# CHAPTER I

# INTRODUCTION

## 1.1 Molecularly Imprinted Polymers (MIPS)

### 1.1.1 Historical concept of MIPs

Molecularly imprinted polymers (MIPs) are cross-linked organic polymers containing specific functionalized recesses making from the addition of template molecules in the polymerization process. The artificially generated recognition sites contained in the cavities are able to specifically rebind a target molecule in preference to other closely related compounds.[1] MIPs are developed under the basic of antibody formation theory.[2] From the efforts to produce the antibody *in vitro* which later was called bio-imprinting, the molecular imprinting approach was then performed by the antibody formation on silica base. The resulting sodium silicate polymer was prepared in the present of a dye. It was found that the silica prepared in present of a dye would bind the pattern molecule in rebinding experiment, after the dye was subsequently removed. The rebinding mechanism involved is based on molecular recognition. Therefore, they are often called synthetic antibodies in comparison with immunosorbents. Indeed, both have comparable selectivities but the imprinting polymers offer better handling and stability as well as cheaper and easier to prepare.

The molecular imprinting using organic polymers was firstly prepared by Wulff and Klotz research groups in 1972.[2] To obtain the fixed distance between functional monomer and template, the reversible covalent bonds for monomer-template

interaction was used in Klotz's work while Wulff interested in the molecular imprinting of metal ion. Since these initial works, MIPs used for several classes of compounds have been tailored.

Owning to their recognition property, these materials have found widespread use in several fields. Most of the works so far have involved the development of MIPs in sample preparation by using the polymers as sorbent in solid phase extraction for cleaning-up the analyte from sample matrices and/or enhancing the concentration of the target analyte prior to analysis.[3] The application of MIPs in separation of mixtures by using the polymers as solid phase adsorbents for liquid chromatography becomes another famous application of MIPs in the adsorbent field. Moreover, the MIP approach was investigated through out for bioassay[4-6], biosensor[7-10], organic synthesis[11-13] and catalysis[12-14] application.

### 1.1.2 Molecular imprinting procedure

As MIPs are the synthetic polymeric materials with specific cavities designed for a template molecule. The imprinting procedure is generally based on the linkage of suitable monomers containing functional groups to template molecule by chemical interactions, which can be described as follows; the functional monomer was firstly connected to the template and become template functional monomer complex. Next, these complexes were frozen by polymerization with cross-linker leading to a highly cross-linked three-dimentional net work. Once the polymerization has taken place, the template molecule was extracted and binding sites with shape, size and functionalities complementary to the target analyte was established. The schematic diagram of polymerization procedure is shown in **Figure 1.1**.

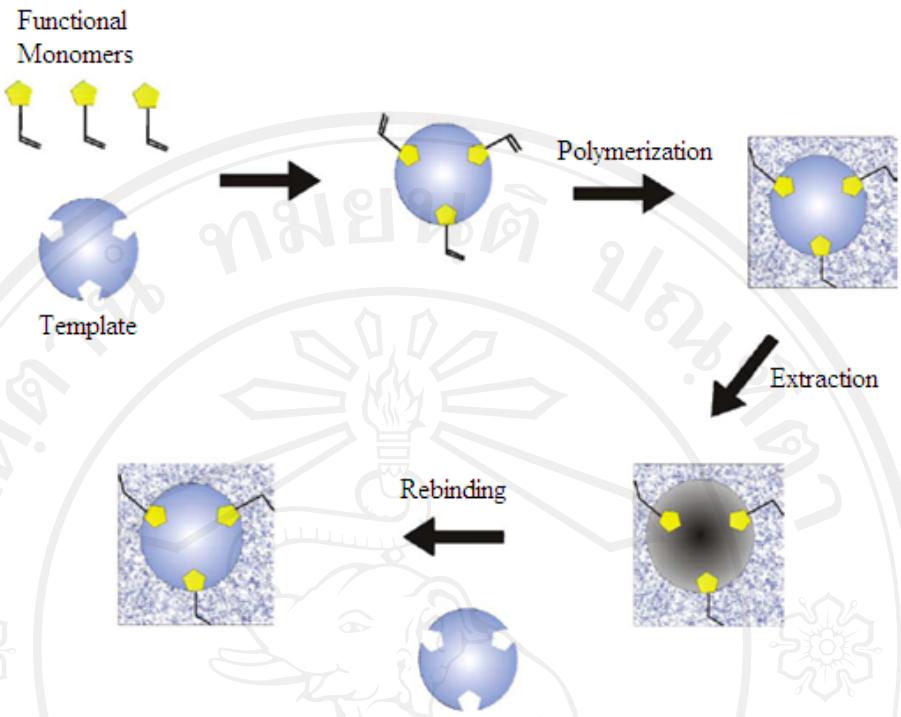



**Figure 1.1** Schematic representation of the molecular imprinting procedure[15]

The resulting imprinted polymers are stable, robust and resistant to a wide range of pH, solvents and temperature. Therefore, the behavior of MIPs emulates the interactions established by natural receptors to selectively retain a target molecule (i.e. antibody-antigen) but without the associated stability limitations. In addition, it is important to point out that MIPs synthesis is also relatively cheap and easy, making them a clear alternative to the use of them as natural receptors.

Molecularly imprinted polymers (MIPs) may be prepared according to a number of approaches that are different in the way template is linked to the functional monomer and subsequently to the polymeric binding sites. Three different approaches to prepare MIPs have been reported which are covalent, non-covalent and semi-covalent approaches (**Table 1.1**).

**Table 1.1** Approaches for imprinting molecules

| Imprinting approach | Imprinting process | Rebinding process |
|---|---|---|
| Covalent |  |  |
| Non-covalent |  |  |
| Semi-covalent |  |  |

Where

T means Template

M means Functional monomer

☐ means Covalent interaction

⬚ means Non- covalent interaction

**1.1.2.1 Covalent imprinting approach**

In covalent approach, the chemical interaction between the template and functional monomers before polymerization are reversible covalent bonds such as carboxylic ester, boronic ester and Schiff base. The templates are then removed by the cleavage of the corresponding covalent bonds. In rebinding performance, the corresponding covalent bonds are re-formed with the target analytes. In this approach, the stability of template-monomer interaction is high. The functional monomers can be employed in the exact stoichiometric ratio to the template molecule. Therefore, homogenous population of binding site is found and the non-specific sites are not

existed. However, the difficulty of designing as appropriate template-monomer complex in which covalent bound formation a cleavage are readily reversible under mild conditions makes this approach rather restrictive. The choice of template and functional monomers are limited.

**1.1.2.2 Non-covalent imprinting approach**

The non-covalent approach is based on the formation of non-covalent interactions between template molecule and the functional monomer such as hydrogen bonding, π-π interactions, hydrophobic or van der Waals interaction and ionic or electrostatic interactions. In this way, the functional monomers are added into the template solution to perform the non-covalent interaction between the template and functional monomer by self-assembly before polymerization. This approach is by far the most used for the preparation of MIPs since they are usually more easily employed. The experimental procedure is rather simple and a wide variety of monomers able to interact with almost any kinds of template are commercially available. However, with non-covalent interactions the binding constants are generally rather low compare to covalent interactions so that the imprinting procedure requires the functional monomers to be present in large excess. Therefore, a high amount of monomer is used in this approach. The binding sites in the resulting polymer thereafter are not exclusively located inside the cavities. The resulting non-specific binding sites become a main drawback derived from this synthetic approach. Accordingly, for the applications that the high specificity is necessary required such as catalytic and preparative chromatographic purposes, this approach should be avoided.

#### 1.1.2.3 Semi-covalent imprinting approach

It has already been mentioned that covalent binding during the imprinting procedure has the advantages of stability and exact stoichiometry, whereas readily reversible non-covalent interactions are better suited for the equilibration of the free cavities with the substrate. This intermediate option is the semi-covalent approach. In this case, the template is also covalently bound to a functional monomer. The template was then removed by cleavage of the covalent bond, same as the covalent approach. After the template removal, the functional monomers are left only in the specific cavities which can give the template rebinding based only on non-covalent interactions. Under this concept, the exact binding sites can be produced and the rebinding procedure is easier. However, the disadvantage of this approach is often the stability of the some covalent bond, which is responsible for splitting percentages of only around 20%. Furthermore, some problems arise when considering that different kinds of interaction during imprinting and rebinding imply different bond distances between the binding sites in the cavity and the template molecule. For this reason, the choice of functional monomers and templates are still limited.

### 1.1.3 Constituents of MIP synthesis[16]

The challenge of designing and synthesizing a molecularly imprinted polymer (MIP) can be a daunting prospect to the inexperienced practitioner, not least because of the sheer number of experimental variables involved, the nature and levels of template, functional monomer(s), cross-linker(s), solvent(s) and initiator, the method of initiation and the duration of polymerization. Fortunately, a good number of “rules of thumb” have emerged in the literatures that are helpful in this regard, however

most of these literatures is based on the basics of free radical polymerization processes, the commonly polymerization method in MIPs synthesis. In the following, attention is drawn to a number of factors pertaining to the template molecule and the selection of suitable functional monomers, cross-linkers, solvents and initiators for general polymerization procedures.

**1.1.3.1 Template**

In all molecular imprinting processes the template is of central importance in that it directs the polymerization of the functional groups pendent to the functional monomers. For a variety of reasons, not all templates are directly amenable to use as template in the MIP production. In terms of compatibility with free radical polymerization, templates should ideally be chemically inert under the polymerization conditions, thus alternative imprinting strategies may have to be required if the template can participate in radical reactions or is, for any other reasons, unstable under the polymerization conditions.

The criteria for consideration of template are as follows:

(1) The template should not contain the functional groups which are polymerisable.

(2) The template should not contain functionality that could potentially inhibit or retard a free radical polymerization such as a thiol group or a hydroquinone moiety.

(3) The template should be stable at moderately elevated temperatures (for example, at or around 60 ºC if AIBN is being used as the chemical initiator) or upon exposure to UV irradiation.

**1.1.3.2 Functional monomers**

The molecular imprinting step is crucial for obtaining optimal selectivity; specific cavities must be designed for the template molecule. For this reason, the monomers are chosen considering their ability to interact with the functional groups of the template molecule. Overall, binding sites are created which have a memory for the template both in terms of shape and matching functionality. Functional monomers are responsible for the binding interactions in the imprinted binding sites and, for non-covalent molecular imprinting protocols, are normally used in excess relative to the number of moles of template to favor the formation of template, functional monomer assemblies (template to functional monomer ratios of 1:4 and upwards are rather common for non-covalent imprinting). It is clearly very important to match the functionality of the template with the functionality of the functional monomer in a complementary fashion such as hydrogen bond donor with hydrogen bond acceptor, in order to maximize complex formation and thus the imprinting effect is high. However, it is also important to keep in mind the reactivity ratios of the monomers to ensure that copolymerization is feasible. Incidentally, it is also worth noting that complexation of a template by a functional monomer can also influence the reactivity of the monomer to some extent, as a result of perturbations to the electronics and/or the steric of the monomer. Scores of functional monomers with chemically diverse structures and polarities are commercially available and many more can be prepared by rational design. The chemical structures of the more important functional monomers classified by functionality of the function are shown in **Figure 1.2**.

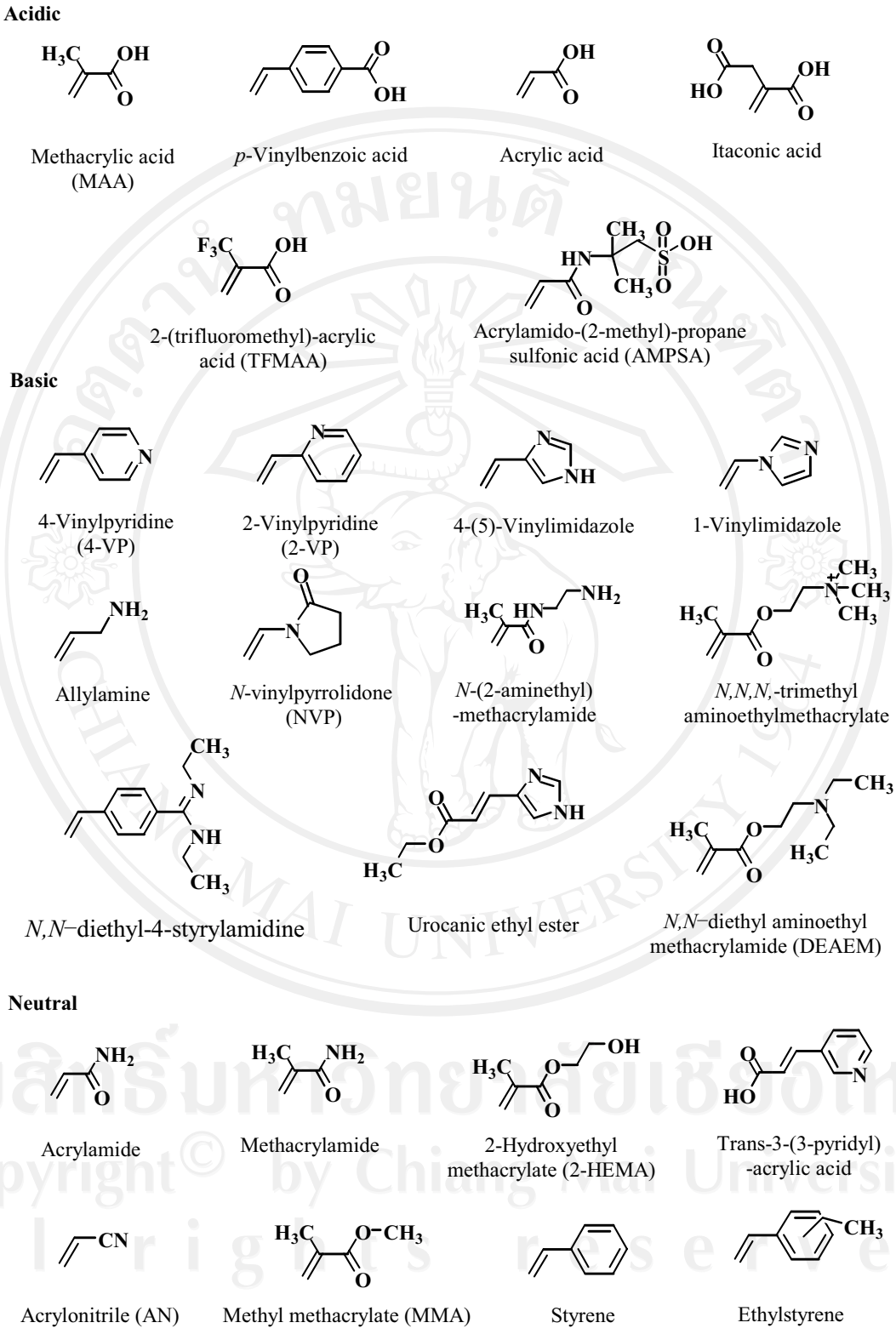



**Figure 1.2** Chemical structures of commonly used functional monomers in MIPs

### 1.1.3.3 Cross-linkers

In an imprinted polymer the cross-linker fulfils three major functions. First of all, the cross-linker is important in controlling the morphology of the polymer matrix, whether it is gel-type, macro-porous or a micro-gel powder. Secondly, it serves to stabilize the imprinted binding site. Finally, it imparts mechanical stability to the polymer matrix. Much has been written about the effect of the cross-linker on the molecular recognition behavior of imprinted polymers, but from a polymerization point of view, high cross-link ratios are generally preferred in order to access permanently porous (macro-porous) materials and in order to be able to generate materials with adequate mechanical stability. Polymers with cross-link ratios in excess of 80% are often in general. For the same reason that one should match the reactivity ratios of functional monomers in a mixture of polymerization to ensure smooth incorporation of the co-monomers, the reactivity ratio of the cross-linker should ideally also be matched to that of the functional monomer(s). The reactivity ratios of cross-linkers may not be known in which case approximations can sometimes be made through studying the values of structural analogues. It should also be borne in mind that there may well be chemically distinct vinyl groups in multi-functional monomers with distinct reactivity ratios, for example different vinyl groups may be incorporated at differential rates into the polymer. Quite a number of cross-linkers compatible with molecular imprinting are known, many of which are commercially available and a few of which are capable of simultaneously complexing with the template and thus acting as functional monomers. The chemical structures of several well-known cross-linkers are shown in **Figure 1.3**.

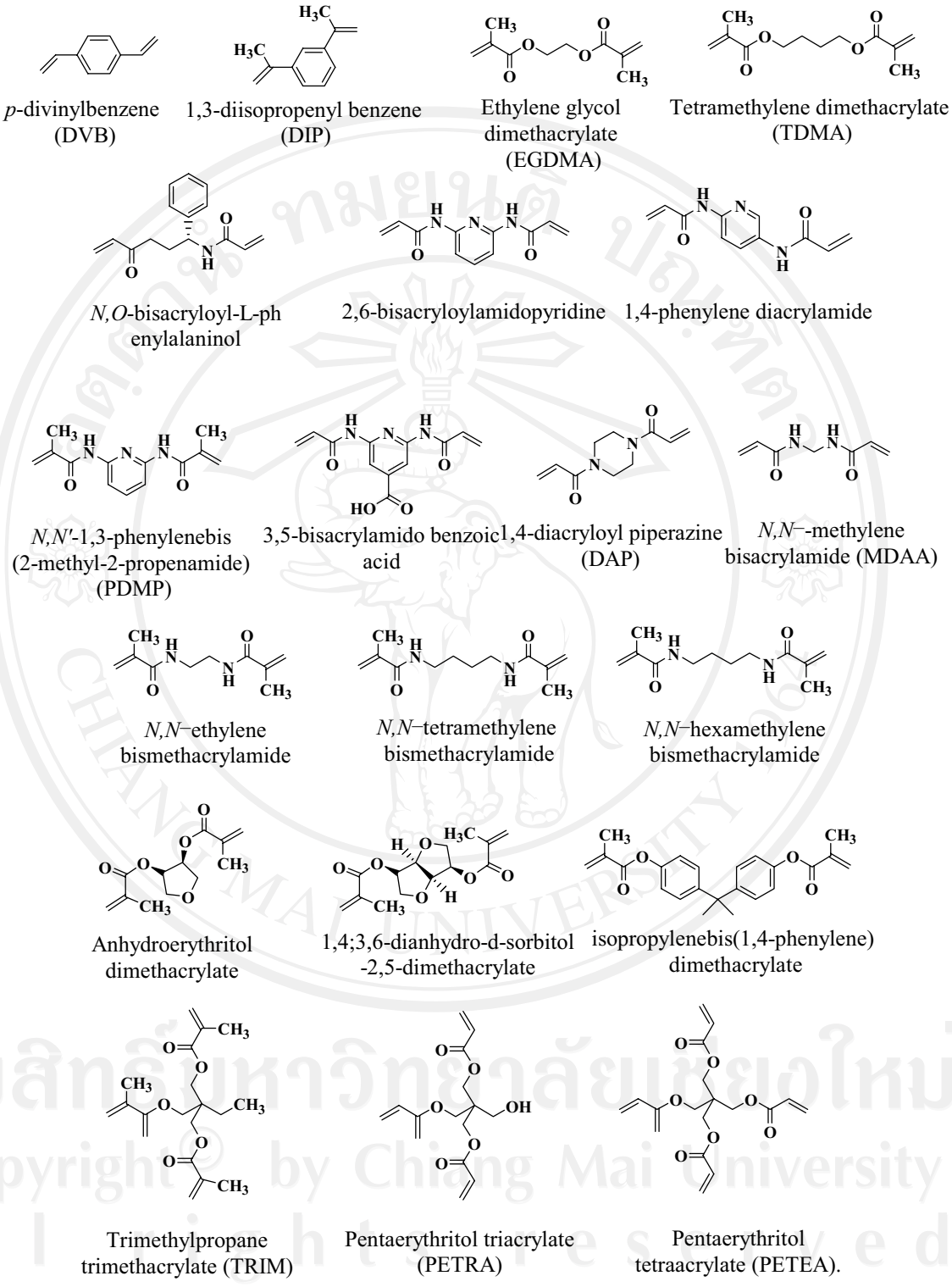



**Figure 1.3** Chemical structures of commonly used cross-linkers in MIPs

**1.1.3.4 Solvents (Porogens)**

The solvent serves to bring all the components in the polymerization such as template, functional monomer(s), cross-linker and initiator into one phase. However, it serves a second important function in that it is also responsible for creating the pores in macro-porous polymers. For this reason it is quite common to refer to the solvent as the "porogen". When macro-porous polymers are being prepared, the nature and the level of the porogen can be used to control the morphology and the total pore volume. More specifically, use of a thermodynamically good solvent tends to lead to polymers with well developed pore structures and high specific surface areas, use of a thermodynamically poor solvent leads to polymers with poorly developed pore structures and low specific surface areas. Increasing the volume of porogen increases the pore volume.

Besides its dual roles as a solvent and as a pore forming agent, the solvent in a non-covalent imprinting polymerization must also be judiciously chosen such that it simultaneously maximizes the likelihood of template, functional monomer complex formation. Normally, this implies that apolar, non-protic solvents, e.g. toluene, are preferred as such solvents stabilize hydrogen bonds, however if hydrophobic forces are being used to drive the complexation then water could well be the solvent of choice.

**1.1.3.5 Initiators**

In principle, any of the methods of initiation described earlier can be used to initiate free radical polymerizations in the presence of templates. However, there may well be drivers for selecting one over another arising from the system under study.

For example, if the templates were photochemically or thermally unstable, the intiators which can be triggered photochemically and thermally, respectively, would not be attractive. Where complexation is driven by hydrogen bonding then lower polymerization temperatures are preferred, and under such circumstances photochemically active initiators may well be preferred as these can operate efficiently at low temperature. The chemicals structures of free radical initiators generally used in MIPs are shown in **Figure 1.4**.

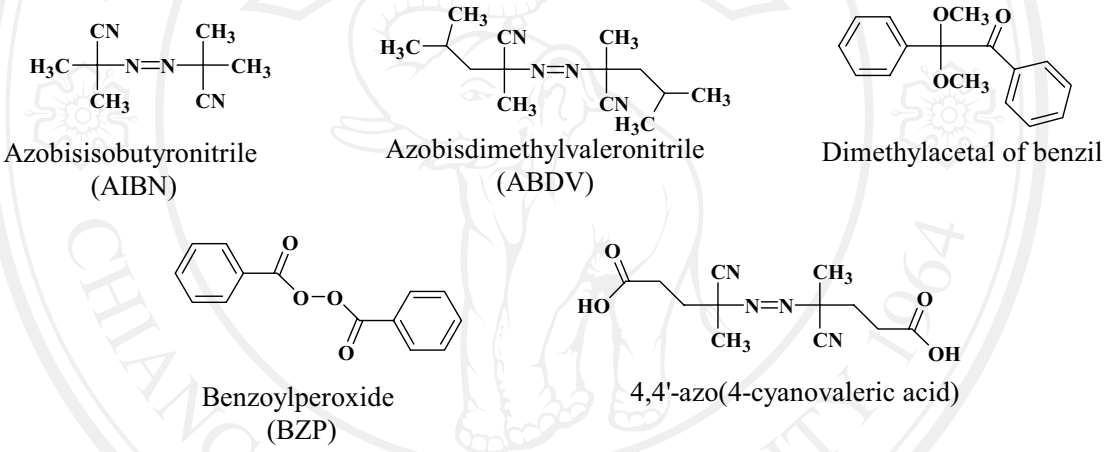



**Figure 1.4** Chemical structures of commonly used initiators in MIPs

## 1.2 Solid phase extraction

Solid phase extraction (SPE) is an extraction method that uses a solid phase and a liquid phase to isolate one, or one type, of analyte from a mixture solution. The principle of SPE is similar to that of liquid-liquid extraction (LLE), involving a partitioning of solutes between two phases. The SPE involves partitioning between a liquid (sample matrix or solvent with analytes) and a solid (sorbent) phase instead of two immiscible liquid phases as in LLE. The analytes are adsorbed onto the sorbent

with several kinds of interaction depend on the type of sorbent. Next, the undesired components are washed away, and then wash off the desired analytes with another solvent into a collection tube. Underneath this extraction procedure, the analytes present in the sample solution are concentrated on a solid sorbent and then desorbed with a small volume of a suitable organic solvent. In other words, this technique is envisaged to perform the solvent exchange of target analyte using small volume of organic solvent. It is currently the most common method of sample treatment before analysis.

The growth of SPE has largely been at the expense on liquid-liquid extraction (LLE) where the advantages of SPE over LLE can be perceived. It consumes less organic solvents and a winder range of extraction mechanisms can be utilized. With SPE, many of the problems associated with LLE can be prevented, such as incomplete phase separations, less-than-quantitative recoveries, use of expensive, breakable and specialty glassware, and disposal of large quantities of organic solvents. SPE is more efficient than LLE. It yields quantitative extractions that are easy to perform, is rapid, and can be automated. Solvent use and lab time are also reduced.

In SPE technique four types of sorbent formats exist: free disks (which are generally 47 mm in diameter or the standard filtration size), disks in syringe barrels-cartridge (which vary in size from microsized disks in 1 ml syringes to a 6 ml syringe), a 96-well microtiter plate configuration that uses the 1-ml disk, and the SPE pipette tip. The stationary phase is generally contained in a glass or plastic column above a frit or glass wool. The column might have a frit on top of the stationary phase and might also have a stopcock to control the flow of solvent through the column. Commercial SPE cartridges have 1-10 ml capacities and are discarded after use. The

main format in SPE is the syringe-barrel and cartridge type. The commercial solid phase extraction cartridge is a small plastic or glass open-ended container filled with adsorptive particles of various types and adsorption characteristics. The cartridge type is still the most popular format with typically 40-60 μm diameter packing material.

There are several types of stationary phases on SPE as same as are used in liquid chromatography columns. It can be performed using either silica based or organic resin based sorbents, with suitable physical characteristics and chemical properties. The nature of the base material and the additional functional groups both affect the way that the sorbents are used. The sorbents in all cases are three-dimensional polymeric materials which are manufactured under conditions designed to provide a very porous but rigid material with a high surface area.

The extraction method of SPE is mainly determined by the sorbent used in the extraction column. Hydrophobic and hydrogen bonding energies are rather small compared with electrostatic interaction energies. Although it can be stated that a more selective extraction may be performed by using higher energy interactions, nowadays a large number of sorbents are available and the most frequently used group of sorbents are;

- Chemically modified silica gel
- Polymer sorbents
- Graphite or porous carbon

The various types of sorbents used in different extraction methods are shown in **Table 1.2.**

**Table 1.2** Commercial sorbents in various types of extraction methods in SPE

| **Extraction method** | **Sorbent** | **Polarity** | **Sample matrices** | **Elution solution** |
|---|---|---|---|---|
| Reversed phase | C18, C8, C2, carbon, cyclohexyl, cyano | Packing and analyte are non-polar | Polar, often aqueous | Non-polar solvent or mixed solution |
| Normal phase | Silica, Foricyl, carbon, cyano, amino | Packing and analyte are polar | Non-polar, often an organic solvent | Fresh solvent |
| Ion exchange | See below | Packing and analyte carried opposite electronic charge | aqueous | a) Methanol only<br>b) pH adjusted methanol<br>c) pH adjust water containing strong counterion or strong buffer |
| Anion exchange | SAX,$NH_2$ | Positively charged | Acids or negatively charged analytes | Adjust pH to 1-2 until below $pK_a$ of analyte |
| Cation exchange | SCX, carboxylic acid | Negatively charged | Bases or positively charged analtyes | Adjust pH to 1-2 until above $pK_a$ of analyte |

SPE is routinely used in many different areas of analytical chemistry. It is an increasingly useful sample preparation technique to clean up a sample before using chromatographic or other analytical methods to quantify the amount of analyte(s) in the sample. Some of the main fields are environmental and pharmaceutical analysis where cleaning and concentration of the sample are important steps in the analytical protocol. SPE is used most often to prepare liquid samples and extract semivolatile or nonvolatile analytes, but also can be used with solids that are pre-extracted into solvents.

SPE is the very popular technique currently available for rapid and selective sample preparation. The versatility of SPE allows use of this technique for many purposes, such as purification, trace enrichment, desalting, derivatization and class fractionation. The last few years have been characterized by a wide interest in this technique and many publications describing SPE methods have been published.[17] This period is connected with the intensive development of research procedures for novel types of sorbent materials and lasted from the late 1960s until the beginning of the 1980s. The introduction of a wide spectrum of sorbent materials into analytical procedures gave a new stimulus for the development of SPE methodology.[18]

Commercial SPE sorbents are excellent for sample extraction, concentration, and cleanup. They are available in a wide variety of chemistries, morphologies, and sizes. Selecting the most suitable sorbent for each application and sample is important.

After the appropriated sorbent was chosen, the SPE cartridge was then proceeded by any of the following techniques.

a) Positive pressure

SPE tubes can be processed individually using a syringe and an adapter (**Figure 1.5a**). The liquid sample is placed in the SPE tube, and the processor or syringe is used to provide positive pressure to force the liquid through the tube. Positive pressure from an air or nitrogen line also may be used to force the solutions through the tube.

b) Sidearm or vacuum flask

A solution also can be processed through a single SPE tube using a vacuum flask and rubber stopper (**Figure 1.5b**). The vacuum pulls a solution through the SPE tube. The solution then can be collected in a test tube located inside the flask.

c) Centrifuge

Several SPE tubes can be processed using a centrifuge (**Figure 1.5c**). The solutions are placed in the SPE tubes and the centrifuge forces the solutions through the tubes into test tubes. Appropriate spin rates must be determined. They can vary depending on the type and mass of the packing in the tube and the volume of sample.

d) Vacuum manifold

Multiple tubes can be processed simultaneously using a 12- or 24-port vacuum manifold (**Figure 1.5d**). SPE tubes can be processed individually or can be combined using an adapter to provide different selectivities. Small volumes are processed directly in the SPE tube. Larger volumes can be accommodated by using a reservoir with an adapter. For very large samples, a large volume sampler is available, which allows unattended sample processing.

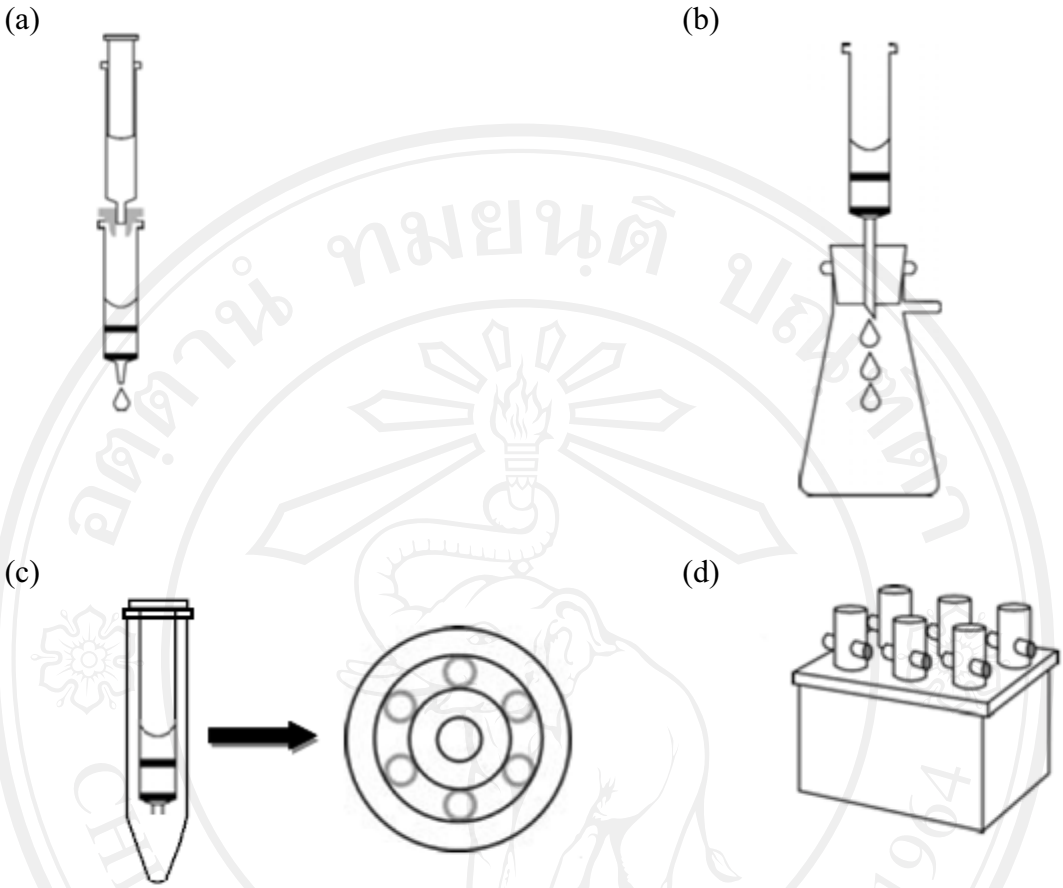



**Figure 1.5** The hardware and accessories for processing samples in SPE



### 1.2.1 Solid phase extraction procedure

The solid phase extraction process can provide samples that are in solution, free of interfering matrix components and concentrated enough for detection. Solid phase extraction is achieved through the interaction of three components: the sorbent, the analyte and the solvent. The analyte must be attracted more strongly to the sorbent than to the matrix. The best solid phase extraction mechanism and procedure are defined by the characteristics of the analyte in the sample. The steps of the solid phase

extraction process are consisted of conditioning, sample loading, washing and elution as described below and shown in **Figure 1.6**.

#### 1.2.1.1 Conditioning

The cartridges should be conditioned to wet and settle the sorbent, activate the packing materials, and remove any residual process materials. The conditioning solution volume of 1-2 folds of column volume is recommended. The packing sorbent should remain wet before adding sample.

#### 1.2.1.2 Sample loading

After the cartridge is conditioned, the compound of interest and the impurities in the sample are loaded onto the SPE cartridge by passing the sample completely through the cartridge. In this step, the components of the sample which can be either the compounds of interest or the sample impurities will bind to the sorbent depend on the type of the selected sorbent. However, for the selective extractions where on sorbent during sample loading step only selected components are retained while the remaining compound (impurities) aren't retained.



#### 1.2.1.3 Washing

For the washing step, the impurities remained on the cartridge from previously step are rinsed throughout with wash solutions. In this step, the wash solutions are passed quickly and completely through the cartridge. The wash solutions are usually strong enough to remove the impurities, but weak enough to leave the compounds of

interest behind on the cartridge. The limit amount of washing solution is to a maximum of 1 to 2 column volumes.

**1.2.1.4 Drying**

For the immiscible solvent using in the next step, the drying process is required to avoid emulsion problem. The analytical-grade inert gas such as nitrogen is applied, or draw vacuum through cartridge until packing appears dry.

**1.2.1.5 Elution**

The selective elution where the adsorbed compounds of interest are eluted in a solvent that leaves the strongly retained impurities behind. The extraction solvent is passed through the cartridge at a slow, drop wise rate to allow initial soak time on the sorbent to ensure maximum extraction efficiency. The strong solvent or buffer solution is generally used to obtain a more complete extraction by eluting with multiple small volumes. The total amount of the extraction solvent should not exceed 1 to 5 column volumes.

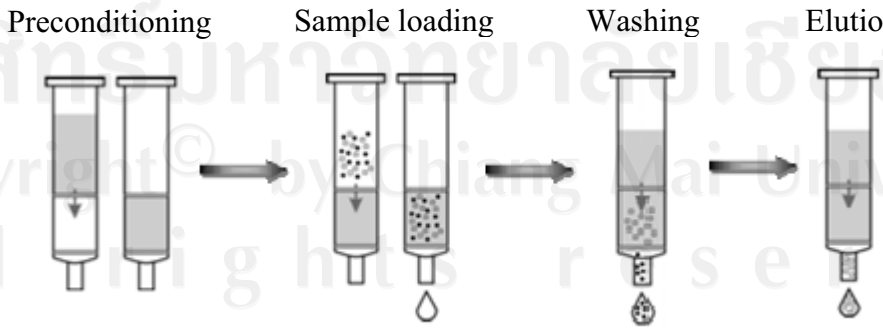



**Figure 1.6** SPE general procedures

**1.3 Molecularly imprinted polymers in solid phase extraction**[3,19-21]

The use of MIPs as selective sorbent materials allows performing a customized sample treatment step prior to the final determination. Thus, their use in solid-phase extraction, so-called molecularly imprinted solid-phase extraction (MISPE), is by far the most advanced technical application of MIPs. The first application of MISPE was carried out by the group of Sellergren in 1994 for the extraction of pentamidine present at low concentration in urine.[22] Since this work, MIPs have been largely applied to the selective extraction or to the clean-up of target analytes from various complex matrices. The principle of selective extraction on MIPs is the same as on an immunosorbent. In the most common approach, the MIP particles are packed into a disposable cartridge or a small-size column between two frits. After a conditioning step, the sample is percolated through the MIP and a washing step allows the removal of the interfering compounds retained by non specific interactions. This step must be optimized in order to keep the target analytes strongly retained inside the specific cavities of the MIP. The analytes desorption is achieved by percolating a solvent able to disrupt the interactions between the monomer residues and the analytes in order to recover them. However, the nature of the different solvents involved in a MISPE procedure can be very different from those used in immunoextraction. Due to the use of biological reagents, immunosorbents are particularly well adapted to the direct percolation of aqueous samples, the washing and the desorption steps mainly consisting in the use of hydro-organic mixtures. In return, due to the synthesis of the polymers in organic media, the MIP-based extraction procedures mainly consist in the use of organic solvents.

Today, MIPs are being applied to the selective extraction or clean-up of target analytes from various complex matrices. Many examples in the pharmaceutical domain deal with the extraction of a drug from plasma, serum and urine. In the environmental field, MIPs were mainly developed for the selective extraction of a particular class of pollutants including phenolic compounds in river water, triazines and phenylureas from soil or plant extracts, surface waters and food matrices.

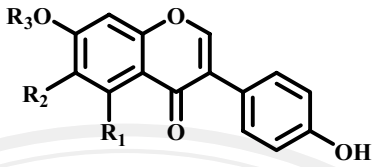
**1.4 Isoflavones**

Isoflavonoids belong to a large group of natural substances present in plants. More than 3000 flavones and more than 700 known isoflavones exist in plants. Their structure based on a 3-phenylbenzpyrone (3-phenylchromone) group. The structure differs in the degree of methylation, hydroxylation and glycosylation. The synthesis of isoflavones in plants is based on a carbon skeleton of an isoflavonoids and on different oxidations of three central atoms. The isoflavones are mostly present in the *O*-glucosidic form, the malonate or the methylmalonate hemi-esters forming the glucosidic part. The isoflavone *C*-glucosides are distinguished from the isoflavone *O*-glucosides, as the name implies, by possessing a sugar which is carbon-carbon linked via its anomeric (C-1) carbon to the C-6 and/or C-8 of the isoflavone nucleus.

Interest in soybeans and soy-based products has grown significantly in the past few decades due to the association of soybean consumption with variety of health protective effects.[23] Soybeans are known to contain a large number of bioactive phytochemicals such as isoflavones, saponins, phytosterols, protease inhibitors, inositol hexaphosphates, sphingolipids, phenolic acids, and Bow-man-Brik trypsin inhibitors.[24,25] Isoflavone enriched extracts have been evaluated in the prevention of a

wide range of health problems associated with menopause, cardiovascular disease, osteoporosis, and in breast, prostate, and colon cancers.[26] Isoflavones are widely distributed in the plant kingdom, but accumulate predominantly in plants of the *Leguminosae* family. The best natural source of isoflavones is soybeans, which have been a major part of the traditional diet for Eastern Asian populations for centuries. The global annual consumption of soybeans has increased from 114 to 170 million tons during the past decade.[27] Soybeans contain 1.2-2.4 mg of total isoflavones per gram of sample.[28] This hundred percent variation in soybeans is due to variation in genotypes, environment, location, post harvest storage and assay procedures.

There are three aglycon forms of isoflavones commonly found in soybeans which are daidzein, genistein, and glycitein. These three isoflavones can also exist in conjugated forms with glucose (daidzin, genistin, glycitin), malonylglucose (malonyldaidzin, malonylgenistin, malonylglycitin), and acetylglucose (acetyldaidzin, acetylgeinstin, acetylglycitin) units. Thus 12 free and conjugated forms of isoflavones have been isolated from different soybean samples. The chemical structures of these soybean isoflavones are shown in **Figure 1.7**.




| **Name** | **$R_1$** | **$R_2$** | **$R_3$** |
|---|---|---|---|
| Daidzein | H | H | H |
| Glycitein | H | $OCH_3$ | H |
| Genistein | OH | H | H |
| Daidzin | H | H | Glu |
| Glycitin | H | $OCH_3$ | Glu |
| Genistin | OH | H | Glu |
| Acetlydaidzin | H | H | Glu-$COCH_3$ |
| Acetylglycitin | H | $OCH_3$ | Glu-$COCH_3$ |
| Acetylgenistin | OH | H | Glu-$COCH_3$ |
| Malonyldaidzin | H | H | Glu-$COCH_2COOH$ |
| Malonylglycitin | H | $OCH_3$ | Glu-$COCH_2COOH$ |
| Malonylgenistin | OH | H | Glu-$COCH_2COOH$ |

**Figure 1.7** The chemical structures of isoflavones commonly found in soybeans[29]

**1.5 Analytical separation and detection methods for Isofalvone**

Over the years many sample pre-treatment methods have been developed to determine isoflavonoids in various sample types.[30] There are three main types of isoflavonoid-containing matrices: plants, food and liquid samples such as biological fluids and drinks. The solid samples are usually first homogenized, which may be proceeded by (freeze-) drying or freezing with liquid nitrogen. The next step is analyte isolation. Commonly used methods for isolation of the isoflavones include a simple mixing, Soxhlet extraction, or ultrasonication techniques using different organic solvents. On the other hand, several new extraction procedures, such as supercritical fluid extraction (SFE), accelerated solvent extraction (ASE), etc., have been developed in the last several years as an alternative to the traditional procedures.[31] However, solvent extraction (SE) which may be followed by SPE is still the most widely used technique, mainly because of its ease of use and wide ranging of target analyte.

Analysis of isoflavones in biological samples is complicated due to low efficiency of chromatographic separation and time consuming sample preparation. A high performance liquid chromatography (HPLC) coupled with an electrochemical UV-vis diode-array detector (DAD) and/or mass spectrometric are generally used for determination of the isoflavones. Furthermore, a combination of highly effective chromatographic techniques with effective isolation/purification techniques is suitable for the quantitative analysis of the compounds.

**1.6 Aim of this research**

1.6.1 To synthesize MIPs selective to genistein using fragment templates.

1.6.2 To optimize the conditions of MISPE for extraction of genistein from soybean extracts.

1.6.3 To apply these synthetic MIPs in the extraction of genistein from soybean extracts.