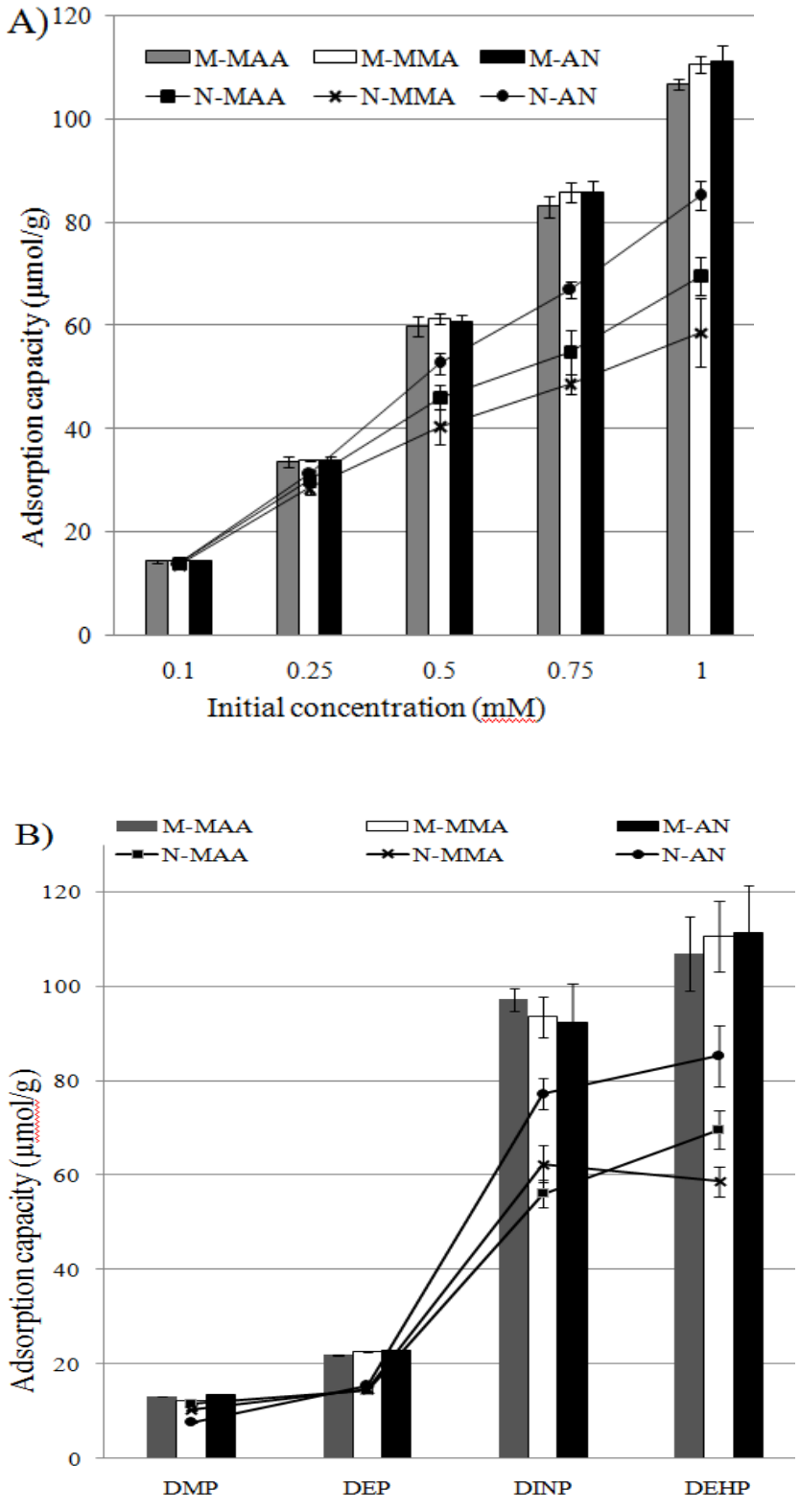


Fig. 2.4. (A) Adsorption dynamics and (B) competitive binding selectivity of the MIPs and NIPs. The result was triplicate independent variable values. Solvent: methanol/water (1:1, v/v); volume: 3 mL.

2.3.2.4 MISPE procedure

Binding adsorption. Prior to testing the real sample pretreatment in a micro-column, a series of experiments were carried out; using the procedure described above (see **section 2.2.5.4 (A)**). According to the values (**Fig. 2.5A**), both of the MIPs and NIPs rebinding amounts were very competitive in this stage due to an increase of sorbent particles, which create more imprinted and non-imprinted binding sites for the substrate interaction. As the column was injected with 1 µmol of substrate, the effects of M-AN, M-MMA, and M-MAA were 96%, 94%, and 92%, respectively, where N-AN, N-MMA, and N-MAA were 94%, 86%, and 91%. In this case, the value was not statistically different between MIP and NIP produced by MISPE procedure, but a significant difference was noted in a suspension method (**Table 2.1**). Probably, it is because we used 50 mg of MIPs particles in a MISPE method, while we only used 20 mg of MIPs in a suspension method, and while the same amount of analyte (100 µmol mL^{-1}) was maintained in the investigation. Therefore, the imprinting effect showed significance in the suspension method (polymer saturation) rather than in the MISPE. From the data illustration, we concluded that increasing particles could induce more binding efficiency of both MIPs and NIPs. **Fig. 2.4A** also showed no significant difference when we employed a low concentration of analyte (0.1 mM), but it started illustrating statistically different when the initial concentration of analyte was constantly increased. Moreover, once the concentration had been raised to 3 µmol, a substantial increase of adsorbed amount of template onto the polymers was still obtained, indicating that MIPs had great capability and functionality in the adsorption process. Despite the fact that M-AN was seen have a slightly higher amount of uptake, all the MIPs, generally, displayed strong responses of affinity; the average adsorbed amount of MIPs was approximately 95%.

Competitive binding. As already mentioned in section **2.2.5.4 (B)**, the DEHP template and its analogues were mixed into the same stock solution of methanol to a final concentration of 40 µmol mL^{-1} , and 0.4 µmol aliquot was then selected to run through MISPE column. Based on the chromatograms on high performance liquid chromatography (**Fig. 2.5B**), almost the entire DMP and DEP phthalate volume was eluted from the micro-column after loading the mixture solution, which illustrated that the DMP and DEP poorly interacted with M-AN while DEHP content was almost recovered after loading. This proved that M-AN has excellent binding cavities to its template. According to the results, the average adsorbed amount of DEHP, DINP,

and DEP, DMP through MIPs was at 97.98%, 90.22, 11.55 and 10.83%, respectively, which enabled us to draw a conclusion that the shape, size and functional structure of a template could shape the effectiveness of a polymer's functionality and discrimination. The synthetic MIPs also showed higher than the NIPs in term of binding amount uptake. In short, the MISPE method also firmly supported the suspended method (see **section 2.3.2.3**).

Real sample Test. To demonstrate the suitability and potential application of this method for sample pretreatment, M-MAA, M-MMA, and M-AN were used for MISPE to evaluate the recovery rate of spiked baby powder (refer to section 2.5.4 (C)). The standard calibration curves were established by plotting the chromatographic peak areas versus the constant concentrations (0.0025, 0.005, 0.1, 0.2, 0.4, 0.6, 0.8, 1 μg mL^{-1}) through diluting the stock solution in methanol. The standard solutions were then directly injected in triplicate. The results showed good linear regression equation and correlation coefficient (y= 9.154x + 1.923, R^2 0.998). The limit of detection (LOD) and quantitation (LOQ), based on signal-to-noise ratio of 3:1 and 10:1, were 0.045 μg mL^{-1} and 0.139 μg mL^{-1}. This level of sensitivity enabled us to effectively determine trace residues in the target matrix. The analytical results shown in **Table 2.2** demonstrated that the average recoveries of DEHP ranged from 89.06-97.98% and the relative standard deviations (RSD) values were less than 6.47% in this real sample analysis. Furthermore, the continuous column system of these three MIPs could be renewed by cycling the adsorption and desorption of DEHP. Each of them was tested 10 times with 1μg mL^{-1} and 5μg mL^{-1}, respectively, these tested MIPs were found to possess a robust usability without any quality loss (Recovery: 92.13 - 98.32%, RSD: 1.14 - 4.7 %), reflecting that MIPs have an outstanding stability for recycling state.

Table 2.3 compares the proposed method constituted by MISPE-HPLC with other previously reported methods. Though the LOD of this method is higher than those previously published reports for phthalates determination, probably because it does not couple with a mass spectrometry (MS) or a flame ionization detector (FID), it is still below the acceptance limit calculated as per USFDA tolerance criteria (3.5μg mL^{-1} for adults and 0.3μg mL^{-1} for neonates and infants). Compared to the recovery rates, there is no any significant difference amongst its compared groups.

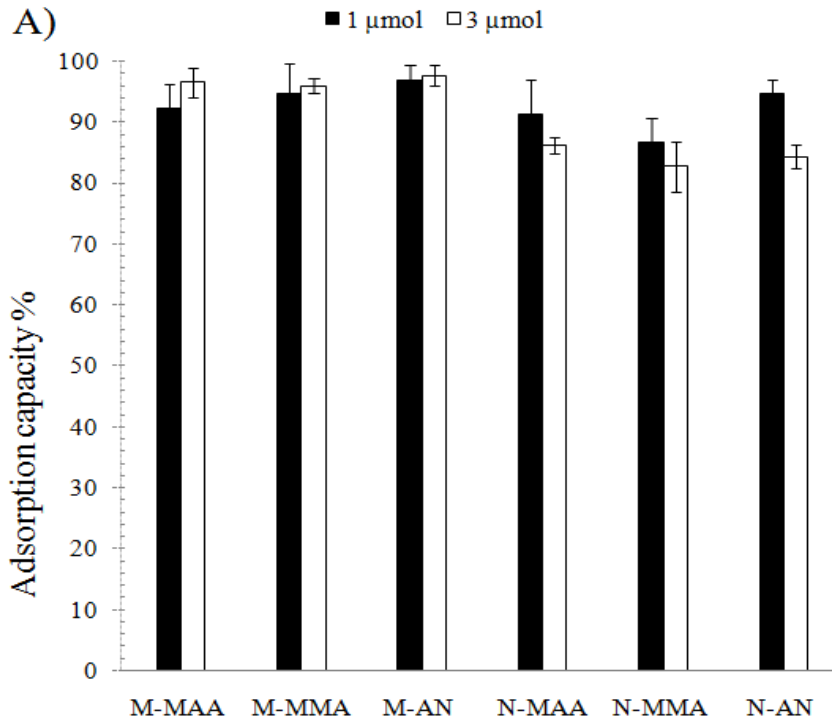
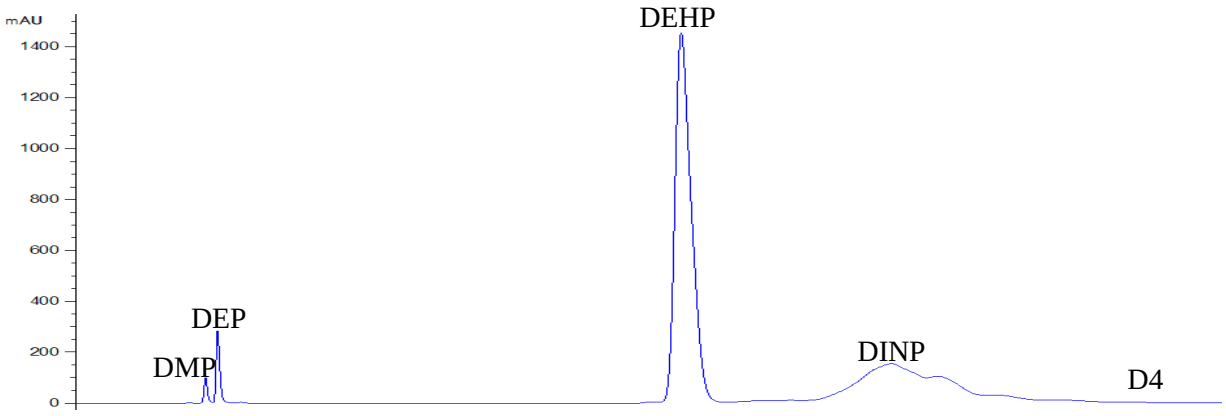


Fig. 2.5. (A) The percentage of binding adsorption efficiency through MISPE (n=3).

B)



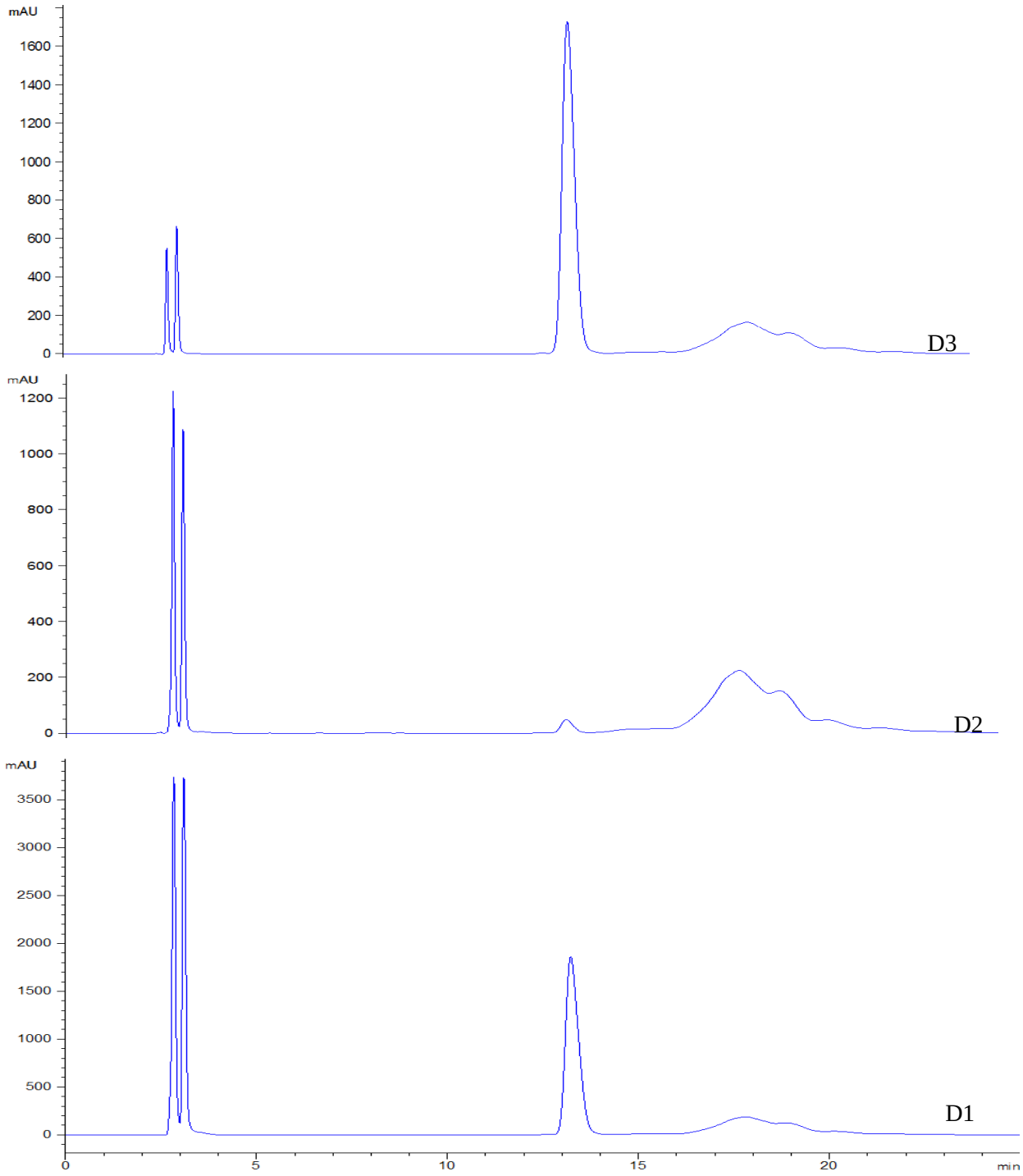


Fig. 2.5. (B) chromatograms on high performance liquid chromatography: D1, mixture solution before adsorption; D2, mixture solution after adsorption; D3, eluate of M-AN; D4, eluate of N-AN. Note: we changed to the column (250 x 4.60 mm, 5 micrometer, phenomenex, USA) due to the unavailability of the previous one.

Table 2.2 Recovery of DEHP phthalate in an incurred baby formula using different sorbents (n=3; incurred level ranging of 100-400 ng mL^{-1})

Sorbents	Analyte DEHP							
	100 ng mL^{-1}		200 ng mL^{-1}		300 ng mL^{-1}		400 ng mL^{-1}	
	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)
M-MAA	89.06	5.26	91.76	4.35	91.41	6.16	92.38	5.13
M-MMA	91.81	6.47	93.59	4.43	96.32	2.28	95.11	2.49
M-AN	93.59	2.37	94.78	3.21	97.98	1.47	96.81	3.18

Table 2.3 Comparison of different methods for phthalates determination

Method	Template	Sample	LOD	Recovery (%)	Ref.
MISPE-GC-MS	Dipentyl phthalate (DPP)	Cow milk	na*	93.73	[Kang, et al., 2012]
MISPE-GC-MS	Diheptyl phthalate (DHpP)	Wastewater	na*	94.60	[Chen, et al. 2007]
MISPE-GC-MS	DBP	Soybean milk	na*	91.50 - 106.40	[He, Lv, Zhu, & Lu, 2010]
MI-SPME-GC-MS	DBP	Reservior water	2.17 ng mL^{-1}	94.54 - 105.34	[He, et al., 2010]
MISPE-GC-FID	DEHP	River water	0.011 μg mL^{-1}	94.98 - 99.35	[Shaikh, et al., 2012]
MISPE-HPLC	DEHP	Infant formula	0.045 μg mL^{-1}	93. 59 - 97.98	This work

* not available

2.4 Conclusion

In this study, various types of DEHP-imprinted polymers derived from allyl-β-cyclodextrin were prepared by bulk polymerization techniques. M-MAA were characterized by FT-IR; M-AN characterized by SEM. Water has an influence on binding solutions; higher volume of water content induces stronger uptake of DEHP template when M-MAA was taken as a model experiment. Though M-AN has a slightly higher uptake than M-MAA and M-MMA, all of them were found to produce strong effects on specific binding capacity and selectivity towards the

target analyte, and they are highly superior to its references (M-AA, M-β-CD) in binding specificity. Additionally, they can separate the template from its structural analogues in a competitive binding adsorption. When the MISPE made of M-MAA, M-MMA and M-AN, also known as the binary functional monomers of MIPs, were used for the incurred baby formula analysis, the data indicated that they can be used as selected adsorbents in SPE techniques for real sample analysis. The MIP in this proposed method is simple and cost-effective to prepare, and requires small quantities of solvents for isolation and enrichment of analyte in the analytical procedure. All in all, the binary functional monomer-synthesized MIPs have yielded higher bindings toward target analytes than single ones, and the production of MIPs fabricated with allyl-β-cyclodextrin as functional monomers was efficient separation of hydrophobic molecules.

CHAPTER 3 USING MOLECULARLY-IMPRINTED POLYMERS AS THE EXTRACTED SORBENTS OF CLENBUTEROL AHEAD OF LIQUID CHROMATOGRAPHIC DETERMINATION

3.1 Introduction

Clenbuterol hydrochloride (4-amino-a-(t-butylaminomethyl)-3,5-dichlorobenzyl alcohol hydrochloride, CLEN), a class of therapeutic drugs of β_2-adrenergic agonists, is primarily used in human and veterinary medicine to treat asthma and other pulmonary disorders; however, when orally overdosed, it acts as a repartitioning agent by shifting nutrients from the adipose tissue towards the muscle tissue, and functions as a growth promoter (Ricks et al., 1984). Owing to the health concerns about its toxicity reported by numerous researchers (Pulce et al., 1991; Brambilla et al., 1997; Garay et al., 1997; Ramos et al., 2003; Babosa et al., 2005), the use of CLEN has been prohibited in meat-producing animals by many countries, including USA, European Union (EU), China, etc. The symptoms of consumers after ingesting food poisoning from this tainted meat were typically described as gross tremors of extremities, tachycardia, nausea, headaches and dizziness (Babosa et al., 2005). Of the β_2-agonist sympathomimetic class, CLEN is the only member of drug allowed for the therapeutic use within Europe on animals as a bronchodilator; however, the FDA has classified it in group I drugs with no allowable extra-label uses in any food-producing animal species (Crescenzi et al., 2001). To ensure that meat products are suitable for human consumption, the maximum residue limit (MRL) of CLEN in the livers of cattle and horses proposed by EU is fixed at 0.5 μg/kg (De Washch et al., 1998). Therefore, a reliable, sensitive method is required to determine a trace amount of CLEN in meat products below the parts per billion (ppb) levels.

Several techniques have been developed for extracting CLEN from complex matrices. The conventional pre-treatment ones are mainly reliant on liquid-liquid extraction (LLE) (Keskin et al., 1998; Zhang et al., 2003; Courtheyn et al., 1991; De Washch et al., 1998), or solid-phase extraction (SPE) (Horne et al., 1998; Li et al., 2010; Bruins et al., 1999). But these two classic methods have time-consuming cleanup steps and require large quantities of toxic organic solvents in order to obtain a homogeneous liquid phase containing an analyte of interest. There are many newly developed pre-treatment methods of β_2-agonists CLEN determination, including molecularly imprinted matrix solid-phase dispersion (MI-MISPD) coupled with high performance liquid chromatography (HPLC) ultraviolet

detection (Qiao & Du, 2013), surface molecularly imprinted polymer and micro-extraction in a packed syringe (SMIPs-MEPS) (Du et al., 2014), matrix solid-phase dispersion (MSPD) and molecularly imprinted solid-phase extraction (MISPE) (Crescenzi, 2001) molecularly imprinted polymer-capped CdTe quantum dots (Huy et al., 2014; Qian et al., 2006; Ying et al., 2014), which were reported. However, although there are many different procedures each claiming its own benefits, there is still a strong need for a simpler, effective and efficient method of selectivity for the extraction of the trace amount of analyte in complex mixtures.

Molecularly Imprinted Polymers (MIPs), possessing the advantages of high selectivity, easy preparation, and high chemical stability, are synthetic polymers which display specific recognition sites for a target analyte (Mahony et al., 2005; Beltran et al., 2010; Hiratsuka et al., 2013; Prieto et al., 2011; Prieto et al., 2011). Molecularly imprinted solid-phase extraction (MISPE) cartridges have been employed to separate pesticides (Muldoon & Stanker, 1997; Lv et al., 2007; Zhu et al., 2002), drugs (Mullett & Lai, 1998; Shi et al., 2007) and illicit food additives (He et al., 2010; Kang et al., 2012) from different biological matrices. The main compositions engaged in the creation of MIPs are the template molecule, the functional group, and the cross-linking agent.

Cyclodextrin (CD) was frequently utilized in the enantiomer separation and drug delivery systems for their unique property to form inclusion compounds with other small molecules (Tsai & Syu, 2005). In recent times, β-cyclodextrin (β-CD) and its derivatives were typically chosen as either a single (Tsai & Syu, 2005; Ning et al., 2001; Xu et al., 2008) or a combined use as binary functional monomers (Kang et al., 2012; Xu et al., 2010; Xu et al., 2007; Chen et al., 2007) to synthesize β-CD-MIPs; the synthesized MIPs, additionally, have been demonstrated to obtain greater adsorption properties with addition of β-CD (Kang et al., 2012).

The objective of the present study was to develop a rapid and sensitive technique to identify a trace amount of CLEN in pork liver samples. The MIP was synthesized using CLEN as the template, and the combined use of allyl bromine-β-cyclodextrin (allyl-β-CD) and methacrylic acid (MAA), allyl-β-CD and methyl methacrylate (MMA), allyl-β-CD and acrylonitrile (AN), as the binary functional monomers; ethylen glycol dimethacrylate (EGDMA) as a cross-linker. An array of assays was performed to evaluate the selective binding efficacy of prepared MIPs, and finally, MIPs were applied to test the spiked liver sample with CLEN constituent to determine the recovery rates through the MISPE procedure.

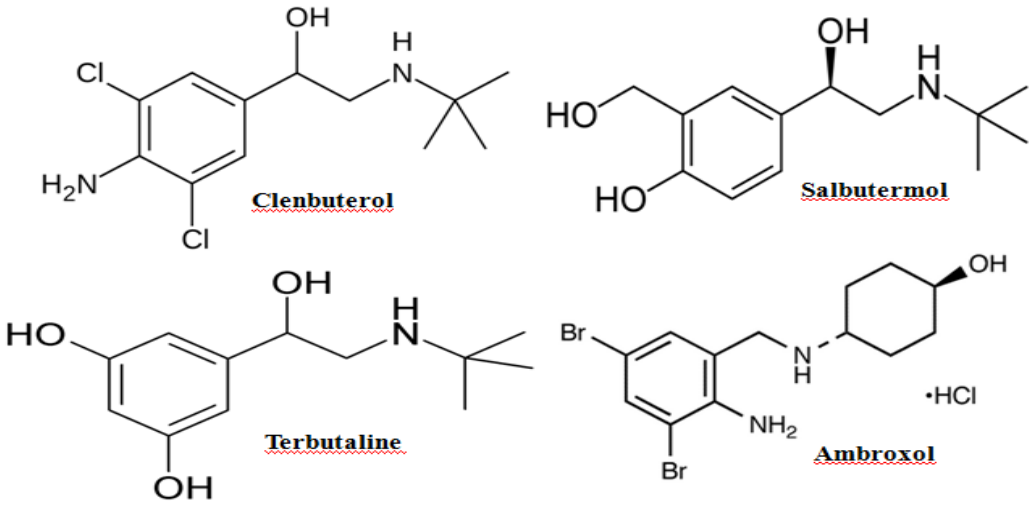


Fig. 3.1. Chemical structures of CLEN and its analogues.

3.2 Experimental Section

3.2.1 Chemicals

The analytical working standard clenbuterol hydrochloride (CLEN, 98.5%) was ordered from The Laboratory Labor Dr. Ehrenstorfer-Schafers (Augburg, Germany). Salbutamol, terbutaline sulfate, ambroxol hydrochloride, β-cyclodextrin (β-CD, 96%), allyl bromide (98%), methacrylic acid (MAA, 99%), methyl methacrylate (MMA, 99%), ethylen glycol dimethacrylate (EGDMA, 98%), azo-N,N-bisisobutyronitrile (AIBN, 99%), N,N-dimethylformamide (DMF, 99.5%), and were procured from Alladin Chemistry (Shanghai, China). Acrylonitrile (AN, 99%), and HPLC grade methanol were purchased from Gracia Chemical Technology (Chengdu, China), and Tianjin Sayfo Technology (Tianjin, China), respectively. Ultra-pure water, used for molecularly imprinted polymers (MIPs) synthesis and HPLC mobile phase, was produced from a laboratory water purification system (Ultra pure UF model, Shanghai, China).

β-CD and AIBN reagent were re-crystallized from water and methanol, respectively. The selected re-distillation of MAA, AN, and MMA functional monomers and already prepared CLEN stock solution (1 mg mL^{-1} in HPLC grade methanol) were stored in the refrigerator at $4^{o}C$ prior to their use. All the reagents used in this work were of high-purity grade.

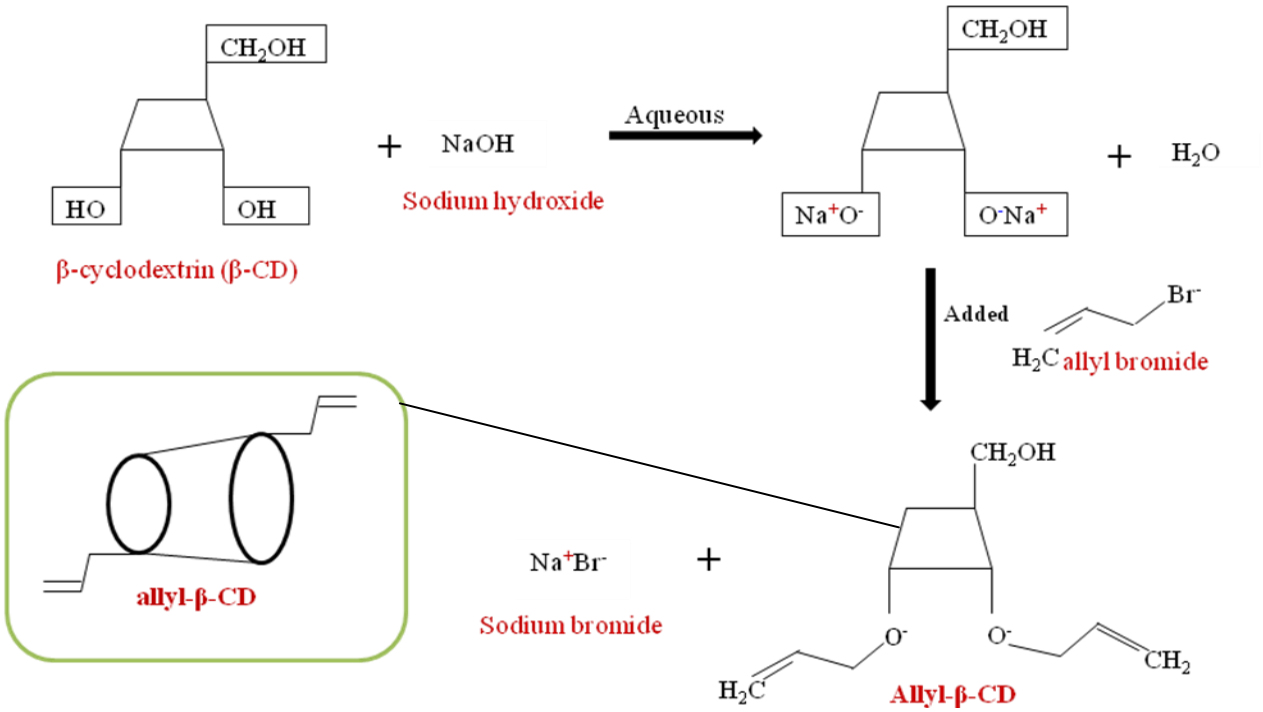
3.2.2 Instruments

The chromatographic analyses were performed on an Agilent 1200 HPLC system, equipped with a UV-visible variable-wavelength detector (225 nm monitored), binary pump (G1312B), degasser (G1322A), autosampler SL, and temperature-controlled compartment (Santa Clara, CA, USA). The reversed-phase column was XB-C18 100A 4.6 x 100 mm (1.8 micrometer, phenomenex, USA). The mobile phase composition of acetonitrile and water was blended in a ratio of 4:1 with addition of 0.5% phosphoric acid (H_3PO_4). The flow rate was run at 1 mL min^{-1} and the controlled temperature was 30^oC. The injection volume of 10 μL and running time of 4 min were observed to result in a sufficiently good response and separation of CLEN compound.

Fourier Transforms Infrared (FT-IR) Spectra (Nicolet, USA) was adopted to characterize the synthetic properties of MIP formations and Scanning Electron Micrograph (SEM) (Hitachi, Japan) were used to characterize the properties of synthetic MIP morphology.

3.2.3 The preparation of allyl-β-CD interaction

The reaction of allyl bromide and β-CD was prepared as follows: 20 g of sodium hydroxide (NaOH) was first dissolved with 20 mL of water in a 100 mL glass beaker. The solution was continually stirred until it was well-mixed before 20 g of β-CD was poured into it. This solution was mechanically stirred in the water bath at 60^oC for 2 hours. Afterwards, 4 mmol allyl bromide was put in the solution and continually stirred under nitrogen gas for 2 hours in the inert atmosphere at 60^oC. The solution was finally transferred back to the water bath for 30 min prior to desiccating in the oven at 60^oC. After a few days, the rigidly dried yield of reaction mixture obtained was crushed and ground into fine powder and washed 5 times with absolute ethanol (5 min each time), in order to remove the residues from the resulting product. The below diagram showed the chemical reaction process for the preparation of allyl-β-CD interaction.



3.2.4 The synthetic polymerization of MIPs

The procedure of synthesized MIPs was largely modified from the previous report (Kang et al., 2012). 2.80 g of allyl-β-CD synthetic stock compound was first dissolved in 40 mL DMF for 30 min prior to adding 2 mmol CLEN to the solution. The reactive solution was processed for 2 hours at a controlled temperature of 60^{o}C under continuous magnetic stirring with nitrogen gas purging. A 12 mmol functional monomer (MAA, AN, MMA) was then supplied to the solution, allowing 1 hour for reaction. Afterwards, 60 mmol EGDMA was supplemented for another 1 hour, followed by adding 40 mg AIBN as an initiator; the resultant solid-state reaction mixture was obtained after about 30 min and was then incubated in a water bath at 60^{o}C for 24 hours. The bulk solid, removed from the thermal bath, was continually desiccated in an oven for two days before being crushed and ground into free-flowing powder through a 400 mesh steel sieve. Fine particles were collected and washed with hot water to minimize possible contaminants. They were subsequently Soxhlet extracted with methanol and acetic acid (9:1, v/v) until no CLEN was detected by UV-vis spectrophotometry. The MIPs were finally washed with methanol to get rid of acetic acid residue prior to testing its binding characteristic capabilities.

Non-imprinted polymers (NIPs) were simultaneously prepared under the same conditions except that the CLEN template was removed.

3.2.5 Binding assays

3.2.5.1 The influence of adsorption solvent on the binding factor

Varying methanol and water ratios of 10:0, 8:2, 6:4, 5:5, 3:7, 1:9 (v/v) were chosen as a source of solvent to evaluate the binding assays of MIPs and NIPs. Several studies described MAA monomers as having a great effect on binding adsorption(Kang et al., 2012; Beltran et al., 2010; He et al., 2010; Shi et al., 2007), thus, MAA-linked allyl-β-CD MIPs (M-MAA) were selected as models amongst the prepared MIPs to evaluate their binding performance for CLEN in different testing solvents. 20 mg particles of polymers were inoculated for 1 hour in 3 mL of 0.1 mg mL^{-1}CLEN solution in 10 mL conical flasks by shaking at 140 rpm at the controlled temperature of 30^{o}C.

After the defined time of inoculation, the solvent was removed by filtering through 0.22 μm for HPLC analysis. The percentage of bound substrate to the polymer matrices was calculated as the following equation:

% Bound = [(initial amount – free amount after adsorption)/ initial amount] x 100

3.2.5.2 The binding specificity

All the synthetically imprinted and non-imprinted polymers were tested for their rebinding capacity with a target binding molecule of CLEN in solvent media. The amount of template bound to polymers (Q, mg g^{-1}) was determined by equilibrium binding experiments and calculated according to the equation (Lv et al., 2007):

$$Q = \frac{V(Ci - Ca)}{m}$$

Where V (mL), the volume of the solution; Ci (mg mL^{-1}), the initial concentration; Ca (mg mL^{-1}), the free concentration after adsorption; m (g), the dried mass of polymers.

The distribution co-efficient (Kd, mL g^{-1}) of CLEN was utilized to understand its binding affinity between polymers and the solution. The higher the amount of Kd, the

greater the role the polymers have played. The following equation for distribution co-efficient is from a previous study (Zhu et al., 2002):

$$\mathrm{Kd} = \frac{Q}{Ca}$$

To understand the significant variation of the amount of uptake between MIPs and NIPs, the specific binding capacity ΔQ (mg g^{-1}) was applied according to:

$$\Delta Q = Q(MIP) - Q\ (NIP)$$

The molecular imprinting factor (IF) was employed to validate the imprinting result. IF was calculated as follows:

$$\mathrm{IF} = \frac{Kd\ (MIPs)}{Kd\ (NIPs)}$$

3.2.5.3 Binding dynamics and competitiveness

To study the binding dynamics of each synthetic polymer, 20 mg MIP particles were inoculated for 1 hour in 3 mL of a defined amount of CLEN solution ranging from 0.001 – 0.1 mg mL^{-1} in 10 mL flasks by shaking at 140 rpm at the temperature of 30^{o}C.

A series of structural analogues of CLEN, including salbutamol, terbutaline sulfate, ambroxol, were tested under the same conditions to characterize the polymers' competitive binding affinity and efficiency, as well as functional recognitions of its structurally-related compounds. The performed concentration of solution was 0.1 mg mL^{-1} in all circumstances. **Fig. 3.1** shows the chemical structure of CLEN and its analogues.

3.2.5.4 Procedure for preparation of Molecularly Imprinted Solid-Phase Extraction (MISPE)

A. Binding adsorption. An aliquot (50 mg) of MIPs and NIPs was packed into a micro-column (pipet) with a piece of cotton as a stopcock, followed by a thin layer of quartz crystal sand on the top of the polymers. The stock solution of CLEN was prepared in methanol and water (3:7, v/v) with the concentration of 10 μg and 100 μg mL^{-1}. The

polymers were pre-conditioned with 3 mL methanol and 3 mL water prior to loading the sample. After conditioning, 1 mL of stock containing 10 µg or 100 µg was load into the pipet; a total of 3 mL adsorption solvent was used per sample. It was later washed out with 3 mL hexane in order to displace any non-adsorbed components in the column; hexane was described to have a good effect of washing for CLEN (Qiao & Du, 2013). 3 mL elution of methanol containing acetic acid in a ratio of 9:1 (v/v) was run to remove the template material. The eluate was continually collected in a test tube, and dried out under nitrogen gas streaming. It was then re-dissolved with 1 mL methanol for HPLC analysis. The elution solvent was eluted through the column with a flowing rate of 1 mL min^{-1}.

B. Extraction method. The persistence of CLEN in plasma and urine is reported to be low, but it persists in a liver at much higher levels than in other edible tissues and is detectable in the liver for up to two weeks after the withdrawal of the drug from animal's feed (Meyer & Rinke, 1991). Hence, the liver is the tissue of choice for detecting illegal use of CLEN. The liver organ was purchased from local markets and used as our sample. ~ 3 g of the ground liver was weighed into a 50 mL polypropylene centrifuge tube and 10 mL water was then added to the sample. The mixture was blended by a vortex agitator for 5 min then 0.5 g of sodium chloride (NaCl) and 5 mL hexane were added to the solution and it was homogenized once again prior to sonicating for 10 min. This solution was separated by centrifuge at 3500 rpm for 5 min. The collected supernatant was decanted into a test tube, and 3 mL methanol was mixed to precipitate the fat and protein contents and it was subsequently filtered before loading for a testing process. The recovery experiment (R%) was evaluated by spiking blank liver with various concentrations of CLEN standard solutions (0.005, 0.1 µg, 1 µg , and 10 µg g^{-1} liver), and the blank control sample was carried out by the same sample preparation procedure except that CLEN standard solution was removed. The recovery calculated as follows:

$$\% \text{ R} = \frac{Cmean}{Cspike} \text{ x } 100$$

Where C_{mean} is the mean of the fortified liver concentration, and C_{spike} is the spiked concentration.

3.3 Results and Discussion

3.3.1 Characterization of the synthesized MIPs

The MAA-linked allyl-β-CD MIP (M-MAA) was taken as a demonstrated model for characterization by FT-IR spectroscopy (**Fig. 3.2**). A very sharp peak at 1730.75 cm^{-1} was a carbonyl group (C=O) from MAA and EGDMA, 1159.55 cm^{-1} was C-O stretch, and 1636.96 cm^{-1} indicated the aromatic ring from CLEN compound. A large vibration from around 1450 cm^{-1} to 1250 cm^{-1} was a carbon-carbon single bond (C-C), confirming that template and functional monomers were presenting in the polymeric networks (**Fig. 3.2A**). Additionally, a very broad band between 3600-3000 cm^{-1} was O-H stretching and 2957.06 cm^{-1} was C-H stretch which is compatible to the β-CD constituent functional structure. For non-imprinted polymers (**Fig. 3.2B**), it was observed that the region and shape of the major bands were similar to those of imprinted polymers.

According to SEM micrographs, the imprinted and non-imprinted polymers were remarkably different in texture. M-MAA imprinted polymers looked rough, friable and porous while N-MAA was smooth and compact (**Fig. 3.2C**). The irregular rough, porous MIPs surface is most likely formed by the template removal during Soxhlet apparatus operation creating the particular sites of rebinding cavities.

We chose allyl-β-Cyclodextrin (allyl-β-CD) and MAA functional monomers as binary functional monomers because the characteristics allyl-β-CD is having hydrophobic inside while having hydrophilic outside cavity, which enable them to form non-covalent host-guest inclusion complexes with organic and hydrophobic compounds, and MAA is having electrostatic attributes that can interact with other guest molecules via hydrogen bonds (H^+-O^-). Therefore, when we mixed allyl-β-CD and MAA together, the imprinted polymers will increase stronger binding interactions with analyte through hydrophobic effects and hydrogen bonding. In the pre-polymerization process for M-MAA synthesis, when allyl-β-CD molecules were mixed with CLEN template in DMF, the possibility of aromatic rings of CLEN would be either trapped inside or outside the hydrophophic core of ally-β-CD stereo-shape cavities based on its chemical nature. Whenever the benzene ring was inside, the hydroxyl group (-OH) of CLEN will make stronger hydrogen bond in the presence of additional MAA monomer. Thus, the allyl-β-CD and MAA were complementary. The position and mutual conformation of allyl-β-CD and MAA were firmly fixed through the crossing-linking agent. This imprinting process is illustrated in **Fig. 3.2D**.

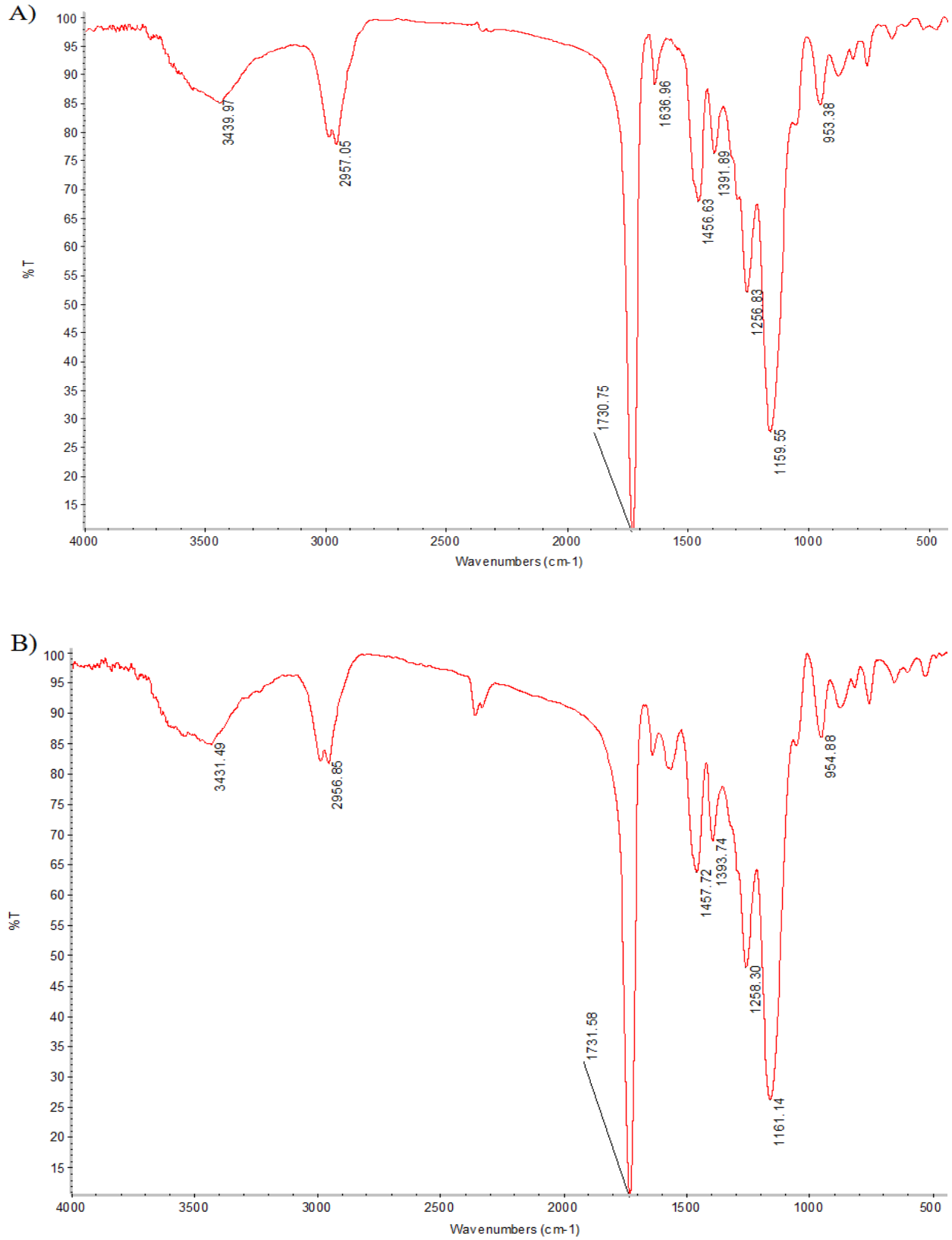


Fig. 3.2. FT-IR spectrum of (A) M-MAA and (B) N-MAA.

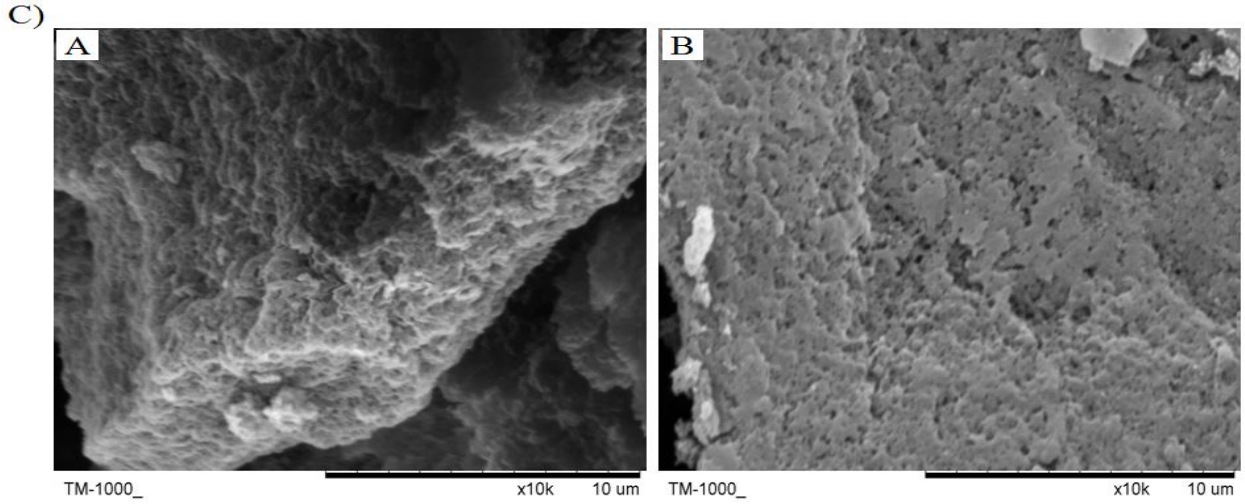


Fig. 3.2. (C) SEM micrographs of selected MIPs: (A) M-MAA, MAA-linked allyl-β-CD MIPs and (B) N-MAA, MAA-linked allyl-β-CD NIPs;

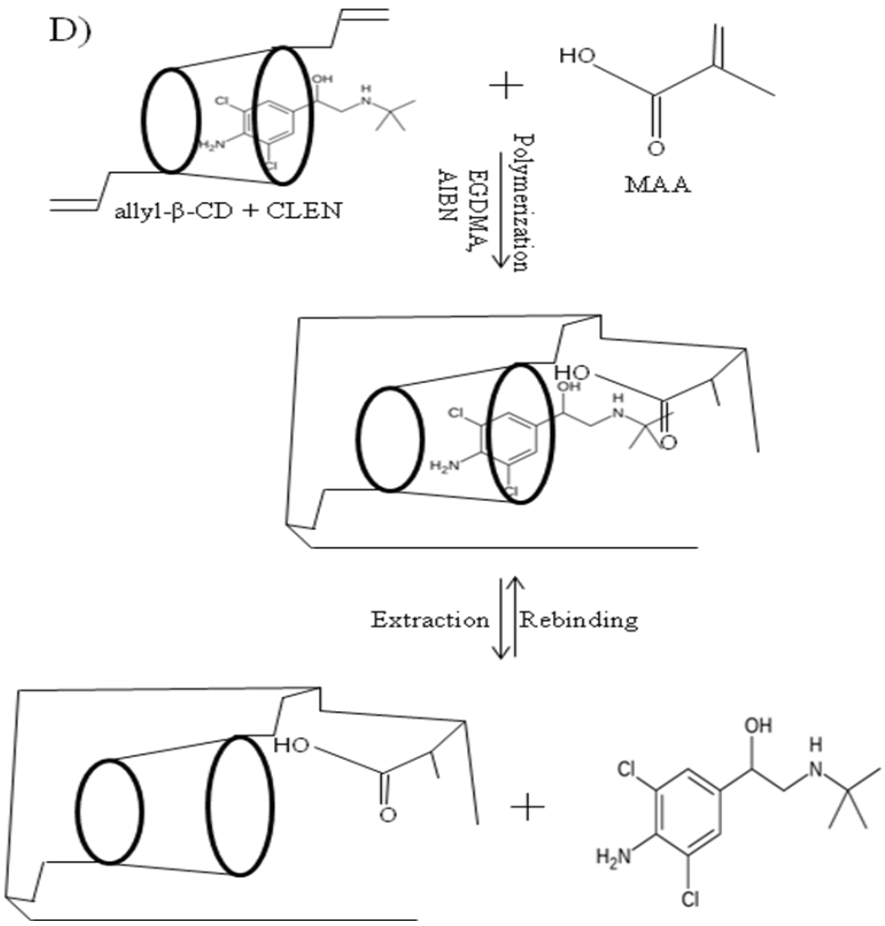


Fig. 3.2. (D) Schematic representation for the preparation of CLEN imprinted MAA-linked allyl-β-CD polymer.

3.3.2 Binding assays

3.3.2.1 The influence of adsorption solvent on the binding factor

As previously mentioned in **section 3.2.5.1**, MAA monomers were demonstrated to have greater yield in binding affinity by numerous reports; therefore, M-MAA and N-MAA were taken as a model in this investigation. **Fig. 3.3** shows that the greater the volume of water involved in the adsorption solvent, the greater the effect of the polymers. Hence, an optimal result was obtained when the water content was higher than methanol. Moreover, there was not a remarkable difference of adsorption capacity between the MIPs and NIPs being noted, probably because the analyte concentration employed was relatively low; therefore, there is no doubt that the analyte was readily capable of interacting with polymers in spite of the absence of the imprinting binding sites. In view of this, we eventually decided to use methanol and water (3:7, v/v) adsorption solvent for our further work.

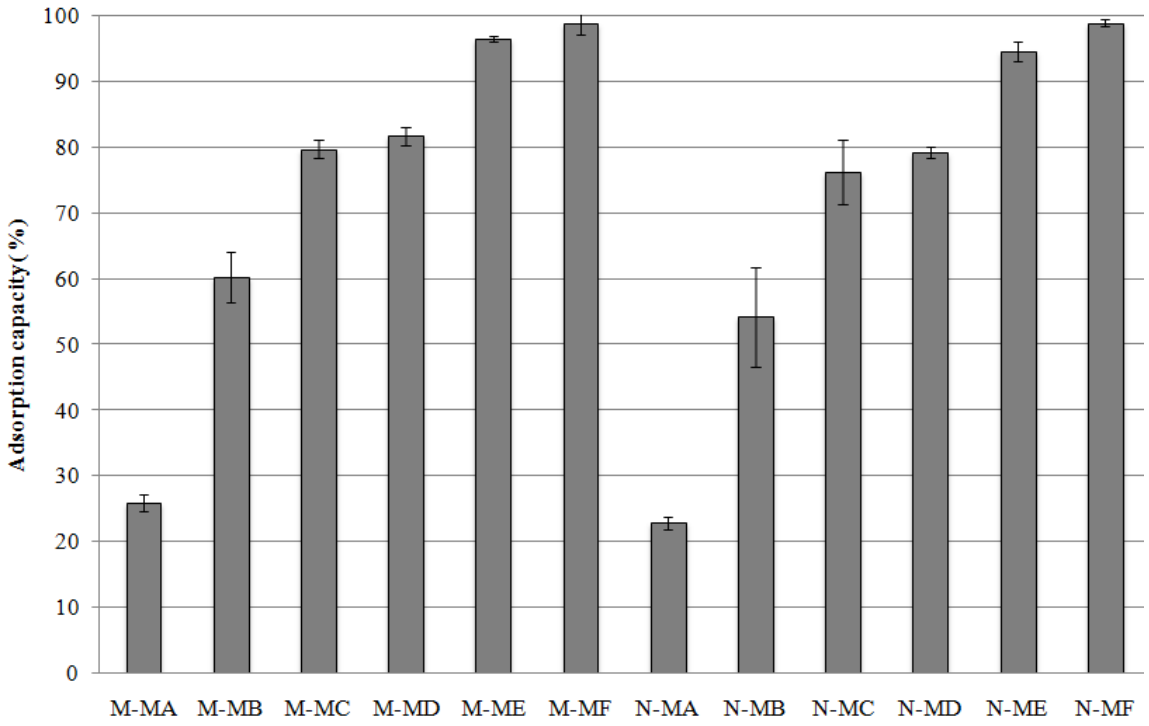


Fig. 3.3. The influence of solvent on binding factor. MA, MB, MC, MD, ME, and MF denoted methanol and water solvent, corresponding to a ratio of 10:0, 8:2, 6:4, 5:5, 3:7, and 1:9 (v/v), respectively. Initial Cont. 0.1 mg mL^{-1} CLEN, M: imprinted, N: non-imprinted, n=3.

3.3.2.2 The binding specificity

The procedure to evaluate the binding specificity was previously described (see **section 3.2.5.2**). **Table 3.1** showed the response of binding performance with various synthesized MIPs. Based on these values, there was a clear indication that polymers made of MAA monomers appeared to be of great functionality in binding forces, compared to its counterparts: AN and MMA. The average of bound substrates of MAA, AN, and MMA was 14.34 mg g^{-1}, 5.11 mg g^{-1}, 4.57 mg g^{-1}, respectively, proving that the most effective and efficient monomers in binding with a template were M-MAA polymers. The values of Q, Kd, ΔQ, and IF of the imprinted polymers were all higher than that of the non-imprinted polymers, confirming that the synthetic MIPs were superior to the NIPs. The adsorption value in this investigation was higher than the previous report (Du et al., 2014). In addition to this, although varying time was assigned, all polymers were found to become saturated in one hour's inoculation; therefore, one hour was selected as the inoculation time for our further experiments.

Table 3.1 Recognition of CLEN uptake on the polymers prepared with different functional monomers*.

Eq.	Time (h)	M-MAA	N-MAA	M-AN	N-AN	M-MMA	N-MMA
Q	1	14.46 ± 0.44	14.18 ± 1.55	4.53 ± 0.24	3.84 ± 1.36	4.29 ± 0.85	4.06 ± 1.69
	3	14.38 ± 1.00	14.24 ± 1.18	4.78 ± 0.38	3.62 ± 2.70	4.79 ± 2.97	3.37 ± 0.10
	6	14.32 ± 1.06	14.06 ± 1.01	5.49 ± 1.22	4.80 ± 1.72	5.13 ± 1.29	4.81 ± 2.28
	9	14.27 ± 0.45	14.23 ± 2.65	4.84 ± 2.32	4.55 ± 0.96	4.78 ± 2.40	3.52 ± 1.12
	12	14.27 ± 2.32	14.11 ± 3.12	5.94 ± 2.49	3.64 ± 0.80	3.87 ± 2.30	3.05 ± 1.88
Kd	1	4034.88	2607.41	64.90	51.58	60.04	55.58
	3	3460.60	2827.39	70.21	47.71	70.30	43.48
	6	3166.23	2231.11	86.56	70.50	77.86	70.84
	9	2941.14	2933.07	71.41	65.36	70.05	46.03
	12	2916.70	2381.73	98.22	48.14	52.19	38.22
ΔQ	1	0.28	0.69	0.23			
	3	0.13	1.16	1.42			
	6	0.27	0.69	0.31			
	9	0.04	0.29	1.25			
	12	0.16	2.29	0.83			
IF	1	1.55	1.26	1.08			
	3	1.22	1.47	1.62			
	6	1.42	1.23	1.10			
	9	1.00	1.09	1.52			
	12	1.22	2.04	1.37			

* Solvent: methanol/water (3:7, v/v). Initial Cont. 0.1 mg mL^{-1} CLEN. M-MAA, MAA-linked allyl-β-CD MIPs; N-MAA, MAA-linked allyl-β-CD NIPs; M-AN, AN-linked allyl-β-CD MIPs; N-AN, AN-linked allyl-β-CD NIPs; M-MMA, MMA-linked allyl-β-CD MIPs; N-MMA, MMA-linked allyl-β-CD NIPs. MIPs: imprinted polymers, NIPs: non-imprinted polymers, n=3, (mean ± SD).

3.3.2.3 Binding dynamics and competitiveness

As described earlier **(3.2.5.3)**, various contents of CLEN solutions were studied. It was observed that MAA functional monomers of synthesized MIPs were greatly capable of binding with various concentrations of CLEN compared to AN and MMA monomers (**Fig. 3.4A**). The recovery rates of M-MAA were approximately 95% in a one-hour inoculation with 140 rpm in a shaker. Conversely, as a constantly increased concentration of CLEN was added into the adsorption solvents, the effectiveness of binding capability of M-AN and M-MMA were gradually decreased, reflecting that M-AN and M-MMA polymeric

networks had poor binding interactions with a template and became saturated when the concentration of CLEN was reached to 0.01mg mL^{-1}. According to these data, we could conclude that M-MAA polymers possessed an outstanding functional binding capability. To further differentiate the effect of imprinted and non-imprinted polymers made of MAA functional group, we next gradually add the extra initial concentration of CLEN from 0.1 mg mL^{-1} up to 0.4 mg mL^{-1}. As shown in **Fig. 3.4A**, the result found that the higher concentration, the lower effect of non-imprinted polymers on interacting with analyte.

The structurally-related molecules of CLEN, including salbutamol, terbutaline sulfate, ambroxol, were chosen to define the CLEN-imprinted polymers' functional competitiveness (refer to section 2.5.3). The adsorbed capacity of M-MAA was much greater than that of M-AN and M-MMA, and also, only M-MAA polymers are able to discriminate their structural analogues, probably because OH residue of MAA is more complementary with OH residue of the template (see **Fig. 3.2D**) and the hydrogen bonds made of MAA (H - O) could form stronger interaction with other molecules than that of AN and MMA. Moreover, Beltran et al. (2010) also reported in the authors' literature review that amongst functional monomers, MAA has been the most widely used functional monomers for MIPs establishment. As a result, the MAA functional monomer was the best choice to construct the strong binding imprinted polymeric networks for the CLEN molecule, whereas AN and MMA did not prove to perform effectively (**Fig. 3.4B**).

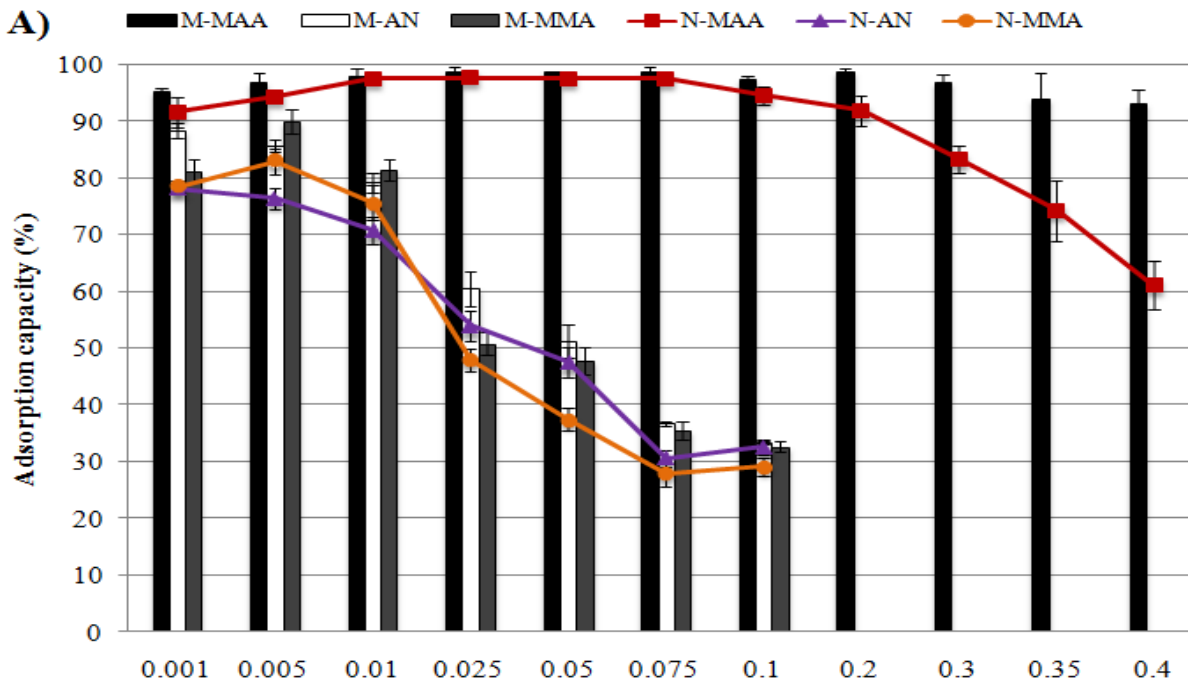


Fig. 3.4. (A) Binding dynamics of polymers with various concentrations.

B)

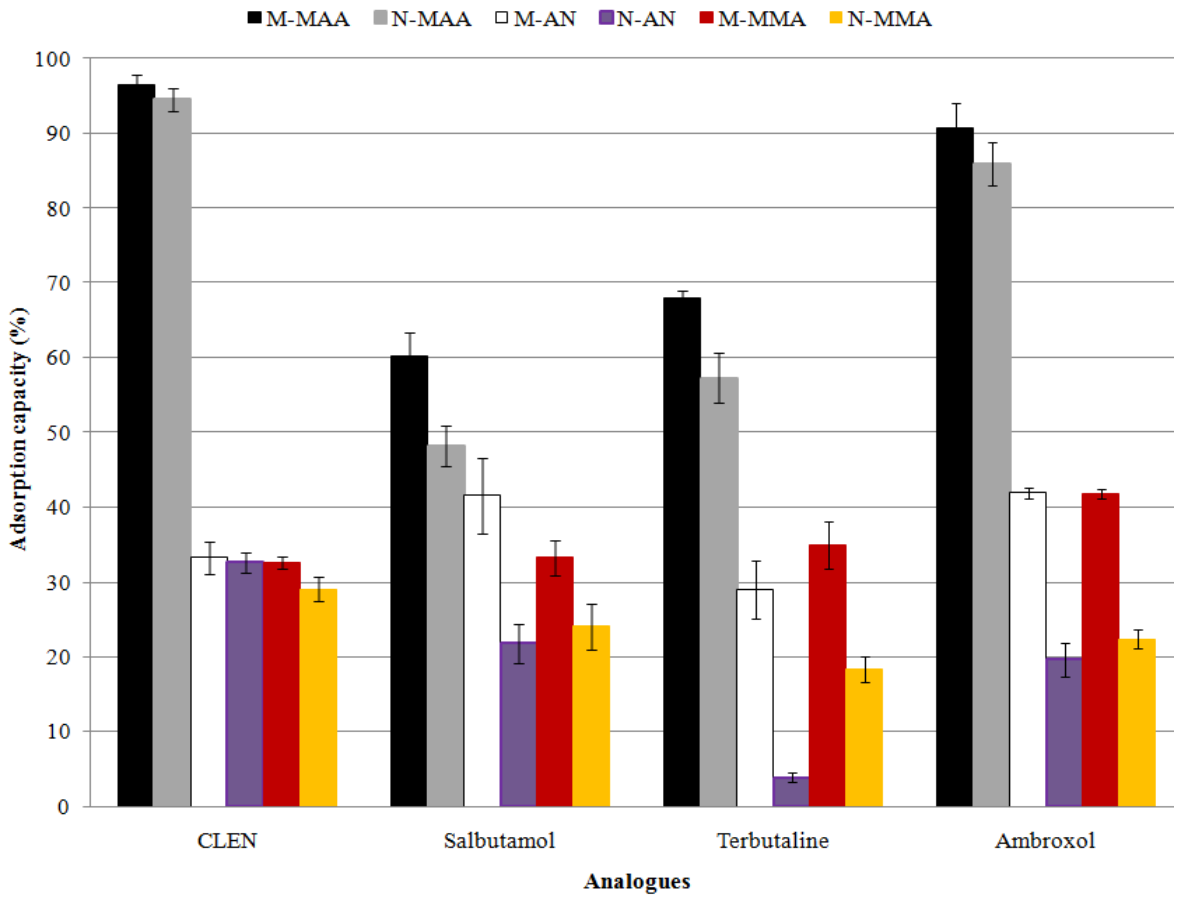


Fig. 3.4. (B) Comparing competitive binding recoveries of template with its analogues. Solvent: methanol/water (3:7, v/v). Cont. 0.1 mg mL^{-1} CLEN (n=3).

3.3.2.4 Validation of method via MISPE procedure

Binding adsorption. Prior to testing the real sample pretreatment in a micro-column, a series of experiments were carried out using the procedure described above (see **section 3.2.5.4 (A)**). When the column was injected with 10 µg mL^{-1} CLEN, all synthesized polymers were performing well in adsorption recovery. This is due to an increase of sorbent particles up to 50 mg, which created more available imprinted and non-imprinted poring spaces for the substrate interaction. However, when the amount of substrate was increased to 100 µg mL^{-1}, only can M-MAA polymers retain the same capable function, confirming that the rebinding sites of M-MAA were much stronger and more efficient than that of M-AN and M-MMA. The binding recovery of M-MAA, M-AN, M-MMA for these two different concentrations of CLEN was 95.03, 73.11, 66.10%, respectively.

The proposed method, constituted MISPE-HPLC method, was validated through specificity, linearity, limit of detection (LOD), limit of quantitation (LOQ), recovery, intra-assay and inter-assay deviation. The specificity of this method was measured by analyzing blank samples. The chromatograms on high performance liquid chromatography (HPLC) shown in **Fig. 3.5** illustrated that there were no interfering peaks from endogenous compounds at the retention time of CLEN and MISPE-HPLC method could enrich CLEN to sufficient purity. The calibration curve of CLEN through HPLC was constructed using the areas of the chromatographic peaks versus the constant concentrations measured at 8 increasing spiked levels of pork liver sample ranged from 5 ng to 1000 ng g^{-1} through diluting the stock solution in methanol. Good linearity of CLEN was obtained with calibration equation of y= 15.52 x + 0.739, (R^2 =0.998). The limit of detection (LOD) and quantitation (LOQ), based on signal-to-noise ratio of 3:1 and 10:1, were 0.047 μg kg^{-1} and 0.144 μg kg^{-1}, respectively. Recovery experiments were executed by spiking blank liver with CLEN standard solutions (0.005, 0.1 μg, 1 μg, and 10 μg g^{-1} liver); analyses were done five replicates for each concentration. The results from the proposed method responded a high recovery rate of CLEN ranging from 91.03-96.76% with the relative standard deviations (RSD) values in a range of 2.54 - 4.45%, which demonstrated that the method was reliable and could be employed for the determination of trace CLEN residues in pork livers. Moreover, the intra-assay precision and accuracy of the method, expressed as the RSD of concentration from known spiked samples at the same day, were ≤ 4.45%, while inter-assay reproducibility in four consecutive days was less than 6.80% with the average accuracy of approximate 94%.

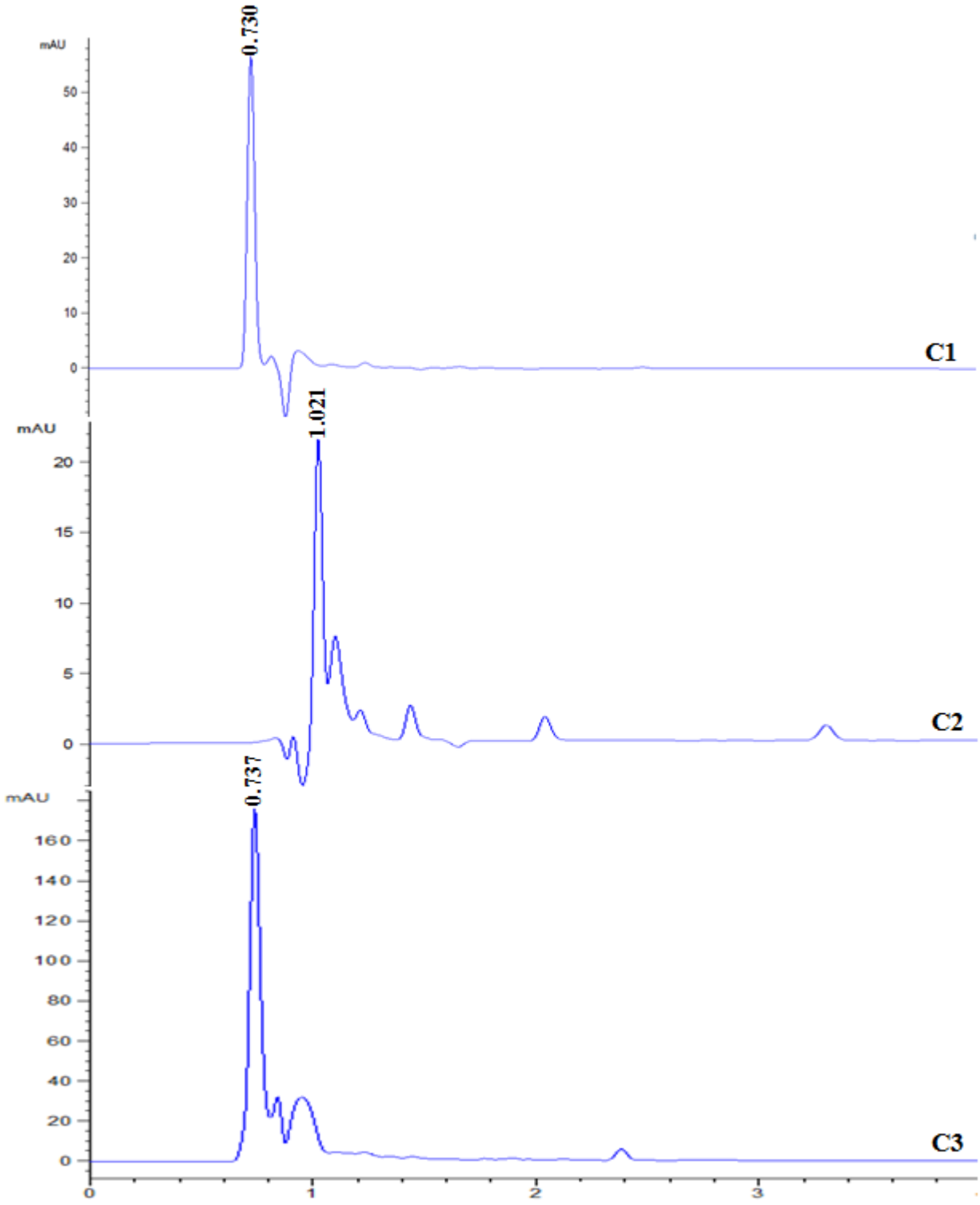


Fig. 3.5. Chromatograms on HPLC: C1, standard CLEN solution (10 μg mL^{-1}); C2, blank pork sample; C3, eluate of spiked pork sample from M-MAA (10 μg g^{-1}).

3.3.2.5 Real sample analysis

To illustrate the suitability and potential of this proposed method, 8 pork liver samples were purchased from the local markets. In the results, there are no any CLEN residues being detected in the livers through this method. Therefore, the accuracy of the method was re-evaluated through spiking with two different concentrations (1 μg, and 5 μg g^{-1} liver) in a real pork sample. The results finally revealed that the accuracy of recovery was attained ~ 95%, reflecting that the proposed method was suitable to be utilized for the determination of trace CLEN residues in pork livers.

3.4 Conclusion

In this study, a new preparation step of imprinting CLEN-imprinted polymer and extraction method to detect the CLEN residues in pork livers were developed. To our knowledge, this is the first report that has exploited hydrophobic interaction and hydrogen bonding based on allyl-β-CD and MAA functional monomers for MIPs fabrication for selective recognition of clenbuterol. We also innovate a new way of extracted method of clenbuterol residue from pork sample and finally, our prepared MISPE procedure is simple and quick; we only need a glass pipette, cotton, and quartz crystal sand. Most importantly, these materials are inexpensive and commercially available everywhere. Based on our MISPE protocol, we spent at most 3-5 minutes for the whole preparation process. Of the three distinctively synthetic polymers, MAA-linked allyl-β-CD imprinted polymers (M-MAA) were found to be superior to its referent members: M-AN, M-MMA, and possessed an effective recognition ability of imprinted polymers. In binding specificity, the average of bound substrates of M-MAA, M-AN, and M-MMA was 14.34 mg g^{-1}, 5.11 mg g^{-1}, 4.57 mg g^{-1}, respectively. Initially, M-MAA and N-MAA were not demonstrated substantial differences at low concentration. However, once the amount of analyte concentration was gradually increased up to 0.4 mg mL^{-1}, the effect of M-MAA can differentiate from that of N-MAA. The recovery from a spiked sample was very high, ranging of 91.03 - 96.76%, RSD $\leq$ 4.45%. All in all, this proposed method could be employed for food quality and safety control analysis due to its simplicity, cost-effectiveness, and rapid detection of CLEN residue in pork livers.

CHAPTER 4 SYNTHESIS AND APPLICATION OF SELECTIVE ADSORBENT FOR PIRIMICARB PESTICIDE IN AQUEOUS MEDIA USING ALLYL-β-CYCLODEXTRIN AND METHACRYLIC ACID FUNCTIONAL MONOMERS

4.1 Introduction

Pesticides, including nonpolar organochlorine insecticides (OCs), relative polar organophosphorus insecticides (OPs), and organonitrogen pesticides (ONs) (referring to carbamates, triazines, and their derivatives) have been applied to a wide range of crops, plants, and vegetables to eliminate pests. Commonly applied pesticides include insecticides, herbicides, rodenticides, and fungicides. Presently, pesticides are reported to be diverse and omnipresent; approximately 1400 pesticides are in use worldwide. All pesticides are mildly or highly toxic by their nature; therefore, they trigger not only health hazards to humans and animals, but also environment through dietary intake or exposure (John & Tsunehiro, 2004). Human health problems depend on the type of the used pesticides and also the extent of exposure. The immediate symptom of poisonings from pesticides include mild headaches, flu, skin rashes, blurred vision and other neurological disorders, but rarely, paralysis, blindness, and even death. Long-term persistence of exposure could induce cancer, infertility, miscarriage, male sterility, birth defects, and effects on nervous system (Moses, 1995). Regarding the public health and environmental concerns, the introduction of new and efficient analytical approaches, therefore, is importantly demanded for controlling the pesticide residues in agricultural products, plants, and environmental samples.

Pirimicarb (N,N-dimethylcarbamate), a class of selective insecticides used against aphids in vegetables and fruits, could be harmful for human health because of its potent toxicity (Machemer & Pickel, 1994). It is also suspected of a carcinogenic agent and mutagens (Li, Hammock, & Seiber, 1991). According to the Chinese food safety regulation of the pirimicarb‘s residues, the allowable maximum limit on vegetables and fruits is 1000 μg kg^{-1} and 500 μg kg^{-1}, respectively (Zhou et al., 2010).

Molecularly imprinting is a technique for the construction of artificial receptor-like binding sites with memory-inside cavities of the molecularly imprinted particles complementary in size, shape, and chemical functionality of the template structure. Non-

covalent bonding, ionic interactions, and hydrophobic interactions are normally employed in the creation of molecularly imprinted polymers (MIPs) (Zhou et al., 2010), which is considered as a class of smart sorbents for analytical separation (Edward & Stanley, 2003). Owing to the potential applications of this technique, MIPs have attracted much significant interest in the areas of chromatographic stationary phases, solid-phase extraction (SPE), artificial antibody mimics, catalysis, and biosensor over the past decades (Zhou et al., 2010).

Recently, several newly developed methods of detection and determination of trace levels of pirimicarb chemical were reported (Zhou et al., 2010; Gao, Wang & Yang, 2009; Gao, Wang, An, & Liu, 2008; Sun & Fung, 2006; Mena, Mart´ınez-Ruiz, Reviejo, & Pingarrón, 2002); individuals claimed its own advantage of their innovative analytical method. However, there is still strongly needed an alternatively simpler and efficient method of detection and quantitation of the trace amount of analyte. It has been demonstrated that molecularly imprinted solid-phase extraction (MISPE) is an excellent method for pre-concentration of traces of analytes from environmental samples (Sergeyeva et al., 2001; Baggiani, Baravalle, Giraudi, & Tozzi, 2007; Baggiani, Giovannoli, Anfossi, & Tozzi, 2001; Tarley & Kubota, 2005; Carabias-Martı´nez, Rodrı´guez-Gonz-alo, & Herrero-Herna´ndez, 2006; Beltran et al, 2007; Breton et al., 2007) due to the specific recognition ability of the molecularly imprinted polymers (MIPs) for their templates. Therefore, application of MISPE before a liquid chromatography could be a promising area that could reduce or eliminate risk potential interference by unknown matrix components. In this study, we aimed to develop a pre-treatment methodology for pirimicarb isolation and enrichment in a complex matrix environment through exploiting molecularly imprinted polymers as a selective solid-phase extraction (SPE) prior to applying a liquid chromatographic analysis.

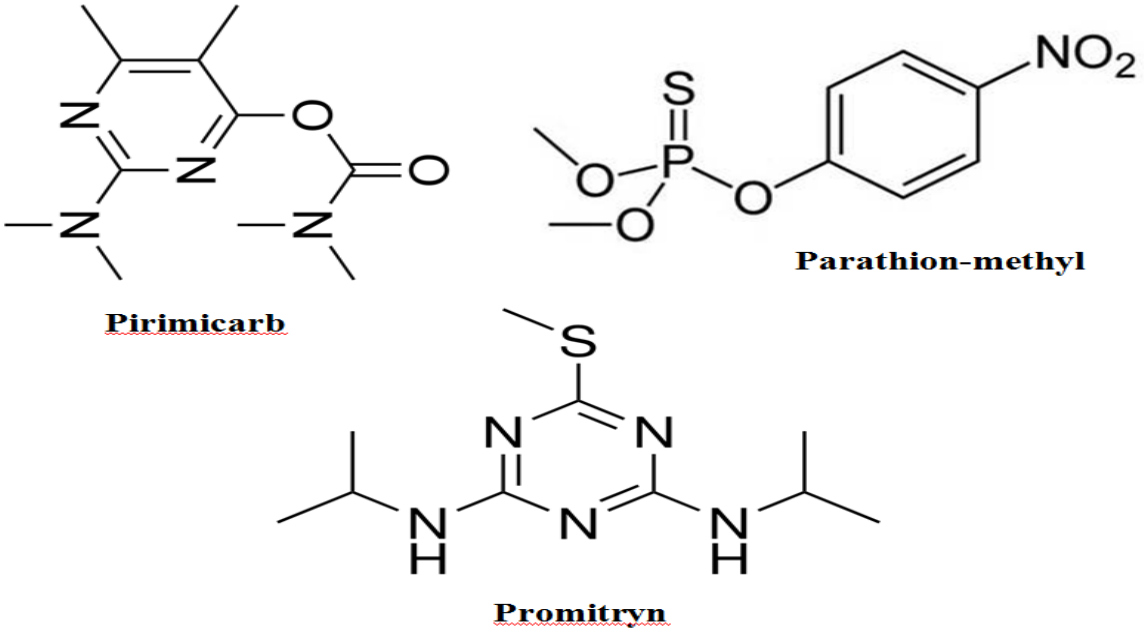


Fig. 4.1. Chemical structures of pirimicarb and its analogues.

4.2 Experimental Section

4.2.1 Chemicals

The analytical working standard pirimicarb (98.7%) was ordered from Sigma. Parathion-methyl, prometryn (99%), β-cyclodextrin (β-CD, 96%), allyl bromide (98%), methacrylic acid (MAA, 99%), ethylen glycol dimethacrylate (EGDMA, 98%), azo-N,N-bisisobutyronitrile (AIBN, 99%), N,N-dimethylformamide (DMF, 99.5%), and were procured from Alladin Chemistry (Shanghai, China). Acrylonitrile (AN, 99%), and HPLC grade methanol were purchased from Gracia Chemical Technology (Chengdu, China), and Tianjin Sayfo Technology (Tianjin, China), respectively. Ultra-pure water, used for molecularly imprinted polymers (MIPs) synthesis and HPLC mobile phase, was produced from a laboratory water purification system (Ultra pure UF model, Shanghai, China).

β-CD and AIBN reagent were re-crystallized from water and methanol, respectively. The selected re-distillation of MAA and AN functional monomers and already prepared pirimicarb stock solution (1 mg mL^{-1} in HPLC grade methanol) were stored in the refrigerator at 4 ^{o}C prior to their use. All the reagents used in this work were of high-purity grade.

4.2.2 Instruments

The chromatographic analyses were performed on an Agilent 1200 HPLC system, equipped with a UV-visible variable-wavelength detector (225 nm monitored), binary pump (G1312B), degasser (G1322A), autosampler SL, and temperature-controlled compartment (Santa Clara, CA, USA). The reversed-phase column was 250 x 4.60 mm (5 micrometer, phenomenex, USA). The mobile phase composition of methanol and water was blended in a ratio of 6:4. The flow rate was run at 1 mL min^{-1} and the controlled temperature was 30 ^{o}C. The injection volume of 10 μL and running time of 11 min were observed to result in a sufficiently good response and separation of pirimicarb compound.

Scanning Electron Micrograph (SEM) (Hitachi, Japan) was employed to characterize the synthetic properties of MIP formations and its morphological characeristics.

4.2.3 The preparation of allyl-β-CD interaction

The reaction of allyl bromide and β-CD was prepared as follows: 10 g of sodium hydroxide (NaOH) was first dissolved with 10 mL of water in a 100 mL glass beaker. The solution was continually stirred until it was well-mixed before 10 g of β-CD was poured into it. This solution was mechanically stirred in the water bath at 60 ^{o}C for 2 hours. Afterwards, 8 mmol allyl bromide was put in the solution and continually stirred under nitrogen gas for 2 hours in the inert atmosphere at 60 ^{o}C. The solution was finally transferred back to the water bath for 30 min prior to desiccating in the oven at 60^{o}C. After three days, the rigidly dried yield of reaction mixture obtained was crushed and ground into fine powder and washed 5 times with absolute ethanol (5 min each time), in order to remove the residues from the resulting product.

4.2.4 The synthetic polymerization of MIPs

The procedure of MIPs synthesizing was as follows: 2.80 g of allyl-β-CD synthetic stock compound was first dissolved in 40 mL DMF for 30 min prior to adding 5 mL methanol containing 500 mg pirimicarb concentration to the solution. The reactive solution was processed for 2 hours at a controlled temperature of 60 ^{o}C under continuing magnetic stirring with nitrogen gas purging. A 12 mmol functional monomer (MAA, AN) was then supplied to the solution, allowing 1 hour for reaction. Afterwards, 60 mmol EGDMA was

supplemented for another 1 hour, followed by adding 40 mg AIBN as an initiator; the resultant solid-state reaction mixture was obtained after about 30 min and was then incubated in a water bath at 60^{o}C for 24 hours. The bulk solid, removed from the thermal bath, was continually desiccated in an oven for two days before being crushed and ground into free-flowing powder through a 400 mesh steel sieve. Fine particles were collected and washed with warm water to minimize possible contaminants. They were subsequently Soxhlet extracted with methanol until no pirimicarb was detected by UV-vis spectrophotometry.

Non-imprinted polymers (NIPs) were simultaneously prepared under the same conditions except that the pirimicarb template was removed.

4.2.5 The influence of adsorption solvent on binding factor

Varying water and methanol ratios of 95:5, 90:10, 85:15, 50:50, 20:80% (v/v) were chosen as a source of solvent to evaluate the binding assays of MIPs and NIPs. 20 mg particles of polymers were inoculated for 1 hour in 3 mL of 0.2 mg mL^{-1} pirimicarb solution in 10 mL conical flasks by shaking at 150 rpm at the controlled temperature of 30^{o}C.

After the defined time of inoculation, the solvent was removed by filtering through 0.22 µm for HPLC analysis. The percentage of bound substrate to the polymer matrixes was calculated as the following equation:

% Bound = [(initial amount – free amount after adsorption)/ initial amount] x 100

4.2.6 The binding specificity

All the synthetically imprinted and non-imprinted polymers were tested for their rebinding capacity with a target binding molecule of pirimicarb in solvent media. The amount of template bound to polymers (Q, mg. g^{-1}) was determined by equilibrium binding experiments and calculated according to the eq. (Lv, Lin, Feng, Zhou, & Tan, 2007):

$$Q = \frac{V(Ci - Ca)}{m}$$

Where V (mL), the volume of the solution; Ci (mg. mL^{-1}), the initial concentration; Ca (mg. mL^{-1}), the free concentration after adsorption; m (g), the dried mass of polymers.

The molecular imprinting factor (IF) was employed to validate the imprinting result. IF was calculated as follows:

$$\mathrm{IF} = \frac{Q\ (MIPs)}{Q\ (NIPs)}$$

4.2.7 Preparation for Molecularly Imprinted Solid Phase Extraction (MISPE) procedure

A. Binding adsorption. An aliquot (50 mg) of MIPs and NIPs was packed into a pipet with a piece of cotton as a stopcock, followed by a thin layer of quartz crystal sand on the top of polymers. The polymers were pre-conditioned with 3 mL methanol and 3 mL water prior to loading the sample. The stock solution of pirimicarb was prepared in water and methanol (95:5, v/v) with different concentrations of 10-200 μg mL^{-1}. After conditioning, a range of varying concentrations (10, 25, 50, 100, 200 μg mL^{-1}) were tested. A total of 3 mL adsorption solvent was used per sample. It was later washed out with 3 mL water in order to displace any non-adsorbed components in the column. 2 mL elution of absolute methanol was run to remove the template material. The eluate was continually collected in a test tube, and dried under nitrogen gas streaming. Finally, the sample was re-adjusted with methanol to a final volume of 1 mL for HPLC analysis. The elution solvent was eluted through the column with a flowing rate of 1 mL min^{-1}.

B. Competitive Binding selectivity. Two different structural analogues of pirimicarb, parathion-methyl and prometryn, were chosen to run through MISPE procedure to find out the polymers' selective binding efficiency between template and its structurally-related analogues. The performed concentration of solution was 0.2 mg. mL^{-1}. **Fig. 4.1** shows the chemical structure of pirimicarb and its analogues. Moreover, a mixed solution of template and its analogues in the adsorption solvent, containing water and methanol (95:5, v/v), in the concentration of 0.05 mg. mL^{-1} was prepared to test, too.

C. Extraction method of vegetable as real sample analysis. The vegetables were purchased from a local market and used as our sample. 1 mL or 3 mL methanol containing 10 μg mL^{-1} in methanol was dispensed into 50 mL centrifuge tube consisting of 3 g of

chopped vegetables. The vegetable was incubated three hours in the ambient atmosphere prior to adding remaining methanol up to 20 mL in each sample. The mixture was blended by a vortex agitator for 5 min, and 1 g of sodium chloride (NaCl) was then added to the solution. The mixture sample was homogenized once again prior to sonicating for 10 min. This solution was separated by centrifuge at 3500 rpm for 5 min. The collected supernatant was decanted into a 50 mL filtered tube, which cotton was used as a stopcock. 5 mL extra-methanol was dispensed to clean all residues remain inside the extracted sample; totally we used 25 mL methanol. The collected sample was evaporated to almost dryness through a rotary evaporator at 70^{o}C before 10 mL water was added to dissolve the sample in the round bottom flask. The sample was sonicated for 5 min in each sample before we decided to filter again through a 50 mL filtered tube. 5 mL extra-water was dispensed to clean all residues remain inside the round bottom flask; totally we used 15 mL water in the process. Finally, to get purer sample by minimizing the residues which could interfere during the running of MIPs micro-column, the sample was decided to filter again through 0.45µm. The recovery experiment (R%) was evaluated by spiking blank vegetable with various concentrations of pirimicarb standard solutions (1, 3.33 and 10 µg g^{-1}), and the blank control sample was carried out by the same sample preparation procedure except that pirimicarb standard solution was removed. The recovery calculated as follows:

$$\% \text{ R} = \frac{C\ mean}{C\ spike} \text{ x } 100$$

Where C_{mean} is the mean of the fortified vegetable concentration, and C_{spike} is the spiked concentration.

4.3 Results and Discussion

4.3.1 Characterization of the synthesized MIPs

The morphological study of polymers was done by SEM. It was evident that imprinted and non-imprinted polymers were remarkably different. Pirimicarb-imprinted polymers looked rough, friable and porous while non-imprinted polymers were smooth and compact. The arrow heads showed the surface area of imprinted and non-imprinted polymers (**Fig. 4.2A**). The irregular rough, porous MIPs surface layer is most likely formed by the

template removal during Soxhlet apparatus operation creating the imprinted sites of rebinding cavities.

Fig. 4.2B illustrated a schematic diagram of host-guest inclusion complex formation process between polymer and template. We can draw from the diagram that the possibility of aromatic rings of pirimicarb can either entrap inside the hydrophobic ally-β-CD core or stay outside depending on its chemical nature. Whenever the aromatic rings were inside, the carbonyl group (C=O) of pirimicarb will interact with MAA functional monomers through hydrogen bonding. Therefore, adding MAA monomers to polymerized solution will enhance stronger binding interaction between the functional monomers and template, so as allyl-β-CD and MAA were complementary. The position and mutual conformation of allyl-β-CD and MAA were firmly fixed by the help of crossing-linking agent.

A)

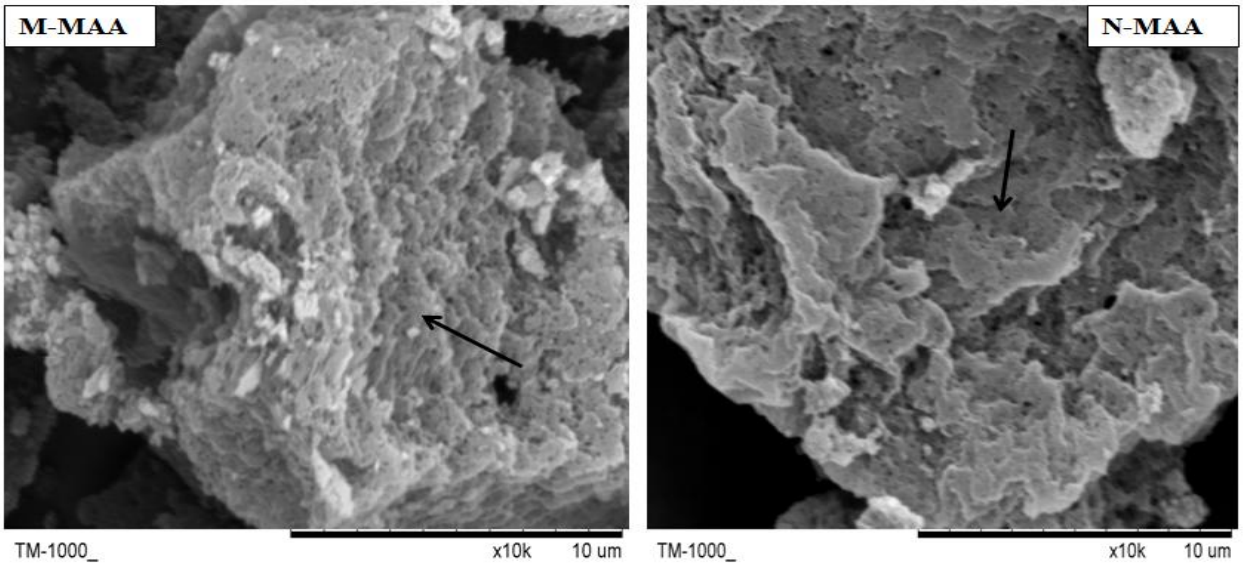


Fig. 4.2. (A) SEM micrographs of MIPs and NIPs.

B)

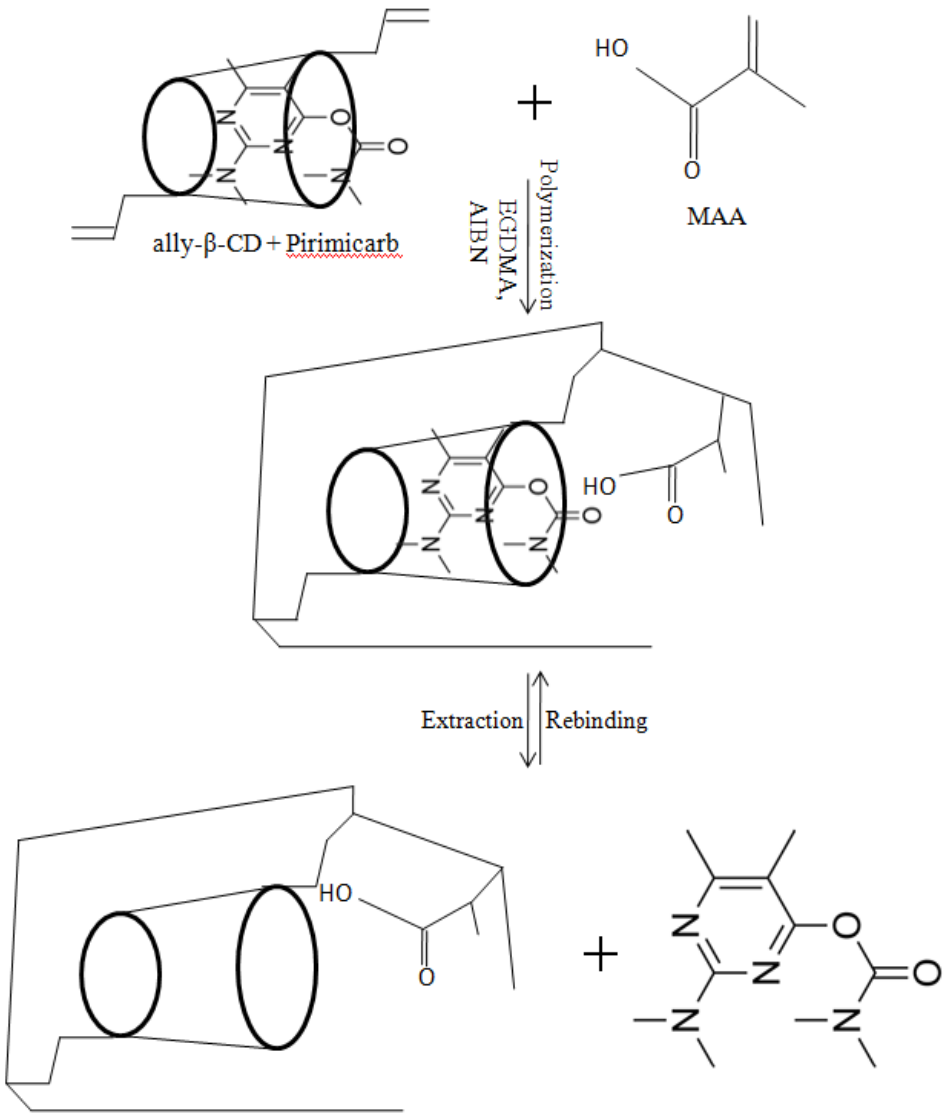


Fig. 4.2. (B) Schematic representation for the preparation of pirimicarb-imprinted polymer made of MAA-linked allyl-β-CD (M-MAA).

4.3.2 Adsorption solvent evaluation

As aforementioned in **section 4.2.5**, varying water and methanol ratios of 95:5, 90:10, 85:15, 50:50, 20:80% (v/v) were chosen as a source of solvent to evaluate the binding assays of MIPs and NIPs. **Fig. 4.3** indicated that the constantly decreased volume of water content in solvent, the less efficiency of binding responses of polymers. On the contrary, gradually added water in solvent could increase binding response because water could

drive the template into imprinted cavities of β-CD through host-guest inclusion complex based on hydrophobic effects. Our results were consistent with the previous reports, emphasizing that water used as an integral part of eluent was indispensible in inducing the capacity factor since the main driving force for the binding of the guest molecule is hydrophobic interaction, not hydrogen bonding or other interactions, for the inclusion complex formation (Asanuma, Hishiya, & Komiyama, 2004). Besides this, Xu et al. (2008) also reported that the main contribution to the recognition ability of the β-CD imprinted polymer was the stereo-shape effect inherent and hydrophobic effects are important role in the process of recognition. In our experiment, we observed that the increasing volume of water, while decreasing methanol content in mixed solvents, would generate higher hydrophobicity in solvents; as a result, the pirimicarb template could easily insert into cavities of β-CD residues through host-guest inclusion complex formation based on hydrophobic effects. Therefore, the most optimal binding adsorption was obtained once water and methanol was matched in a ratio of 95:5, v/v. Moreover, we found that all the pirimicarb-imprinted polymers have much stronger binding affinity for the template than the corresponding referenced non-imprinted polymers (NIPs); this could be ascribed to imprinting effect. The functional monomer of MAA that bond with allyl-β-CD were shown higher binding capacity than that of AN that bond with allyl-β-CD.

In short, we could conclude that water promotes binding interaction between template and functional monomers mainly through internal hydrophobic interaction of β-CD structural properties.

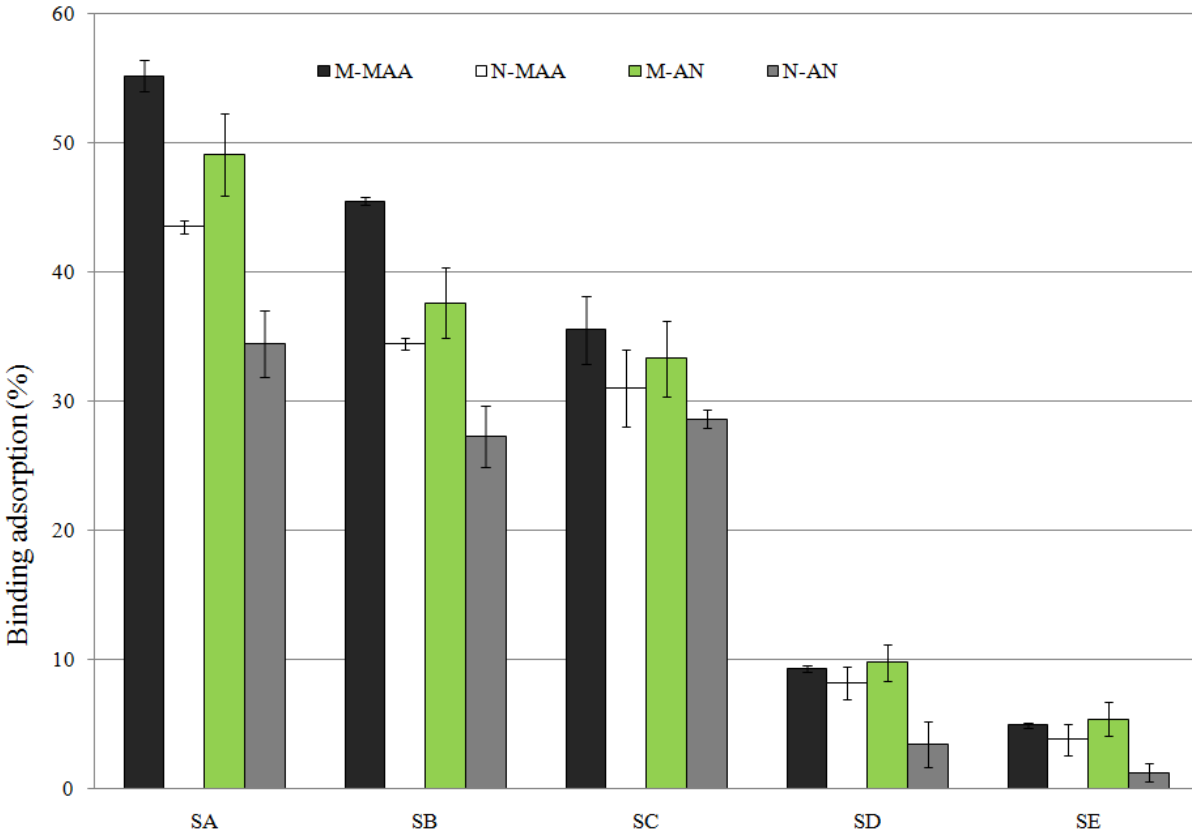


Fig. 4.3. The influence of solvent on binding factor. SA, SB, SC, SD, and SE, were water and methanol solvent, corresponding to a ratio of 95:5, 90:10, 85:15, 50:50, 20:80% (v/v), respectively (n=3).

4.3.3 The binding specificity

20 mg particles of polymers were inoculated for 1 hour in 3 mL of 0.2 mg mL^{-1} pirimicarb solution in 10 mL conical flasks by shaking at 150 rpm at the controlled temperature of 30^{o}C. As shown in **Fig. 4.4**, a sizeable difference of imprinted binding activity between synthesized polymers formed with the presence of template and without template. For example, the capacity factor of binding affinity substantially increased from 13.05 to 16.55 $mg.g^{-1}$ for M-MAA and from 10.33 to 14.73 $mg.g^{-1}$ for M-AN when the polymerization was performed in the presence of pirimicarb template. The molecular imprinting factor of M-MAA and M-AN corresponded to 1.27 and 1.42, respectively.

In all, the amount of substrate bound to M-MAA is found to be much higher amongst its experimental groups.

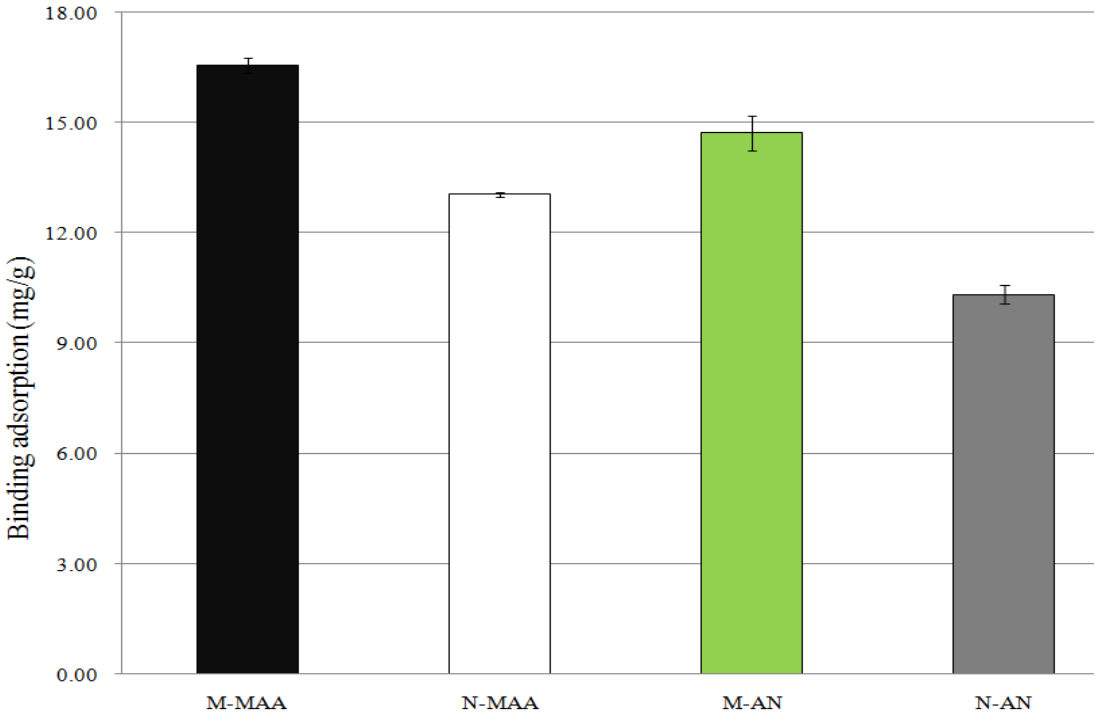


Fig. 4.4. Equilibrium binding experiments of the MIPs and NIPs. Solvent: water and methanol (95:5, v/v); volume: 3 mL (n=3).

4.3.4 Preparation for MISPE procedure

A. Binding adsorption. Prior to testing the real samples in a micro-column, a series of experiments were carried out; using the procedure described above (see **section 4.2.7 (A)**). A range of various concentrations (0.01-0.2 mg L^{-1}) were performed to understand the effects of polymeric individuals. We found that the constantly increased concentrations yield the higher amount of substrate bound to polymers. In this point, we noted that MIPs and NIPs did not show any significant difference in a range of 0.01 and 0.1 mg L^{-1} concentrations; this is, probably, because of an increased amount of adsorbent particles up to 50 mg in MISPE procedure, while only 20 mg in suspension assay, creating more availability of specific and unspecific binding spaces for the substrate contacts. However, once the amount of template was increased to 0.2 mg. L^{-1}, the binding effect of both MIPs and NIPs had shown substantially different binding behaviors (**Fig. 4.5**). The average of selective retention recovery of pirimicarb from micro-column of M-MAA, M-AN, N-MAA, and N-AN were 96.38, 88.88, 83.79, and 76.96%, respectively. In short, the rebinding sites

of M-MAA were observed much stronger and more efficient than that of M-AN; therefore, we decided to choose only M-MAA and N-MAA for our further works.

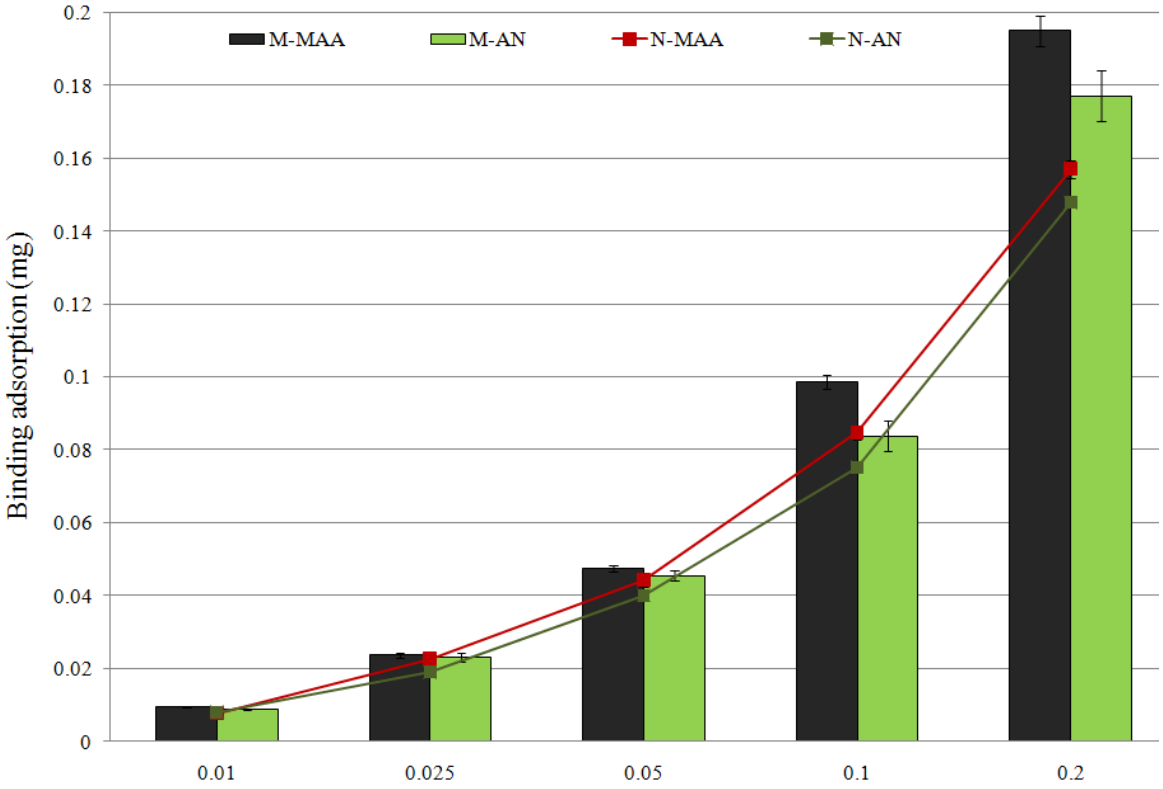


Fig. 4.5. The binding adsorption of the MIPs and NIPs with various concentrations through MISPE (n=3). Solvent: water and methanol (95:5, v/v; n=3).

B. Competitive Binding selectivity. As previously describe in the **procedure 4.2.7 (B)**, structural analogues of pirimicarb, including parathion-methyl and prometryn, were chosen to run through MISPE micro-column. As shown in **Fig. 4.6A**, the effect of selective binding competitiveness towards pirimicarb template is stronger than that of structural analogues, implying that the synthesized polymer is able to discriminate their structural analogues based on its imprinted sites where are able to memorize its guest molecule. Furthermore, it is a clear indication that MIPs also enabled to retain structures closely related to the template molecule through an effect known as cross-selectivity. On the other hand, when the solution was mixed together in a concentration of 0.05 mg mL^{-1} from each side, interestingly, we found that M-MAA and N-MAA were able to interact strongly with all involved substrates in the solution after we reduced from 0.2 to 0.05 mg mL^{-1} of each substrate (**Fig. 4.6B**). According to the chromatograms on high performance liquid chromatography (HPLC) (**Fig. 4.6C**), the content of pirimicarb and its analogues was

almost recovered after loading. This result confirmed that M-MAA was capable of binding affinity towards its template and structural-related to template. Based on this aspect, we could draw a conclusion that the adsorbed effects of MIP could not statistically differentiate from the adsorbed effects of NIPs unless the used concentration was high, more interestingly, no matter how high or low concentrations in the assay, M-MAA is still able to adsorb effectively and efficiently.

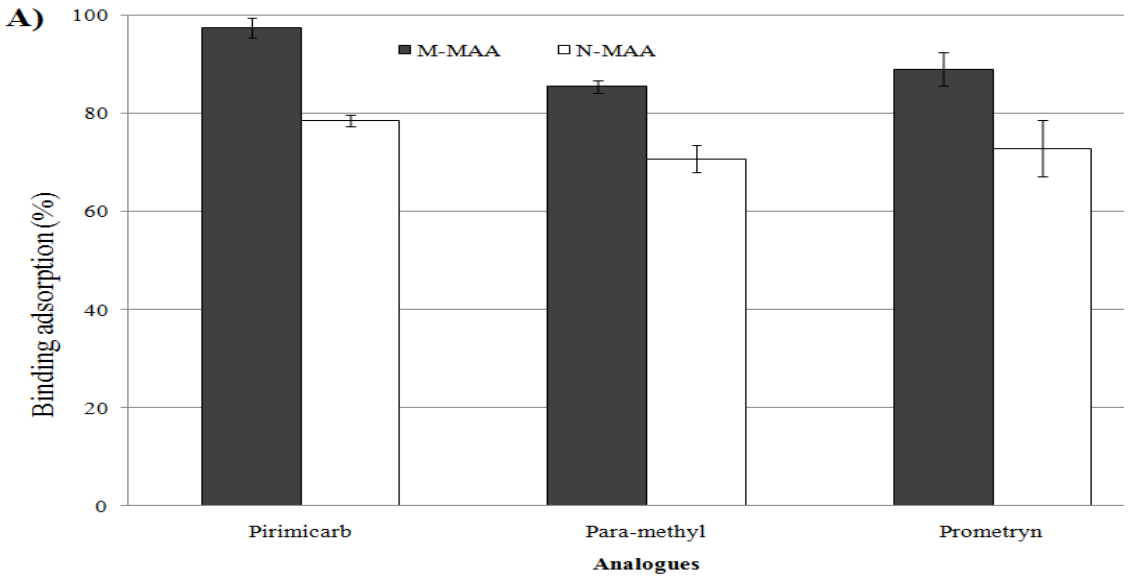


Fig. 4.6. (A) Comparing competitive binding recoveries of template with its analogues. Cont. 0.2 mg. mL^{-1} (n=3).

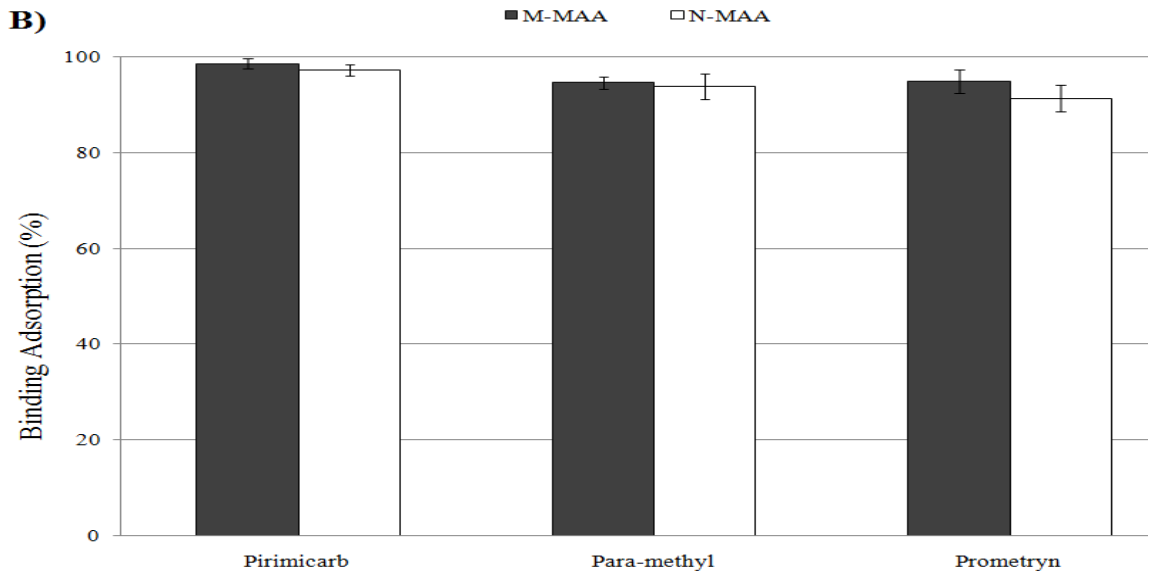


Fig. 4.6. (B) Comparing competitive in mixed solution. Solvent: water and methanol (95:5, v/v). Cont. 0.05 mg mL^{-1} (n=3).

C)

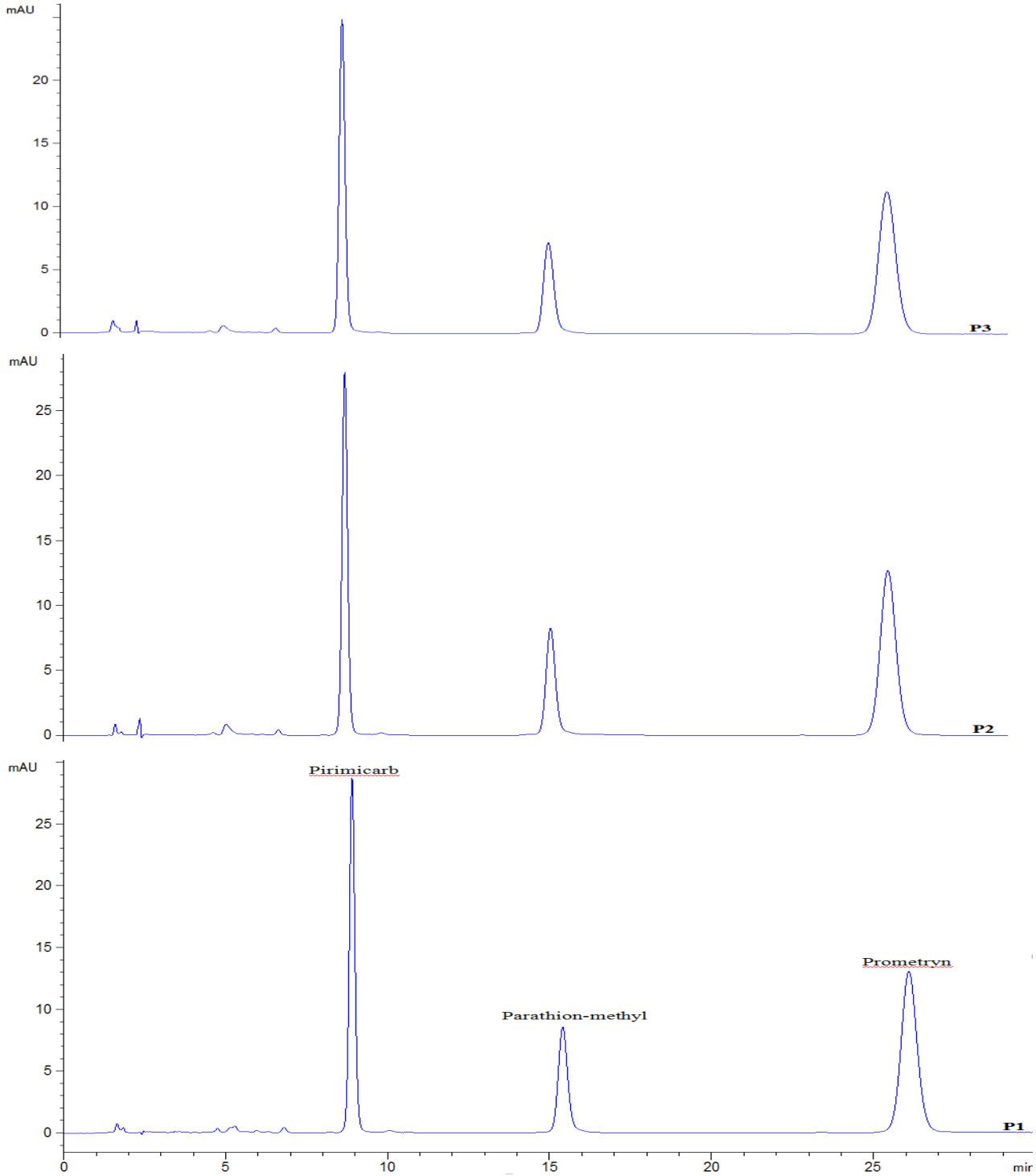


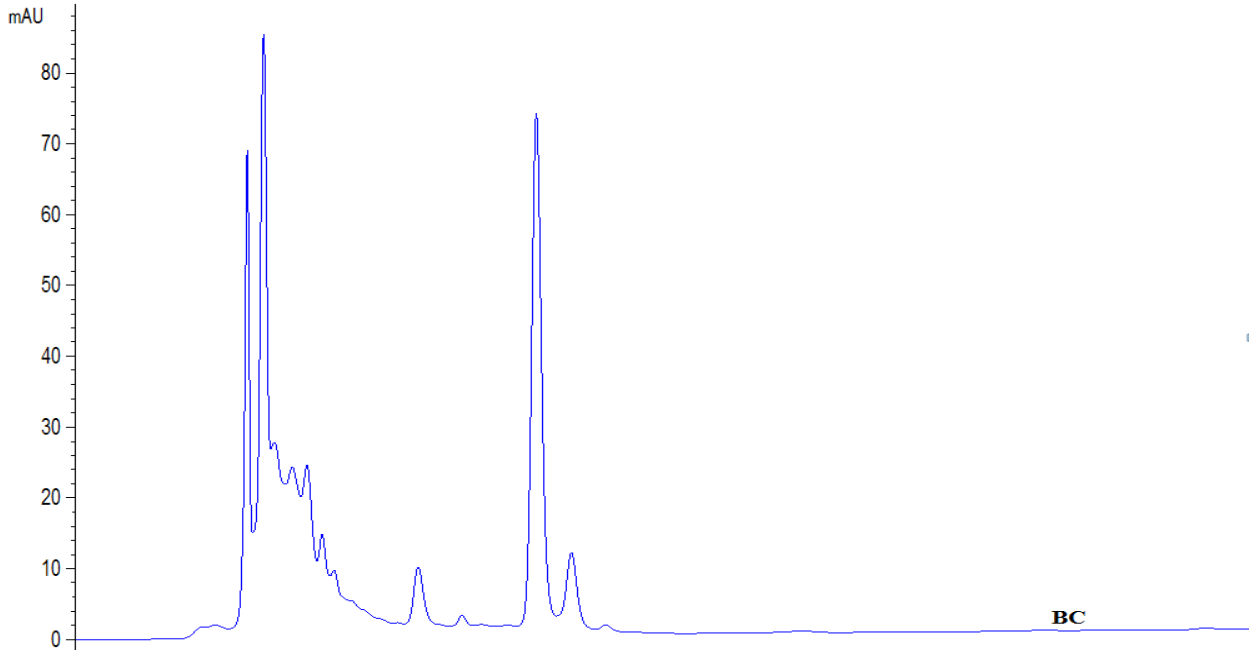
Fig. 4.6. (C) Chromatograms on high performance liquid chromatography: P1, mixture solution before adsorption; P2, eluate of M-MAA; P3, eluate of N-MAA. Cont. 0.05 mg mL^{-1} (n=3).

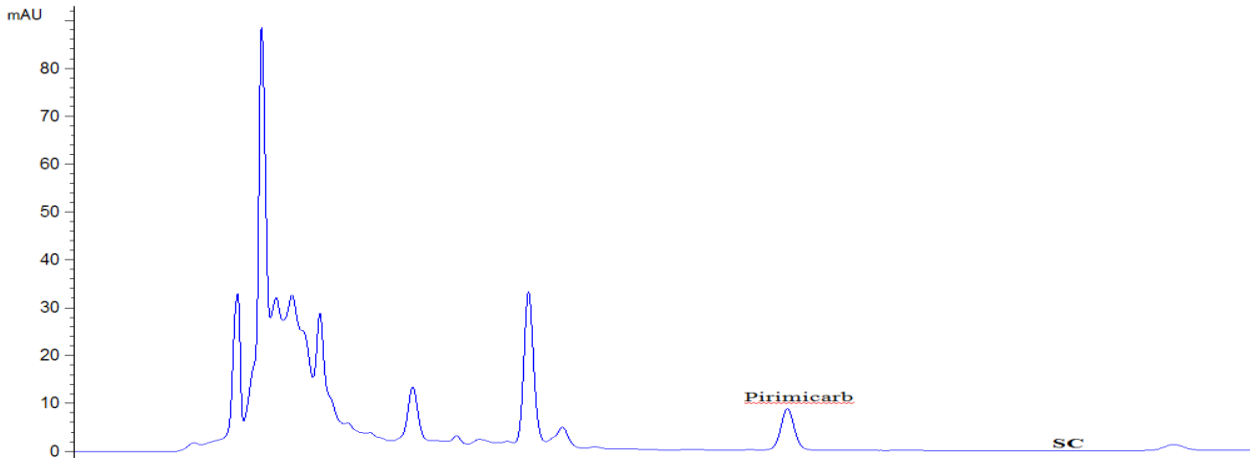
C. Preliminary study of the determination of pirimicarb in spiked fresh leafy vegetables using the imprinted polymers

To demonstrate the suitability and potential application of this proposed method, different types of vegetables; including brassica chinensis var parachinenis, cabbage, broccoli, cauliflower, and lettuce; were purchased from a local market to investigate the pirimicarb residues on vegetables; the extraction procedure was described in the preceding **section 4.2.7 (C)**. In the first hand, we did not find any detection of pirimicarb residues on those vegetables (blank samples) via our method; therefore, the accuracy of the method was evaluated through spiking with three varying concentrations (1, 3.33 and 10 µg. g^{-1}); the determination of spiked concentration was compared with an external standard of pirimicarb. The results eventually revealed that the accuracy of recoveries from samples was obtained in a range of 88.23 - 97.54%, and the relative standard deviations (RSD) values were less than 5.07% in real sample analysis (**Table 4.1)**. **Fig. 4.7** showed chromatograms of the selective recoveries after spiking with pirimicarb on vegetables on HPLC analysis. To sum up, the proposed method was very potential to be employed for the determination of trace pirimicarb residues in fresh leafy vegetable samples.

Table 4.1. Determination of pirimicarb recoveries from spiked vegetables (n=4)

Vegetables	**Analyte**		
	Pirimicarb		
	1 µg. g^{-1}	3.33 µg. g^{-1}	10 µg.g^{-1}
Brassica chinensis var parachinenis	88.23	94.75	95.01
Cabbage	97.54	95.01	96.74
Broccoli	95.91	92.71	92.12
Cauliflower	91.83	96.36	92.33
Lettuce	90.49	95.19	94.03


mAU
80
70
60
50
40
30
20
10
0
BC


mAU
80
70
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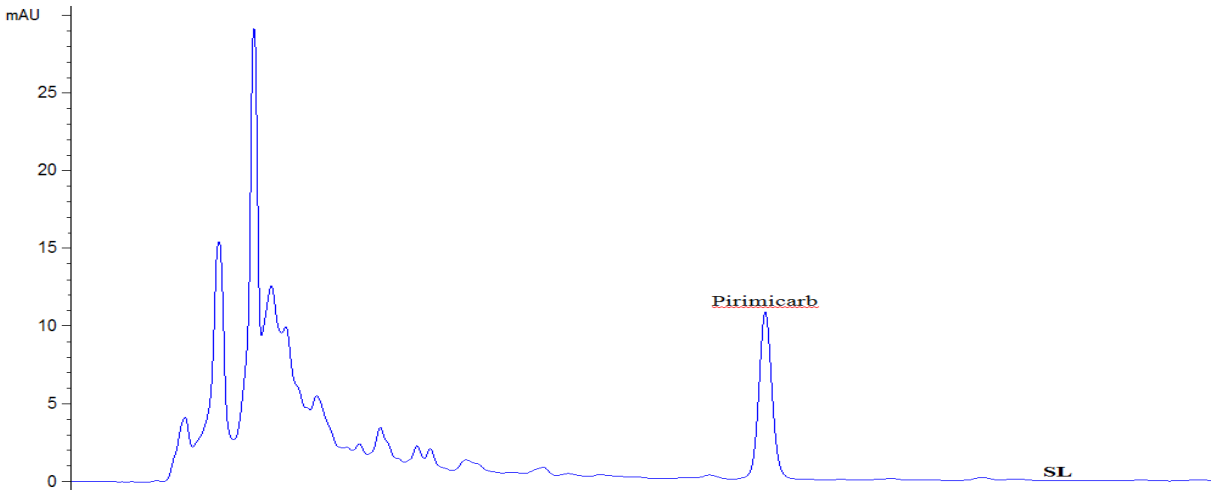
Pirimicarb
SC


mAU
25
20
15
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5
0
Pirimicarb
SL

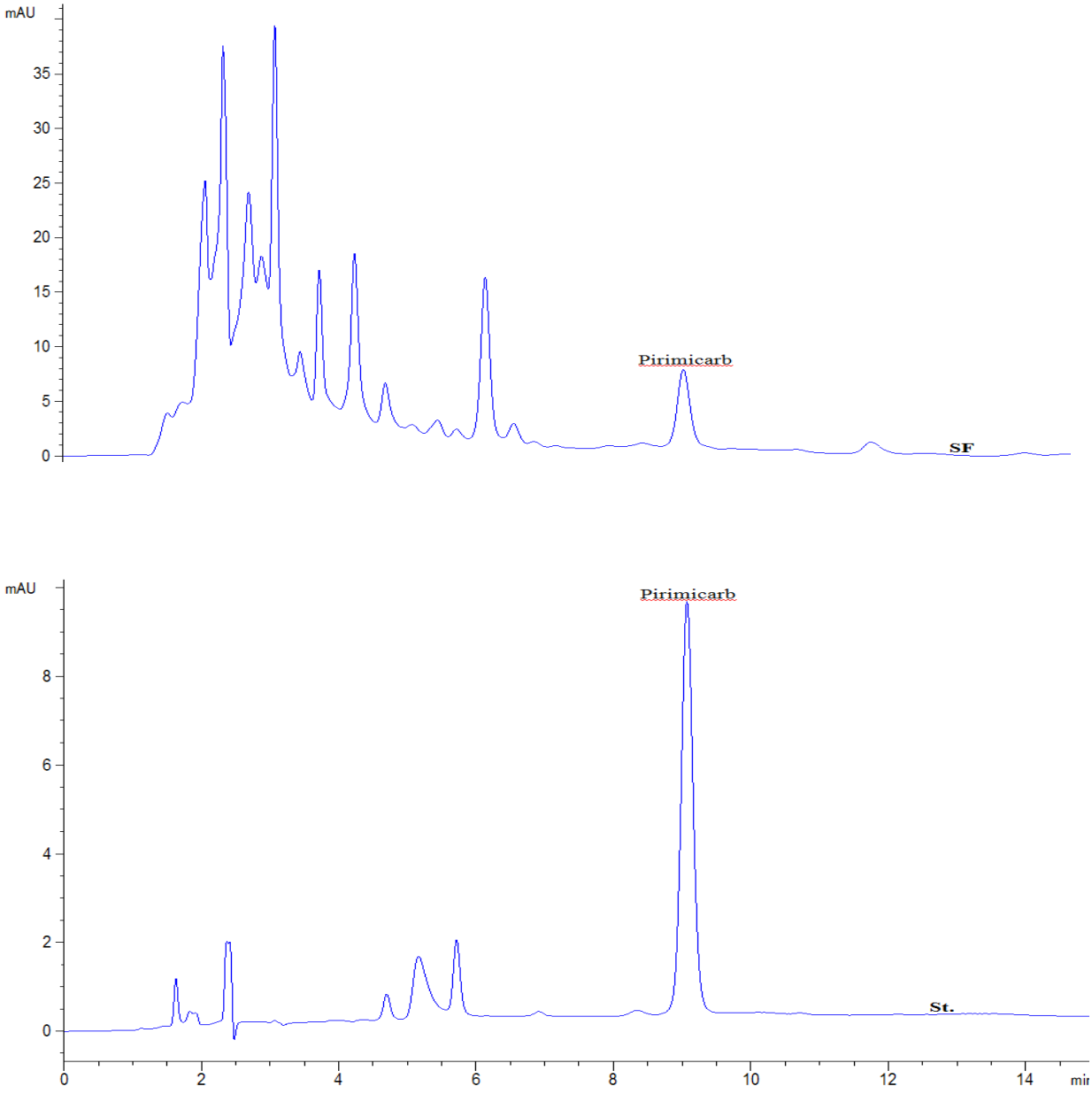

Fig. 4.7. Chromatograms on high performance liquid chromatography: St, working standard solution (3.33 μg. g^{-1}); SF, spiked cauliflower; SL, spiked lettuce; SC, spiked cabbage; BC, blank cabbage eluate of M-MAA (n=4).

4.4. Conclusion

Pirimicarb-imprinted polymers were synthesized by bulk polymerization techniques. The major binding interaction between template and functional monomers were formed by hydrophobic effects due to the fact that the adsorption solvent mostly contributed by water, which generated more micro-environmental hydrophobicity. The combination of allyl-β-

CD and MAA for creation of MIPs illustrated more effective and efficient than that of allyl-β-CD and AN; this led us to draw a conclusion that MAA functional monomers are more applicable than its correspondent referenced counterpart AN for MIPs fabrication. Our study found that the significant difference between MIPs and NIPs could demonstrate only in suspension method, rather than in MISPE procedure because in suspension method we used 20 mg polymers in 0.2 mg. mL^{-1}, causing the polymers to saturate while in MISPE we increased up to 50 mg polymers in 0.2 mg. mL^{-1} or less, which did not cause polymers to saturate. The lower amount of polymers was involved, the higher significant difference between MIPs and NIPs was statistically demonstrated. The MIP in this proposed method is simple, quick, and cost-effective to prepare, and requires small quantities of solvents for efficient isolation and enrichment of analyte in the analytical procedure.

CHAPTER 5 STATE-OF-ART APPLICATIONS OF CYCLODEXTRINS AS FUNCTIONAL MONOMERS IN MOLECULAR IMPRINTING TECHNIQUES: A REVIEW

5.1. Introduction

Due to a versatile tool of cyclodextrins in separation science, the cyclodextrins and their derivatives as functional monomers have been used extensively in imprinting techniques; therefore, we decide to separate the review of cyclodextrin into a single chapter in a purpose to emphazing the usefulness of cyclodextrin in imprinting technique. Molecularly-imprinted polymers containing either cyclodextrin or its derivatives demonstrated superior binding effects for a target molecule. The main goal of this review is to illustrate the exotic applications of imprinting techniques employing cyclodextrin and its derivatives as single or binary functional monomers in synthesizing molecularly-imprinted polymers in areas of separation science by reviewing some of the latest studies reported in the literature.

Scientists all around the world are innovating new technologies and re-inventing existing ones in the never-ending quest to attain lower concentration levels of different analytes in many diverse, highly complex environmental matrices (Betran et al., 2010). In the field of separation science, molecular imprinting techniques are one of the most promising methodologies that have been widely used for synthesizing of potentially artificial receptors for chemical isolation, enrichment and analysis in a wide range of applications, for example, it could be utilized in solid-phase extraction (SPE), chromatographic separation, catalysis, binding assays and sensor amongst others (Xu, Kuang, Feng, & Zhang, 2010). The attractiveness of molecularly-imprinted polymers (MIPs) can be attributed to their stability, durability, reliability, and economic cost of production, making MIPs gain more and more popularity. The major compositions involved in the construction of MIPs are a template molecule (analyte), a functional group, and a cross-linker. **Fig. 5.1** illustrates a general view of the MIPs polymerization.

There are three types of cyclodextrins (CDs), including α (alpha)-cyclodextrin, a 6 membered sugar ring molecule; β (beta)-cyclodextrin, a 7-membered sugar ring molecule; γ (gamma)-cyclodextrin, an 8-membered sugar ring molecule, enabling them to form a cone shape by a number of glucose monomers ranging from six to eight units in a ring (**Fig.**

5.2). Of the three types in terms of purification in water solubility, β-CD molecule is very poorly water-soluble (18.5 g/l, at 25^0C) and could be easily retrieved through crystallization, meanwhile α- and γ-CDs (145 and 232 g/l, respectively) are more soluble, normally requiring a purification by means of expensive and time-consuming chromatography techniques. Cyclodextrins (sometimes its so-called cycloamyloses) are a family of compounds composed of sugar molecules bound together in a ring (cyclic oligosacchrides). Cyclodextrins, all generally recognized as safe by the Food and Drug administration (FDA), are produced from starch by means of enzymatic conversion. They are utilized in food, pharmaceutical, drug delivery, and chemical industries, as well as agricultural and environmental engineering (https://en.wikipedia.org/wiki/Cyclodextrin). In the last decade, cyclodextrins have been frequently exploited in enantiomer separation and drug delivery systems (Tsai & Syu, 2005; Singh, Sharma, & Ranerjee, 2002; Loftssona & Jarvinen, 1999; Sing Muk Ng & R. Narayanaswamy, 2009; Shi, Wu, Qu, Li, & Zhang, 2007; Challa, Ahuja, Ali, & Khar, 2005) due to their unique property to form inclusion compounds with chemical properties of guest molecules or other small ones. The characteristics of cyclodextins are hydrophobic inside while having hydrophilic outside cavity, which enable them to form non-covalent host-guest inclusion complexes with organic and hydrophobic compounds (Tsai & Syu, 2005; Challa, Ahuja, Ali, & Khar, 2005; Connors, 1997; Hapiot, Tilloy, & Monflier, 2006).

In imprinting techniques, amongst the three kinds of cyclodextrins, β-cyclodextrin has been popularly used to prepare molecularly-imprinted polymers because its chemical structure is more suitable in chemical separation and discrimination abilities (Tsai & Syu, 2005; Jian & Kuo, 2000), as seen in **Table 5.1**. Basically, β-cyclodextrin (β-CD) and its derivatives were typically synthesized as either a single (Tsai & Syu, 2005; Zhong, Byun, & Robert Bittman, 2001; Xu et al., 2008) or binary functional monomer (Xu, Kuang, Feng, & Zhang, 2010; Kang, Duan, Li, Kang, & Xie, 2012; Xu, Kuang, Liu, & Deng, 2007; Chen, Chen, & Chung, 2007). **Table 5.1** summarizes the single and binary functional monomers of cyclodextrin in molecular imprinting techniques. The application of adsorbents containing cyclodextrins in separation science have additionally been reviewed elsewhere (Crini, & Morcellet, 2002; Schneiderman, & Stalcup, 2000). It is worthwhile noting that the functional monomers of β-CD and its derivatives have been utilized in molecular imprinting for selective separation with a wide range of chemical compounds, including steroidals (Zhong, Byun, & Robert Bittman, 2001; Cheng, Jiang, Lin, Li, & Dong, 2014; Asanuma, Kakazu, Shibata, Hishiya, & Komiyama, 1998; Asanuma, Hishiya, &

Komiyama, 2000; Davidson, & Hayes, 2002), amino acids (Liu, & Chen, 2006; Qin, He, Li, & Zhang, 2008; Liu, Fang, & Yu, 2013), polysaccharides (Wang & Zhang, 2010), drugs (Xu, Kuang, Feng, & Zhang, 2010; Xu, Kuang, Liu, & Deng, 2007), plant hormone (Zhang, et al., 2010), proteins (Bossi, Bonini, Turner, & Piletsky, 2007; Zhang, et al., 2009), pesticides (Xu, Kuang, Feng, & Zhang, 2010), and plastic additives (Kang, Duan, Li, Kang, & Xie, 2012). Therefore, we mainly decided to compile the analysis of previously published literatures in imprinted polymers attaching a single β-CD or a combined β-CD functional monomers with the aims of highlighting the achievement to date in the development of analytical applications in the review article.

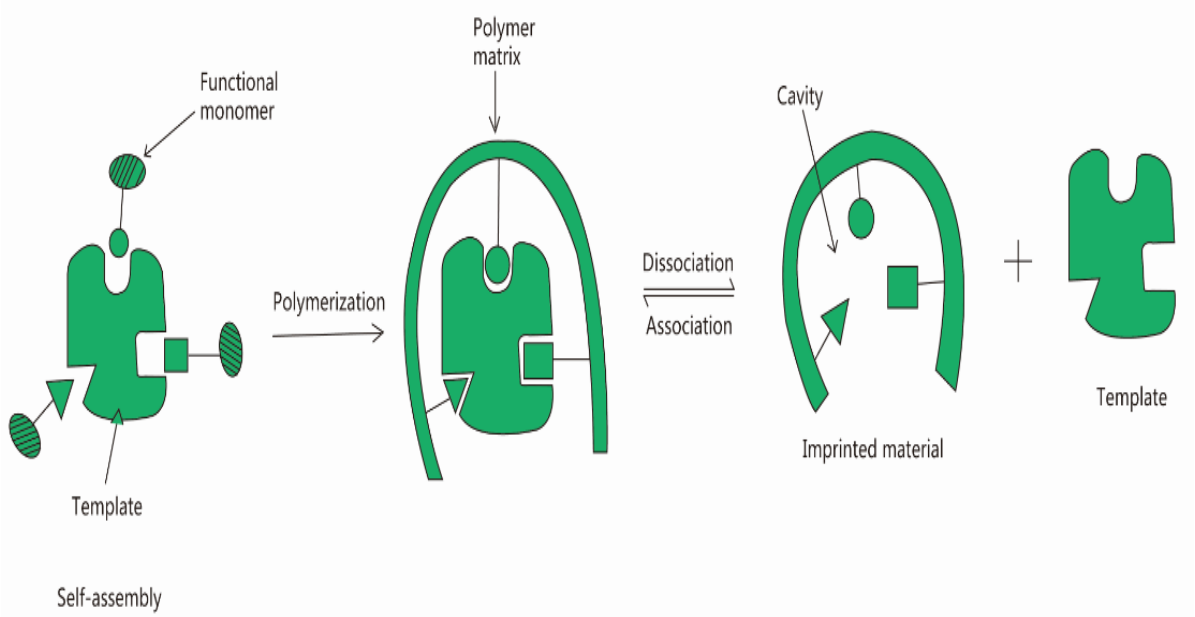

Fig. 5.1. The general view of schematic demonstration of prepared MIPs and rebinding for template.

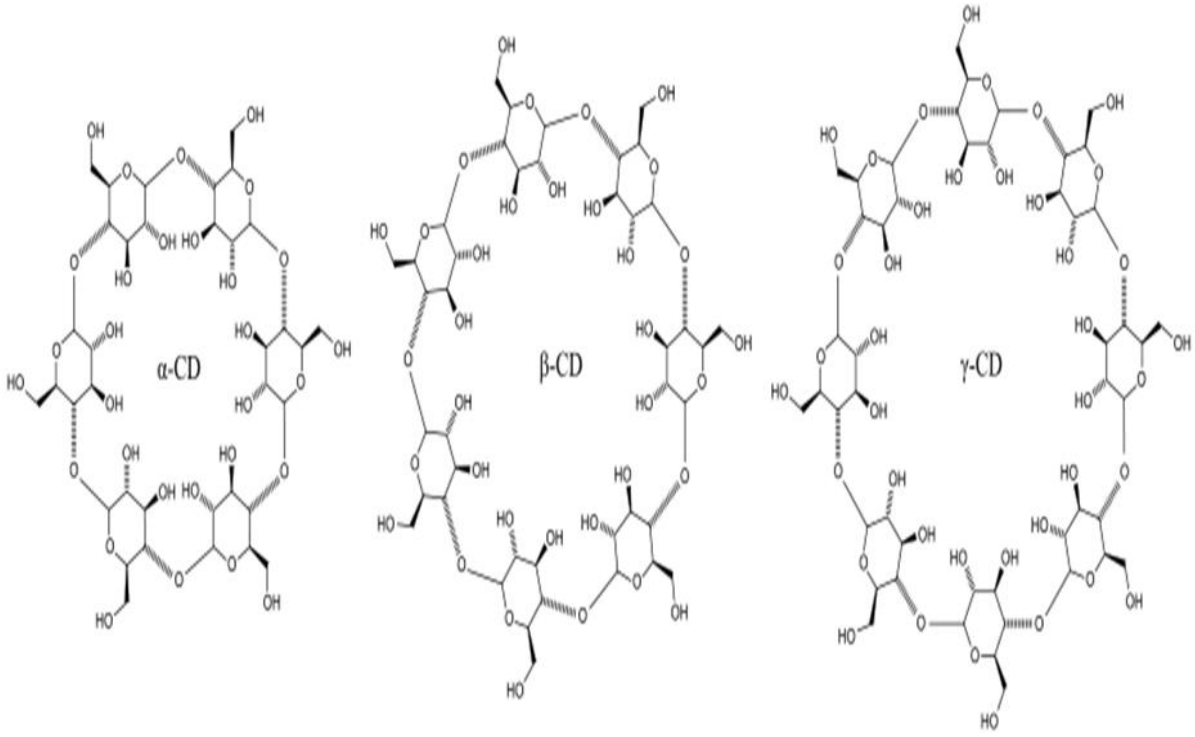

Fig. 5.2. Chemical structures of the three main types of cyclodextrins (Source: https://en.wikipedia.org/wiki/Cyclodextrin)

Table 5.1: The summarization of the single and binary functional monomers of cyclodextrins for MIP synthesis in the field of separation science

β -cyclodextrin (β -CD) /its derivative	Combined monomers	Templates	Cross linkers	Adsorption solvent	Ref.
β-CD	-	4,4'-(1,4-phenylenediisopropylidene) bisphenol (BPP)	Diphenylmethan diisocyanate (DDI)	Methanol + water (80 + 20%)	Xu et al. (2008)
β-CD	-	Creatinine	Epichlorohydrin	Water	Tsai & Syu (2005)
β-CD	-	Cholesterol or stigmasterol	Toluene 2,4-diisocyanate (TDI)	Water + Tetrahydrofuran (5/6, v/v) or Water + Methanol (5/6, v/v)	Hishiya et al. (1999)
β-CD	-	Steroids	Toluene 2,4-diisocyanate (TDI)	Water	Asanuma et al. (2004)
Acryloyl-6-O- α -D-glucosyl- β -CD (Acryloyl-G1-β-CD)	-	Various antibiotics and oligopeptides	N,N'-methylenebiacrylamide (MBAA)	5 mM Tris buffer, pH 8.0	Asanuma et al. (2001)
β-CD	-	N-phenyl-1-naphthylamine (NPN)	Toluene 2,4-diisocyanate (TDI)	Methanol	Sing Muk Ng & Narayanaswamy (2009)
Functionalized Silica-β-CD	-	4-nitrophenol and 2,4-dinitrophenol	-	Water	Yi Fan et al. [2003]
Acryloyl-6-amino- 6-deoxy-β-CD or γ-CD (AABCD or AAGCD)	-	Cholesterol	N,N'-diacryloylpiperazine (DAPA)	2-Propanol (2-PrOH)	Zhong et al. (2001)
Dansyl-modified β -CD (β -CD-en-DNS)	-	Cholesterol	Hexamethylene diisocyanate (HMDI)	Tetrahydrofuran: Water (6/4, v/v)	Cheng et al. (2014)
Bismethacryloyl- β -cyclodextrin (MBA-β-CD)	2-(diethylamide) ethylmethacrylate (DEAEM)	Norfloxacin (NOF)	Ethylene dimethacrylate (EDMA)	Methanol + water (50 + 50%)	Xu et al. (2007)
Allylic bromine-β-cyclodextrin (allyl-β -CD)	Methacrylic acid (MAA)	Dipentyl phthalate (DPP)	Ethylene dimethacrylate (EDMA)	Hexane	Kang et al. (2012)
Bismethacryloyl- β -cyclodextrin (BMA-β-CD)	4-vinylpyridine (4-VP)	Acifluorfen	Ethylene dimethacrylate (EDMA)	Methanol and water (50 + 50%)	Xu et al. (2010)
Functionalized Silica derivative-linked β-cyclodextrin	Acrylamide (AA)	Triptophan	N,N'-methylenebiacrylamide (MBAA)	Phosphate buffer solution (0.01 mol/L Na_2HPO4 and 0.01 mol/L NaH_2PO4)	Qin et al. (2008)
Functionalized Silica beads- acryloyl-β-cyclodextrin	Acrylamide (AA)	Lysozyme (Lys)	N,N'-methylenebiacrylamide (MBAA)	Phosphate buffer solution (0.01 mol/L Na_2HPO4 and 0.01 mol/L NaH_2PO4, pH 7.0)	Zhang et al. (2009)
Silylated-β-CD	4-vinylpyridine (4-VP)	Indole-3-acetic acid (IAA) hormone	Trimethylolpropane trimethacrylate (TRIM)	-	(Zhang, et al., 2010)

5.2 Cyclodextrin (its derivative)-based imprinted polymers

The following collates information about the single functional group of cyclodextrin (its derivates) in the assembly of MIPs from the previously published reports.

Xu et al., (2008) synthesized three molecularly imprinted polymers (MIPs) by exploiting β-cyclodextrin molecule as a functional monomer by bulk polymerization technique for achieving recognition for 4,4'-(1,4-phenylenediisopropylidene) bisphenol (BPP) in aqueous media **(Fig. 5.3)**. The imprinted polymers were prepared in a two-step method. Firstly, authors polymerized MIPs, and secondly they used chlorotrimethylsilane (CTMS) to cap polymers prior to using Soxhlet apparatus with methanol to remove the excess CTMS and extracted the template. According to the result of binding experiments, the MIPs can bind the template of BPP template selectively. Moreover, the authors suggested that the major contribution of MIPs made from β-cyclodextrin functional monomers to the recognition ability of the imprinted polymer is the stereo-shape effect inherent, while the hydrophobic effects are an important role in the recognition process. The synthetic MIPs cross-linked with diphenylmethan diisocyanate (DDI) is more effective than ones crossed-linked with toluene 2,4-diisocyanate (TDI) for imprinting BPP, while Asanuma et al. (1998) found that TDI is more effective cross-linker than hexamethylene diisocyanate for cholesterol, whereas Zhong et al. (2008) indicated that the imprinted polymeric beads prepared with trimethylolpropane trimethacrylate (TRIM) provided the perfect balance of flexibility and rigidity, and better uniform spherical shape than either ethylene glycol dimethacrylate (EGDMA) or divinylbenzene (DVB). From the different viewpoints of results, it can be implied that different templates required different cross-linkers for efficient imprinting ability of MIPs. Apart from this, the authors evaluated the influence of water on the binding of BPP on the polymers by using various ratios of water and methanol as adsorption solvents. They found that constantly increased water content while decreasing methanol in mixed solvent promoted the binding performance because the cavity of β-CD is relatively hydrophobic compared with water, so once the water content increased in the solvent, the hydrophobic effect also increased, more templates could be driven into the cavities of the polymers. Finally, the authors concluded that MIPs were superior to NIPs because there are two interactions forming in MIPs: the imprinting proccess and unspecific binding interaction, whereas there is only one interaction in NIPs, unspecific interaction.

Tsai and Syu (2005) used β-cyclodextrin as a functional monomer, creatinine as a template, and epichlorohydrin as a cross-linking agent to synthesize MIPs with different molar ratios of monomer to template in order to investigate which molar ratio was the most optimal one for polymerization. The authors also used a two-step bulk polymerization method. The imprinted polymers were first polymerized and then they were capped with chlorotrimethylsilane (CTMS). According to the results, the better specific adsorption ability towards the template (creatinine) was achieved once a molar ratio of monomer to template of 3:2 and monomer to cross-linker of 1:10 was matched. The authors suggested that the MIPs made of β-CD illustrated stereo-shape recognition ability and hydrogen binding force for creatinine template. These findings could be inferred that various combinations of molar ratios in MIP polymerization showed varying abilities of binding adsorption in MIPs towards template.

Hishiya et al. (1999) also exploited β-cyclodextrin (β-CD) and either cholesterol or stigmasterol as the template cross-linked by toluene 2,4-diisocyanate (TDI) in dimethyl sulfoxide (DMSO) solvent to prepare MIPs. The author and coworkers proposed that the binding mode of imprinted polymers and template was strongly formed by the apolar alkyl chain at 17-position (17-alkyl residues) and the OH residue at the 3-position (3-OH residue) of the template (cholesterol, stigmasterol) with the OH residue of β-CD, resulting in regulating the depth of penetration of the template in the imprinted cavities of polymers. They also proposed that any steroids contained 17-alkyl residues (cholesterol, stigmasterol, and 4-cholesten-3-one) are bound more strongly than those without the residues (testosterone and progesterone), meanwhile the 3-OH of steroids contributed only a minor role in the binding to the polymers. Moreover, regarding the polymerization solvent, use of sole dry DMSO yielded much more desirable imprinting effects than DMSO/pyridine (1:1, v/v) mixture because the inclusion complex formation is competitively inhibited by the pyridine constituent. On the other hand, several studies were reported elsewhere using a functional monomer of β-cyclodextrin (β-CD) or its derivative and a template of cholesterol (Asanuma, Kakazu, Shibata, Hishiya, & Komiyama, 1998; Asanuma, Kakazu, Shibata & Hishiya, 1997; Asanuma, Hishiya, & Komiyama, 2004].

Asanuma et al., (2004) prepared MIPs by bulk polymerization protocol using a functional cyclodextrin cross-linked with toluent 2,4-diisocyanate (TDI) in dimethyl sufoxide (DMSO) in the presence of hydrophobic biomolecules as templates, and eventually the synthesized polymers were directly applied to the MIPs-packed column stationary phases of high performance liquid chromatography (HPLC) for evaluation. A

significant difference of imprinted binding activity was noted between synthetic polymers formed with the presence of template and synthetic polymers formed without the template; for example, the capacity factor of binding affinity sharply increased from 0.1 to 2.05 when the polymerization was performed in the presence of cholesterol template, and related findings were also presented upon the use of stigmasterol template in place of cholesterol: the capacity factor for stigmasterol rose from 0.42 to 2.5 under the same experimental condition. Furthermore, the authors pointed out that using α-CD as a host molecule in place of β-CD did not work out at all; this led to a conclusion that guest molecules were bound to the β-CD created by the imprinting, not to the cavity created by the cross-linking agents in the polymers. This result is contrary to the previous work studies, reporting that the inclusion complexation resulting from the presence of β-CD and the physical adsorption in polymeric networks introduced by the combined epichlorohydrin cross-linker in the sorption mechanism (Crini et al., 1998). Regarding binding interaction, Asanuma (2004) emphasized that water used as an integral part of eluent was indispensible in inducing the capacity factor since the main driving force for the binding of the guest molecule is hydrophobic interaction, not hydrogen bonding or other interactions, for the inclusion complex formation. Hence, with increasing water while decreasing acetronitrile content in eluent, the increasing selectivity towards the template (cholesterol) was obtained. Ultimately, there are two key points that the authors concluded. First, the synthesized imprinted polymers of β-CD monomer can be successfully applied to the stationary phases of HPLC. Second, the synthesized MIPs can efficiently promote the binding affinity and substrate selectivity towards the target analytes compared to the non-imprinted polymers (NIPs). The proposed method has an advantage in constructing artificial receptors for the chemicals that are water immiscible.

Asanuma et al., (2001) chose water as imprinted surface porosities to study the molecular imprinting of cyclodextrin (β-CD or α-CD) for the recognition of nanometer-scaled guests; the authors synthesized various types of imprinted polymers made of different templates: antibiotics (vancomycin, cefazolin, phenethicillin) and oligopeptides (d-phe-d-phe, l-phe-l-phe, phenylac-phe). To improve the solubility in water, the authors used 6-O-α-D-glucosyl-β-cyclodextrin (G1-β-CD) instead of β-CD to build the vinyl monomer of cyclodextrin (acryloyl-G1-β-CD), which was then cross-linked by N,N'-methylenebiacrylamide (MBAA) for imprinted polymer synthesis. In the guest-binding experiments, the authors demonstrated that the adsorbed amount of synthesized MIPs made of G1-β-CD is more efficient than that of α-CD; for example, vancomycin-acryloyl-G1-β-

CD imprinted polymers could uptake up to 44% of their total contacted template while cefazolin-acryloyl-α-CD imprinted polymers could bind 17% of the total template. On the account of oligopeptide-imprinting, the authors observed that eminent imprinting effect was achieved once hydrophobic moieties (benzene rings) in the oligopeptide were close to imprinted sites, whereas the binding activity of antibiotics-imprinting formed mostly by rigidly fixed imprinting inside MIPs. Compared to the author's previous works in which the template and β-CD were dissolved in dry DMSO in addition of di-isocyanate as a crosslinker (Asanuma, Kakazu, Shibata, Hishiya, & Komiyama, 1998; Asanuma, Kakazu, Shibata, & Hishiya, 1997; Hishiya, et al., 1999), the authors revealed the advantages over the old one as follows: (1) Inclusion complex configuration of CD with a template is much stronger in water than in DMSO, (2) Versatile constituents consisting of substituted carboxylate group (-COO-) or radical amino (-NH2) are existing as templates, whereas these templates are not presented for the previous imprinting in DMSO, (3) All preparatory steps of polymers required no organic solvents. All in all, molecularly-imprinted polymers were successfully prepared in bulk water by use of the vinyl monomers of CD (acryloyl-G1-β-CD).

Apart from the potential application of the molecularly-imprinted solid-phase extraction (MISPE) technique, MIPs could also be exploited as sensors (Nguyen, Hardwick, Sun, & Grattan, 2012; Huy, Seo, Zhang, & Lee, 2014; Liu, Zheng, & Li, 2013; Suriyanarayanan et al., 2012; Rachkov et al., 2000; Blanco-López et al., 2004; Piletsky & Turner, 2002). Numerous articles related to β-cyclodextrin-based imprinting polymers' sensing applications or its derivative as functional sensor were also reported elsewhere (Sing Muk Ng & R. Narayanaswamy, 2009; Cheng, Jiang, Lin, Li, & Dong, 2014; Roche, Ng, Narayanaswamy, Goddard, & Page, 2009; Culha, Lavrik, Schell, Tipple, & Sepaniak, 2003; Shahgaldian, Hegner, & Pieles, 2005). For instance, one of the notably published reports used β-cyclodextrin as a functional monomer, cross-linking toluene 2,4-diisocyanate (TDI), and N-phenyl-1-naphthylamine (NPN) as a template to construct molecularly imprinted polymers as a potential optical receptor for the detection of organic compounds (Sing Muk Ng & R. Narayanaswamy, 2009). It is unusual that the authors used only high-polar methanol as analyte solvent, while other reports usually preferred to use non-polar or low-polar organic solvent for binding affinity. The authors found that the intensity of fluorescence emission spectrum was associated with the activity of binding performance of MIP towards NPN template through batch rebinding analysis. They drew a conclusion from results that the synthetic MIPs efficiently promoted a better sensing signal

by increasing the binding affinity and substrate selectivity to the template molecule as compared to non-imprinting polymers (NIPs), and moreover, the synthesized sensing receptor of MIP possessed a robust usability without any significant decay in intensity with relative standard deviation (RSD) value of 2.24 (n=13), reflecting that the MIPs have an outstanding stability for recycling state. Alternatively, another separate example of a recent research article was reported by Yang Chen et al. (2014). The authors exploited dansyl modified-β-cyclodextrin (β-CD-en-DNS) as a functional monomer to fabricate a chemosensory polymeric cholesterol-imprinted membrane for the analysis of non-fluorescent organic compounds. They demonstrated that forming a complex with a template molecule (cholesterol) by inclusion interaction in an imprinted membrane of a fluorescence chemosensor generated a much greater optical response towards the template than its structurally-related chemical analogues (stigmasterol, estradiol, phenol).

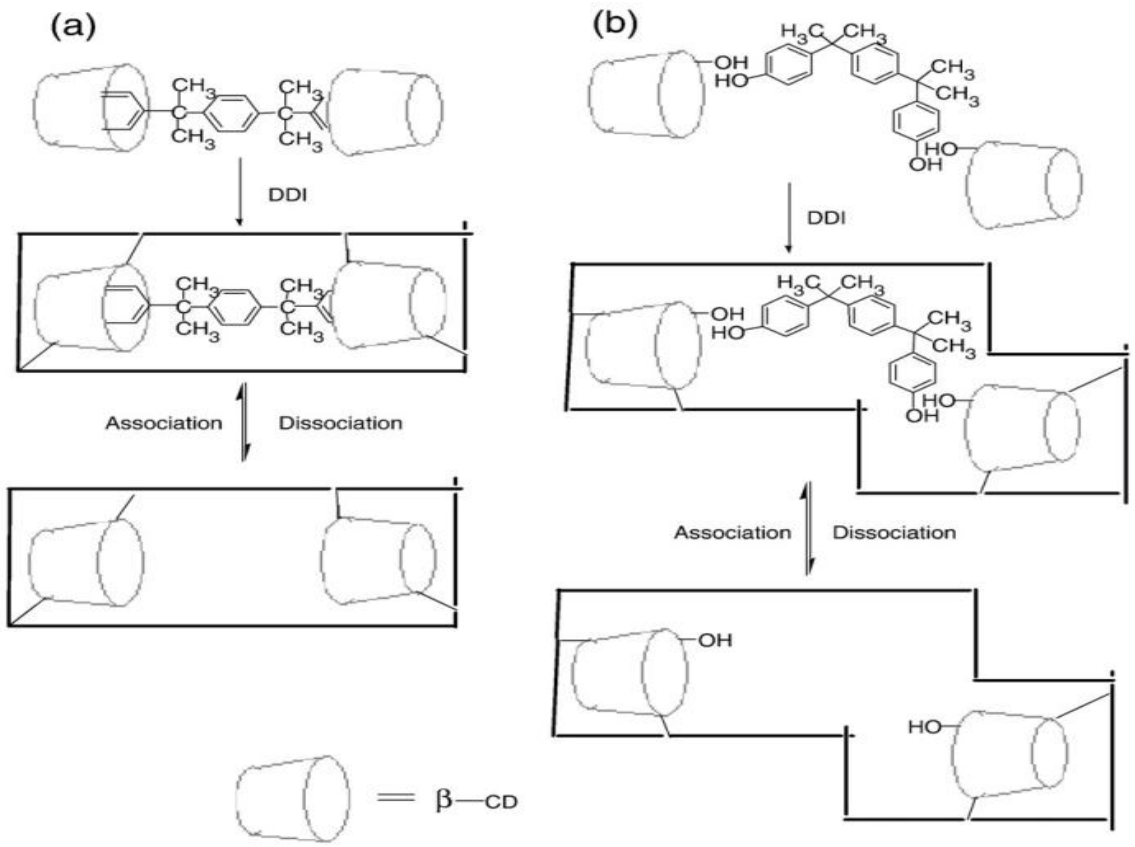

Fig. 5.3. Schematic representation of the two possibly positional preparation processes of single functional monomers of molecularly imprinted polymers and mechanisms of binding specificity (Adapted from Ref. Xu et al., 2008).

5.3 Combination of cyclodextrin (its derivative) and other functional monomers

The following collates information about the combined use of cyclodextrin (its derivatives) and other functional monomers as a binary functional group in the assembly of MIPs from previously published reports.

Zhong et al. (2001) conducted a study on hydrophilic cholesterol-binding molecular imprinted polymers. The author and co-workers used either acryloyl-6-amino-6-deoxy-β-CD (AABCD) derivatives from 6-deoxy-6amine-β-CD (BCD) and acrylic acid (AA) or acryloyl-6-amino-6-deoxy-γ-CD (AAGCD) derivatives from 6-deoxy-6amine-γ-CD (GCD) and acrylic acid (AA) to prepare cholesterol-binding imprinted polymers. The authors found that the binding amount uptake of imprinted polymers (38-50 μmol/g) was approximately 6-fold higher than that of non-imprinted control polymers (6-9μmol/g) according to the binding data illustration. Of the two synthesized polymers, the binding adsorption capacity of AAGCD imprinted polymers was shown to be slightly more than that of AABCD imprinted polymers. In all, the author could give a conclusion that the larger cavity monomer of γ-CD apparently gave a more favorable factor for binding cholesterol template in the polymer matrix.

Xu et al. (2007) attempted to construct MIPs with strong affinity and high specificity toward a fluorinated quinolone, norfloxacin (NOF) through a combination of hydrophobic and electrostatic interaction based on bulk polymerization method. Quinolones are a class of antibiotics commonly utilized to prevent and treat a large variety of infectious diseases in human and veterinary medicine. The authors synthesized norfloxacin-imprinted polymers by a combined use of bismethacryloyl-β-cyclodextrin (BMA-β-CD) and 2-(diethylamino) ethyl1methacrylate (DEAEM) as functional monomers. They found that MIPs using only BMA-β-CD or DEAEM as a functional group were shown to be less effective than that of a combination of BMA-β-CD and DEAEM imprinted polymers in binding affinity and specificity for NOF in aqueous media. In conclusion, the authors demonstrated that the combination of hydrophobic effect and electrostatic interaction between β-CD and adsorption in molecular imprinting process was indispensible for the improvement of the selective ability of the imprinted polymers and the binary functional monomers were superior to the single one in term of MIPs fabrication.

A more recent article of allylic bromine-β-cyclodextrin (allyl-β-CD) derivative combined with methacrylic acid (MAA) as the binary functional monomers to synthesize a dipentyl phthalate-imprinted polymer was reported by Kang et al. (2012). Phthalic acid

diesters (phthalates) are commonly used as a plastic additive to increase the flexibility, transparency, durability and longevity of polyvinyl chloride (PVC) plastics such as food packaging products, medical devices, and toys. They are highly lipophilic (fat soluble) and not chemically bound to the PVC matrices, hence they easily leach out, migrate, or evaporate into the atmosphere and body from the plastic-derived materials when heated, used, or disposed of (Staples, Peterson, & Parkerton, 1997). The phthalates are suspected agents of endocrine disruption by means of carcinogenesis (Naarala & Korpi, 2009; Benson, 2009; Blandeau, 1999), and hormonal and reproductive malfunction (Poon et al., 1997; Foster, Mylchreest, Gaido, & Sar, 2001; Duty et al., 2005). The results displayed that (MAA-allyl-β-CD)-MIPs have specific recognition selectivity and excellent adsorption affinity for a template of dipentyl phthalate (DPP). Moreover, the authors indicated that the newly synthesized (allyl-β-CD)-MIPs showed greater adsorption capacity and better selective adsorption than the MIPs prepared by a conventional method (He, Lv, Zhu, & Lu, 2010).

Xu et al. (2010) employed a combination of bismethacryloyl-β-cyclodextrin (BMA-β-CD) derivative and 4-vinylpyridine (4-VP) as bi-functional monomers for an imprinted polymer synthesis of acifluorfen herbicide via bulk polymerization technique **(Fig. 5.4)**. The authors comparatively studied between a binary functional monomer MIP and a single functional monomer MIP (BMA-β-CD or VP). According to the findings, compared with a single monomer of MIPs, a binary was shown stronger selective binding capacity and imprinting factor for acifluorfen. Moreover, a binary functional MIP could effectively separate an acifluorfen template from its structural analogs. **Fig. 5.4** showed the schematic illustration of the molecular imprinting procedure drawn by the authors. The authors indicated that the combination of hydrophobic effect and electrostatic interaction in imprinting process is a feasible approach for achieving molecular recognition in aqueous media.

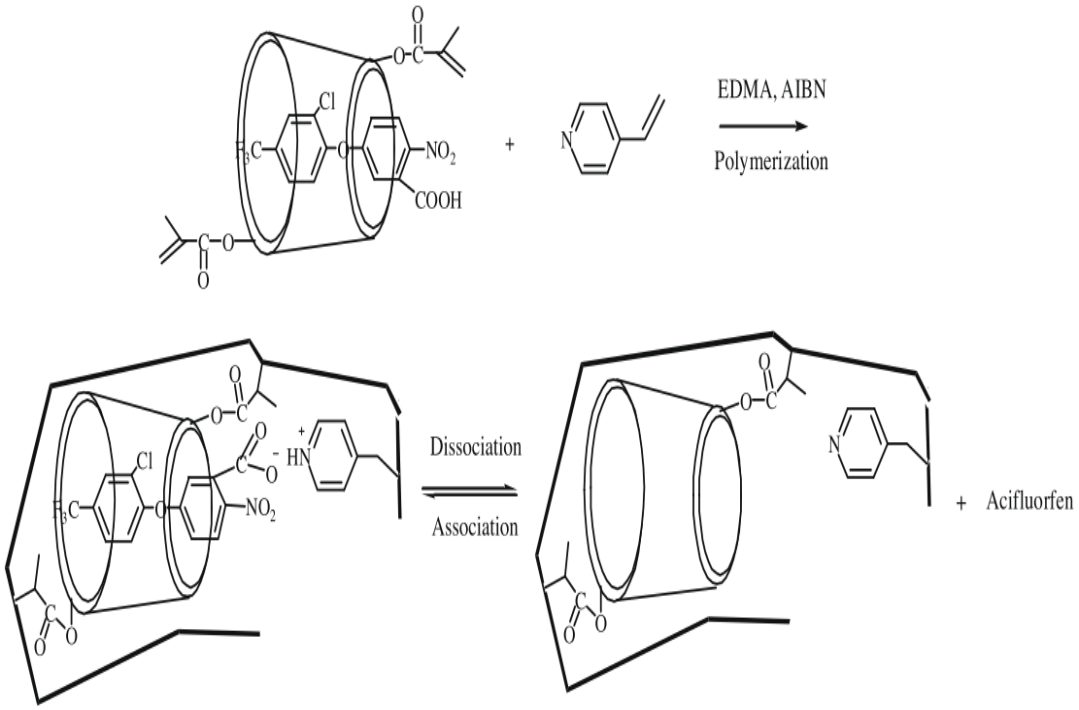

Fig. 5.4. Schematic illustration of the 3-D structure of binary functional monomers of molecularly imprinting procedure (Adapted from Ref. Xu et al., 2010).

Qin et al. (2008) polymerized a series of molecularly-imprinted polymers using single functional monomer which is either bonded β-cyclodextrin (β-CD) or acrylamide (AA) monomer and binary functional monomer which is in combination of bonded β-cyclodextrin (β-CD) and acrylamide (AA) monomers on functionalized silica gel for selective recognition of tryptophan in aqueous media. The author used two methods to assess the adsorption capability of synthesized imprinted polymers: suspension method and MIP-packed-HPLC column. The authors pointed out that the efficient selectivity recognition ability of MIPs made of bonded β-CD and AA monomers was superior to that of MIP made of a single bonded β-CD or AA monomers based on the illustrated results of adsorption experiments. The authors concluded that the bonded β-CD and AA-linked-MIP-packed HPLC column could separate the tryptophan template from its enantiomer in aqueous mobile phase. In addition to this, another separate study on a β-cyclodextrin (β-CD) bonded silica as a selective sorbent was also reported elsewhere (Fan, Feng, Da, & Feng, 2003; Feng, Xie, & Da, 2000). Fan and coworkers synthesized a β-cyclodextrin (β-CD) bonded functionalized silica by using a convenient method and evaluated on the basis of the solid-phase extraction for 4-nitrophenol (4-NP) and 2,4-dinitrophenol (2,4-DNP).

They discovered an addition of inorganic salt (NaCl) to the eluting solution was vital in the formation of an inclusion binding complex between β-CD and analytes. Finally, the authors concluded that the synthesized sorbent attributed a strong capacity to both adsorpbate (4-NP and 2,4-DNP); the recovery rate was in a range of 96% and 99%, respectively, with a 1 L water sample.

Zhang et al. (2009) successfully fabricated with acryloyl-β-CD and acrylamide (AA) as binary monomers and protein lysozyme (Lys) as template on the surface of silica beads to form a unique kind of protein-imprinted polymer **(Fig. 5.5)**. In the binding equilibrium experiment, the authors showed that the hydrogen bonding and hydrophobic reaction between AA-linked acryloyl-β-CD and template (lysozyme) in the immobilized imprinted cavities could improve the efficient adsorption capacity of MIPs almost five times as much as that of the NIPs corresponding counterpart. In the binding selectivity, the overall amount of uptake of template bound to MIPs was higher than that of its closely structural analogues (cytochrome (Cyt), bovine serum albumin (BSA), avidin (Avi), and methylated bovine serum albumin (MBSA)). Moreover, in the study of on-line chromatographic stationary phases, the authors employed the chromatographic stainless steel column (100 mm x 4.6 I.D) packed with AA-linked acryloyl-β-CD MIP and NIP to evaluate their imprinting effect. The results showed that MIP-packed column could separate the template (Lys) from its protein mixtures. In short, the MIP was stronger than that of NIP since NIP could not generate specific recognition sites due to the absence of template protein.

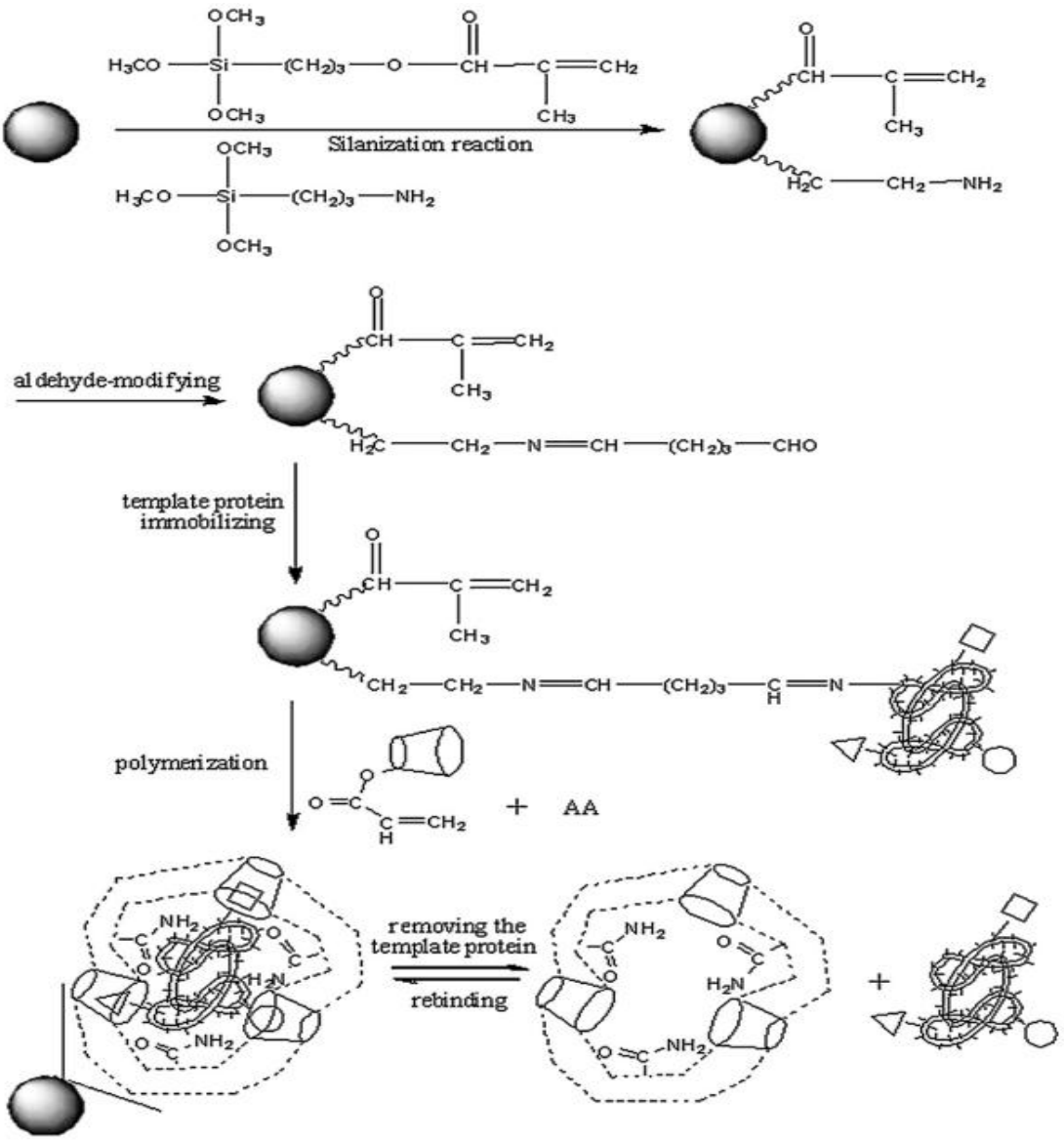

Fig. 5.5. Schematic illustration of the proposed protocol for synthesis of the protein-imprinted polymers (Adapted from Ref. Zhang et al., 2009).

5.4 Conclusion

The versatile tool of cyclodextrins in separation science has made cyclodextrins as functional monomers gain more popularity in the fabrication of imprinting polymers. All the authors claimed the same results of inclusion complex formation between cyclodextrins and templates; the major contribution of MIPs made from β-cyclodextrin to the recognition ability of the imprinted polymer was the stereo-shape effect inherent, while the hydrophobic effects have an important role in the recognition process rather than hydrogen

bonding. Moreover, several findings proved that the quality of adsorbed efficiency of β-CD MIPs is better than that of α-CD (Asanuma, Hishiya, & Komiyama, 2004; Asanuma et al., 2001); therefore, β-CD is perceived as most attractive in MIP fabrication for researchers. More interestingly, the reports which compared studies between single and binary functional monomers of β-CD, claimed that binary (poly-) functional monomers are superior to its single monomers in term of imprinted effects (Xu, Kuang, Feng, & Zhang, 2010; Xu, Kuang, Liu, & Deng, 2007; Qin, He, Li, & Zhang, 2008; Zhang, Li, Hu, Li, & Chen, 2010). Amongst the adsorption solvents, either sole water or water-mixed solvents were the most widely used in β-CD-MIPs (**Table 5.1**). To our knowledge, trio functional monomers in MIP fabrication have not yet been synthesized; maybe it could create another possibility of MIP polymerization.

CHAPTER 6 OVERALL CONCLUSION AND FUTURE PROSPECTS

6.1 Overall Conclusion

Growing food safety concerns have seriously threatened the public heath, living well-beings, and socio-economic development; hence, food safety should place one of the top national interests in governmental policies. To ensure food-is-safe from farm to table is a huge challenge because it requires an active involvement from multiple parties: farmers, food manufacturers, governmental authorities. Farmers are the primary food producers because all agricultural products basically come from them. Afterwards, agricultural foods need to be engineered in the factories producing a variety of semi or final products in order to satisfy diverse demands for consumers. Government is the regulators of the policies and also the supervisors to ensure that all food production chains are conformity with the standard, law and regulations. Even though how much effort of government have put, food scandals related to food poisonings are inevitable because it is impossible to get 100 per cent in detection of food hazards, particularly pathogenic microbes and toxic chemicals. However, with advents of sophistically efficient analytical instruments and methods, we can properly prevent, reduce, or even eliminate all the possible food toxins before consumption.

Molecular imprinting technique is one of the promising methods of identification and quantification of a chemical molecule of interest in food or environmental matrices. This technique has drawn a huge interest in the field of chemical separation science. An exploitation of molecular imprinting technology could produce a product of molecularly imprinted polymers (MIPs). MIPs; physically known as its robustness, long-term stability, reliability, cost-efficiency, and selectivity; have drawn a considerable attraction amongst academia and scientific community. There are a variety of useful applications of MIPs such as off-line or on-line solid phase extraction (SPE), chemical and bio-sensors, catalysis, and drug delivery because MIPs, a synthetically potential artificial receptor-like binding sites with a “memory” for shape and functional group positions of the target molecule, possess a competent ability for selective specificity and recognition for target chemical molecules. Despite various MIPs applications, the most commonly used is an off-line solid phase extraction application due to its simplicity. Utilizations of MIPs-based materials have been applied to a wide range of chemicals, including food contaminants, pesticides, environmental pollutants, preservatives, and antibiotic drug residues for sample clean-up

and pre-concentration, detection, and quantification. A wide range of synthetic MIPs protocols, including bulk, precipitation, multi-step swelling, suspension, emulsion, dispersion, gelation polymerization, have been successfully developed to meet the increasing demands for analytical molecular methods and clinical medicines. Despite the drawbacks of bulk polymerization technique, it is still the most commonly-used protocol for obtaining MIPs synthetic product.

In this thesis, we exploit a molecular imprinting technique to produce a product of molecularly imprinted polymers (MIPs). We synthesized MIPs based on bulk polymerization technique with a two-step process of polymerization, which is a novel development method by our group. The bulk polymers were crushed and ground with a mortar and pestle into fine powder, and then sieved into free-flowing powder through a 400 mesh steel sieve. Fine particles were collected and washed with warm water to minimize possible contaminants. They were subsequently Soxhlet extracted with either methanol and acetic acid (9:1, v/v) or methanol until no template was detected by UV-vis spectrophotometry before the polymers could be used for our experiment. There are two different methods that we employ to evaluate our synthetic polymers: suspension method and off-line molecularly imprinted solid-phase extraction (MISPE) method before HPLC determination. According to our results, we found that suspension method showed more significant values between molecularly imprinted polymers (MIPs) and non-imprinted polymers (NIPs), whereas MISPE did not; this is because we used different amount of polymers in these two methods: suspension method (20 mg) and MISPE (50 mg). Moreover, we found that water plays an important role in binding affinity when CD functional monomers are involved in the MIPs fabrication. The increasing water content in adsorption solvent, the increasing selectivity towards the template was achieved. Finally, Our synthetic MIPs could be applied as a solid phase sorbent to real sample pre-treatment as a sample clean-up, extraction, and enrichment through MISPE procedure because they had strong binding capabilities towards analytes of interest. We have developed the new method of bulk polymerization technique with the two-step reactive process. The resultant imprinted polymer could adsorpt the templates (DEHP, CLEN, and pirimicarb) effectively and efficiency. The affinity of binding force between the polymers and templates were mostly contributed from the hydrophobic effects because mixed adsorption solvent largely came from water as water induced hydrophobicity. To sum up, our synthesized MIPs made of β-CD derivative as functional monomers proved to be a useful tool for modern chemical separation and food safety analysis.

In conclusion, the improvement of analytical method technique of detection of a chemical molecule of interest in food matrices, especially in terms of selectivity and sensitivity, is strongly demanded from public sectors in order to better supervise and control food quality and safety. MIPs are one of the promising analytical tools that could fulfill those demands due to their multi-functional facets in the area of separation science. In short, the development of our current method of MIPs construction and MISPE protocol is simple, rapid, and cost-effective.

6.2 Future prospects

1. Our present studies are mainly focus on the bulk polymerization, known as traditional technique, for MIPs synthesis for food sample pre-treatment and analysis; therefore, we encourage further studies to synthesize MIPs by different techniques such as precipitation, multi-step swelling, suspension technique, etc. in order to find out various ways in MIPs fabrication.

2. We evaluate our MIPs based on off-line molecularly imprinted solid-phase extraction (MISPE) method for sample clean-up and enrichment. One of the alternative promising ways is to use an online HPLC column-packed imprinted polymer chromatography for direct detection and quantitation, resulting in more time- and labor-savings.

3. Because our laboratory is unable to facilitate highly sophisticated instruments such as HPLC-MS, HPLC-MS/MS, GC-MS, etc.; therefore, this is one of our probable research constraints to get a very low amount of detection and accuracy. Regarding this aspect, we suggest other researchers to try to employ those instruments in place of only HPLC machine.

4. Besides MIPs use for sample clean-up and enrichment, one of our main interests in MIPs is drug or bioactive compound delivery for disease treatment. Our synthetic method for MIPs fabrication is reliable and efficient in binding high amount of analytes; hence, we can exploit it as a drug delivery to transport therapeutic drug to particular place of tissue for disease treatment, particularly cancer disease.

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