HACETTEPE UNIVERSITY
HEALTH SCIENCES INSTITUTE

SYNTHESIS OF MOLECULARLY IMPRINTED POLYMERS AS HPLC STATIONARY PHASE FOR 4-AMINOPHENOL

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Basic Pharmaceutical Sciences
Master Thesis

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To the Health Sciences Institute Director's Office,

This work has been accepted as MSc THESIS in the Basic Pharmaceutical Science Programme by our jury.

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CONFIRMATION

This thesis has been approved by the above listed jury members appointed by the Health Sciences Institute Executive Committee.

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ONAY

Bu tez, Sağlık Bilimleri Enstitüsü Yönetim Kurulu'nca belirlenen yukarıdaki jüri üyeleri tarafından uygun görülmüş ve Yönetim Kurulu'nun kararıyla kabul edilmiştir.

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ABSTRACT

Baykara, E., Synthesis of molecularly imprinted polymers as HPLC stationary phase for 4-aminophenol. Hacettepe University Health Sciences Institute MSc Thesis in Basic Pharmaceutical Sciences, Ankara, 2008. Molecularly imprinted polymers (MIPs) are attractive as multi functional and cheap materials which are very promising in recognition for the isolation and separation of target molecules.

This thesis mainly focuses on the synthesis of various molecularly imprinted monolithic high performance liquid chromatography (HPLC) columns in the presence of 4-aminophenol (4-AMP) as template molecule, employing the non-covalent approach. The aim of the study was to achieve the synthesis of polymeric HPLC monoliths capable of separating structurally similar molecules, 4-AMP and paracetamol (P), with high selectivity by in-situ polymerization technique. For this purpose, several 4-AMP imprinted monoliths were synthesized by using only methacrylic acid (MAA) or MAA together with a comonomer, acrylamide (AAM), in the presence of high amount of crosslinker, ethyleneglycol dimethacrylate (EDMA) and these polymeric monolith columns were connected to an HPLC device in order to evaluate their separation capabilities. The good performance of MIP monolith was achieved by selection of appropriate solvents and careful optimization of polymerization conditions. The most efficient molecularly imprinted monolith was the polymer of MAA/EDMA with a selectivity of 5.68 and resolution of 1.91. The existance of comonomer, AAM, reduced both the selectivity and resolution of monoliths due to the weaker interactions between AAM and 4-AMP and formation of monomer-monomer association. The effective binding site densities and dissociation constants were estimated from the frontal analysis and found as 7.95 µmol/g and 1.06 mM, respectively, for the polymeric monolith showing the best performance. BET analysis of the same monolith showed that its surface area was 23.48 m^2/g.

Keywords: monolithic columns, molecular imprinting, in-situ polymerization, 4-aminophenol, paracetamol.

ÖZET

Baykara, E., HPLC sabit fazı olarak molekül kalıplanmış polimerlerin 4-aminofenol için sentezi. Hacettepe Üniversitesi Sağlık Bilimleri Enstitüsü Eczacılık Temel Bilimleri Yüksek Lisans Tezi, Ankara, 2008. Molekül kalıplanmış polimerler (MIPs), hedeflenen moleküle karşı seçicilik gösteren, molekülün ayırımında ve saflaştırılmasında kullanılabilecek çok yönlü ve ucuz malzemelerdir.

Bu tez çalışması, "non-kovalent" yaklaşım kullanılarak, kalıplanacak molekül (template) olan 4-aminofenol (4-AMP) varlığında, çeşitli molekül kalıplanmış monolitik HPLC kolonlarının sentezi üzerine odaklanmıştır. Bu çalışmanın amacı, in-situ polimerizasyon tekniği kullanılarak, yapısal olarak benzer moleküller olan 4-AMP ve parasetamolü (P) yüksek seçicilikle ayırma yeteneğine sahip polimerik HPLC kolonları sentezlemektir. Bu amaçla, sadece metakrilik asit (MAA) ya da MAA ile birlikte akrilamid (AAM) komonomeri kullanarak, yüksek miktarda çapraz bağlayıcı, etilenglikol dimetakrilat (EDMA), varlığında çeşitli molekül kalıplanmış monolitler hazırlanmış ve bu monolitlerin ayırım performansları HPLC ile incelenmiştir. Uygun çözücü seçimi ve polimerizasyon şartlarının optimizasyonu ile iyi performans gösteren MIP monolitler elde edilmiştir. En iyi performansa sahip molekül kalıplanmış monolitin, 5.68 ayırıcılık ve 1.91 seçicilik değerlerine sahip MAA/EDMA polimer sistemi olduğu gözlemlenmiştir. AAM komonomeri varlığının, 4-AMP ile AAM arasındaki zayıf etkileşimlerden ve monomer-monomer etkileşimlerinden dolayı, ayırıcılığı ve seçiliği düşürdüğü görülmüştür. Bağlanma bölgesi yoğunlukları ve ayrışma sabiti değerleri frontal analiz yöntemiyle hesaplanmış ve en iyi performansa sahip polimerik monolit için sırasıyla 7.95 μmol/g ve 1.06 mM olarak bulunmuştur. BET analizi sonuçları, bu monolitin 23.48 m^2/g yüzey alanına sahip olduğunu göstermiştir.

Anahtar kelimeler: monolitik kolon, molekül kalıplama, in-situ polimerizasyon, 4-aminofenol, parasetamol.

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ABBREVIATIONS

4-AMP	4-aminophenol
AAM	Acrylamide
ACN	Acetonitrile
AIBN	2,2'-Azobis(2-methylpropionitrile)
EDMA	Ethyleneglycol dimethacrylate
HPLC	High performance liquid chromatography
IBMA	Isobornyl methacrylate
MAA	Methacrylic acid
MIP	Molecularly imprinted polymer
NIP	Non-imprinted polymer
P	Paracetamol
SEM	Scanning electron microscopy

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1. AIM OF THE STUDY

Paracetamol (acetaminophen) is a widely used non-steroid drug for the management of pain and as antipyresis. Under some improper conditions (heat, pH, temperature), it may degrade to 4-AMP and acetic acid (1). 4-AMP may exist as an impurity in the starting materials of P synthesis and may also form during the synthesis. In the studies with the use of animals, nephrotoxic effect of 4-AMP and its capability of causing methemoglobinemy were observed (2). The amount of 4-AMP is limited to % 0.005 (w/w) in the drug substance by the European, United States, British and German Pharmacopoeias. Therefore, 4-AMP is one of the major impurities, which has to be detected in P. Due to the similar structures of these two molecules, the separation is quite challenging. Chromatographic methods has been widely applied to the separation of 4-AMP from P mostly by using silica based columns. However, these methods are time consuming and the silica based columns have some disadvantages such as poor stability at extreme pH values.

Molecular imprinting is a unique technique particularly for the separation purposes. By molecular imprinting technique, structurally similar molecules, such as 4-AMP and P (Figure 1.1), can be separated selectively based on the created specific cavities complementary to the targeted molecule. Beside, MIPs have the advantages of low cost, easy preparation and high stability. Therefore MIPs can be a good alternative of silica based columns for this purpose. MIPs are prepared widely by bulk polymerization method but resulted polymers are irregular in shape and size. In addition, this method brings a need of crushing, grounding and sieving the bulk polymer in order to be applied to HPLC, which results a loss in their chromatographic performance and it consumes a lot of time. Although other polymerization methods were developed, such as suspension (3) and precipitation polymerization (4), to prepare spherical MIPs, they are still time-consuming due to the grounding and sieving steps.

In order to achieve rapid separations, the use of monolithic columns has attracted many researchers. With their skeleton structure having high porosity resulting in high permeability and with their good efficiency, monolithic columns let high flow rates applied without over pressuring the pump. Therefore, preparation of

molecularly imprinted polymeric monoliths is attracted considerable attention for chromatographic applications. In order to eliminate the time consuming process of grounding and sieving, Matsui prepared molecularly imprinted polymeric monolith by in-situ polymerization technique (5). This technique enabled the easy preparation of molecularly imprinted monolithic columns with good resolution and low back-pressure.

The aim of this study was to prepare a polymeric monolithic HPLC column by in-situ polymerization method, which has high separation capacity for 4-AMP and P molecules. The resulted polymeric monolith was expected to show higher selectivity to 4-AMP than to P achieved by the molecular imprinting of 4-AMP.

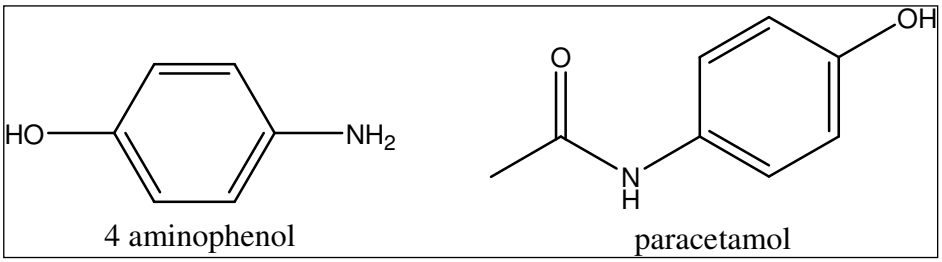

Figure 1.1. Molecular structures of 4-AMP and P.

2. INTRODUCTION

2.1. Molecular Imprinting

Biological macromolecules including proteins, which may serve as enzymes, antibodies and receptors, nucleic acids and saccharides have crucial roles in biological activities for the functioning of living systems. While playing their roles, it is very important for these macromolecules to possess molecular recognition. Enzymes, for example, have excellent molecular recognition abilities owing to that they function based on a system named “lock and key” mechanism in which both the enzyme and the substrate have specific complementary geometric shapes, sizes and chemical functionalities that fit exactly into one another. Molecular imprinting is a very powerful biomimetic approach to mimic such biological systems (6). Molecular imprinting can also be described as a way of making artificial “locks” for “molecular keys”. Many analytical procedures depend on sensitive and reliable natural recognition elements such as antibodies and enzymes. Such host-guest systems have many applications in separation and isolation, in immunoassay-type analyses, in catalytic applications and in biosensor-like devices. Fabrication of MIPs to be used in such applications instead of their natural-counterparts may benefit much by obviation of the stability problems, high costs, need for host animals and in cases where there is no biological recognizing element exists, especially for biosensor-like devices, MIPs are the only alternative. Therefore, there are so many studies conducted or being conducted on recognition systems and MIPs. Thus it is very obvious that this technique will find various application areas in the near future.

Molecular imprinting takes place mainly in three steps. In the first step, monomers carrying certain functional groups are associated with the template molecule through either non-covalent or covalent interactions. In the second step, this complex is polymerized with a high degree of crosslinking, so that the functional groups become fixed in defined positions by the polymer network. In the last step, subsequent removal of the template molecule by solvent extraction or chemical cleavage is carried out leaving cavities that are complementary to the template in terms of size, shape and arrangement of the functional groups. These highly specific

receptor sites are capable of rebinding the target molecule with high specificity. Table 2.1 lists some examples of molecularly imprinted compounds.

Table 2.1. Examples of Some Successfully Imprinted Compounds.

Compound Class	Example	Compound Class	Example
Drugs	timolol (7)	Amino Acids	phenylalanine (15)
	theophylline (8)		tryptophan (16)
	morphine (9)		tyrosine (17)
	ephedrine (10)		
Hormones	cortisol (11)	Carbohydrates	galactose (18)
	enkephalin (12)		glucose (19)
Pesticides	atrazine (13)	Co-enzymes	pyridoxal (20)
Proteins	ribonuclease A (14)	Nucleotide Bases	adenine (21)

Among all widely studied application areas of molecular imprinting method, such as MIPs to be used in sensors (22) and in drug delivery systems (23), the major application area of molecular imprinting technique is chromatographic separations. They serve as molecularly imprinted stationary phases in order to separate even molecules having very minor structural differences.

2.1.1. Historical Evolution of Molecular Imprinting

The origins of molecular imprinting can be traced back to 1890s with the work of Emil Fisher who postulated the idea of “lock and key” mechanism to describe the specifity of enzymes (24). This theory was further developed by Polyakow, who performed a series of investigations on silica for use in chromatography. He prepared silica by the acidification of sodium silicate solution then he dried the gelatinous silica polymer for 20-30 days in the presence of different additives, benzene, toluene or xylene (25). It is reported that the silica placed in a desiccator, containing one of the additives in a beaker, showed different adsorption extends for each of these additives depending on the structure of the additive present during the drying process. This selectivity was suggested to arise from the additives which were considered as templates directly affecting the resultant silica surfaces.

We can now say it was the first time that molecular imprinting was accompanied. (26).

Later, in 1942, Pauling and Campbell reported the “production of antibodies in vitro” (27). Pauling and Campbell employed two dye stuffs, methylene blue and 1,3-dihydroxy-2,4,6 tri(p-azophenylar- sonic acid) benzene, and the polysaccharide Pneumococcus Polysaccharide Type III as templates. Polyclonal (non-immunised) γ-globulin serum was incubated together with high concentrations of antigen under denaturing conditions. After a slow renaturing of antibodies in the presence of the antigen, the precipitated antibodies were redissolved after removal of the antigen and analyzed for selectivity. It was reported that the resulting antibody could efficiently precipitate the polysaccharide antigen, but not a related sugar antigen, polysaccharide Type I (26). This procedure is very similar to todays so called *bioimprinting*.

Following Pauling’s works, a senior student of him Frank Dickey prepared silica gels by polymerization of sodium silicate using a dye molecule as a template (28). This work seems similar to the work of Polyakov mentioned before but there is an important difference; he introduced the template in the pre-polymerization mixture that is before the polymerization starts, on the contrary, Polyakov introduced the template after silica matrix was formed. In this respect, Dickey’s method is more similar to present methodology. Therefore, he is named as the inventor of molecular imprinting. Dickey used 4 different dyes in his work and reported rebinding experiments done after removal of the template molecule as; resulting silica gel is much more selective to the dye molecule which was used as a template in the polymerization solution when compared to other three dye molecules as related to a control gel prepared in the absence of dye.

After some publications on imprinted silica, the interest in this subject declined due to the limitations in stability and reproducibility of the imprinted silica materials. This temporary decline continued till 1972 when Günter Wulff presented the first examples of molecular imprinting in synthetic organic polymers. In their first report on this field, Wulff and Sarhan copolymerized D-glyceric-(p-vinylanilide)-2,3 O-p-vinylphenylboronate and divinylbenzene. Subsequent hydrolyses of the glycerate moiety disclosed imprints that show chiral recognition of D-glyceric acid (29). This work was the first example of molecular imprinting by

using covalent bonds and it is one of the most important marks on molecular imprinting. Today it is known as “covalent approach” to molecular imprinting and Wulff is named as the inventor of this approach. “Covalent approach” is explained in part 2.1.2 in further detail. Group of Wullf reported 30 more original works on molecular imprinting in the following years.

The most important development on molecular imprinting is obviously the introduction of general “non-covalent” approach by group of Mosbach in the early 1980s (30). This approach has significantly broadened the scope of molecular imprinting and is explained in part 2.1.3 in more detail. Today most of the studies conducted on molecular imprinting technique uses “non-covalent” approach.

2.1.2. Covalent Approach

In order to prepare synthetic polymers showing enzyme-like catalytic activity, a number of prerequisites have to be fulfilled. First, a cavity has to be made with a defined shape similar to the shape of template molecule. By the way, functional groups acting as binding sites have to be introduced. To achieve these prerequisites, Wulff has suggested a novel strategy, called as covalent approach to molecular imprinting. Covalent approach, also called as pre-organized approach, includes formation of strong, reversible, covalent arrangements of the monomers with the template molecule before polymerization. Thus, the template molecules need to be derivatized with the monomers before the actual imprinting is performed. After polymerization completes, cleaving the covalent bonds that hold the template molecules to the macro-porous polymer matrix let recognition sites complementary to the analyte remain in the polymer as summarized in the Figure 2.1. However this approach includes a salient disadvantage. That is, the functional monomer and template pairs being able to form reversible covalent bonds are very limited. Since it is not always easy to break a covalent bond, especially without damaging the cavity formed, the problem of removal of template from the polymer matrix arises. Therefore it can be applicable only to limited monomer and template pairs, such as 4-vinylphenylboronic acid as monomer and monosaccharide derivatives as templates (31).

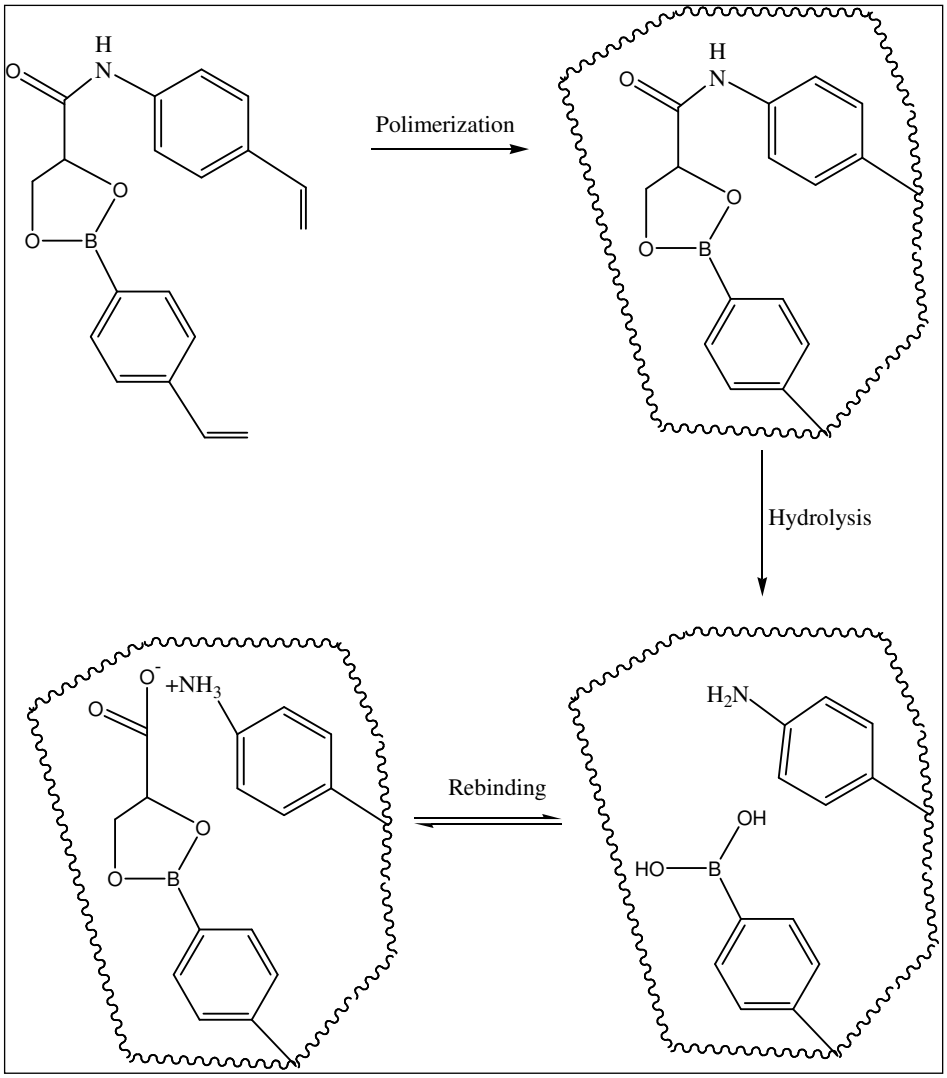

Figure 2.1. Schematic representation of covalent approach to molecular imprinting.

2.1.3. Non-Covalent Approach

Non-covalent approach, also called as self assembly approach, involves formation of non-covalent arrangements before polymerization. Non-covalent interactions are the basis of reversible binding and recognition events in biochemical systems that rely on salt bridges, hydrogen bonds and hydrophobic interactions. The binding energies of non-covalent interactions are weak when compared to covalent bond. However, when multiple non-covalent interactions occur at the same time, they can lead to strong binding sites. For non-covalent imprinting, the most important interactions are van der Waals forces, hydrogen bonding, ionic interactions

and hydrophobic interactions. Due to this diversity and ease of formation of non-covalent interactions, unlikely to covalent approach, non-covalent approach can have many monomer and template pairs by which a successful molecular imprinting could be done. Besides, non-covalent interactions offer another important advantage, that is, it is much simpler and less complicated to remove the template from the polymer matrix after polymerization completes. Mostly a simple extraction by washing away the template molecule with an appropriate solvent would be enough. Therefore, today it is the most preferred system used in molecular imprinting studies. However, in order to achieve a good molecular imprinting by using non-covalent approach, the desired template molecule should have at least more than one functional group and the functional monomer should present in large excess to ensure the required binding energy. Figure 2.2 shows an example of preparation of MIPs by non-covalent approach.

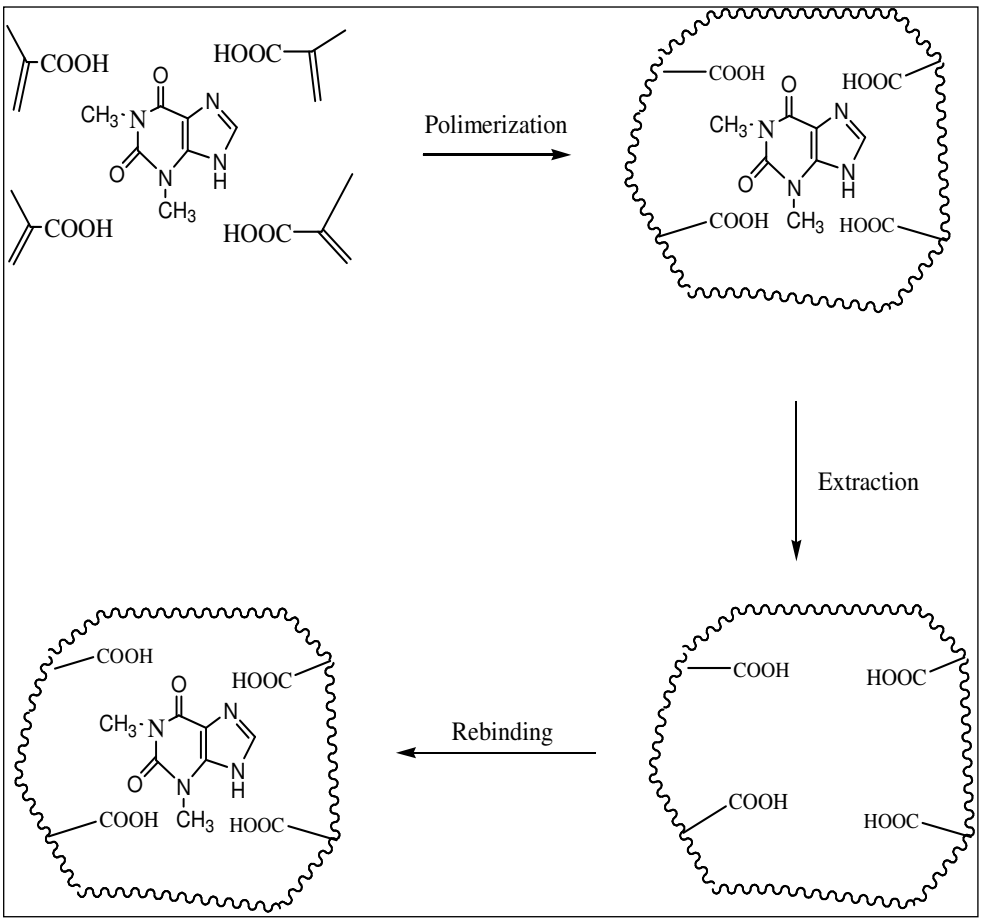

Figure 2.2. Non-covalent imprinting of theophylline.

Non-covalent imprinting mechanism can be outlined as follows: in the first step, functional monomers, added in large excess, and template molecules form non-covalent interactions. Then polymerization is initiated and after polymerization completes, a highly crosslinked polymer containing template molecules inside its cavities is obtained. Afterwards, the template molecule is extracted from the polymer matrix leaving specific cavities functionally and structurally complementary to template molecule.

2.2. High Performance Liquid Chromatography

HPLC is a form of liquid chromatography used to separate compounds in a mixture by means of some chemical interactions between the analyte and the chromatographic column. The basic operation principle of HPLC (Figure 2.3) is to force the analyte through a stationary phase packed into the column by pumping a mobile phase at high pressures. Retention time of an analyte, time that analyte spends in the column, mainly depends on the nature of the stationary phase and the composition of the mobile phase. Molecules which have different natures in one or more way show different behaviors in the column leading to different retention times so that separation takes place.

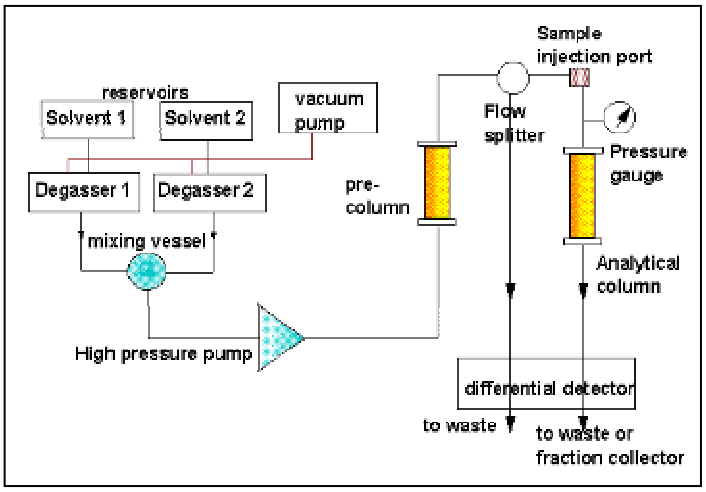

Figure 2.3. Schematic representation of a general HPLC device.

HPLC is a very sensitive technique, it is readily adaptable to accurate quantitative determinations, suitable for separating non-volatile species or thermally fragile ones and it has a wide-spread applicability to substances that are of prime interest to industry, to many fields of science. Because of these unique properties, HPLC is the most widely used technique among all analytical separation techniques.

2.3. HPLC Stationary Phases

Because column is the part of the system that separation occurs, it has seen much more changes than any other part of the system since HPLC has been available starting from 1960s. The most important improvements have occurred in packing material design and stationary phase chemistry. There are hundreds of commercial columns for HPLC with different materials and different morphologies in the market. However it can easily be said that silica based columns are dominating HPLC column market. There are mainly three types of column packing material; silica based columns, polymer based columns and metal oxide based columns. For a number of reasons, chromatographers aim to improve the chemical and the thermal stability and thus the longevity of the columns, especially for reversed phase applications.

2.3.1. Silica Based Stationary Phases

As mentioned before, silica based stationary phases are the most widely used materials in HPLC columns because of their special features. The most important characteristic of silica which makes it so popular is that its surface can easily be modified. That is it has a controllable surface chemistry due to the silanol groups, Si-OH, on the surface. Modifications of silica will be explained in details later. The other important feature of silica is its high surface area which is very crucial for better chromatographic separations. In addition, it can be produced with higher homogeneity compared to other materials.

The main disadvantages of silica based HPLC columns are their low chemical stability, low thermal stability and low mechanical strength compared to metal oxide

based columns. At lower pH values than 3, the siloxane bonds between ligands, for chemically modified silica columns, and the silica surface break down, and at higher pH values than 8, silica backbone collapses. Therefore the optimum pH range for silica based columns is 2.5-7.0. Beside, it would be good to avoid working at temperature values over 60°C in order to prevent loosing stationary phase especially for bonded phase columns.

Pure Silica Gel

Pure silica gel is an adsorbent with outstanding properties. It can be used for adsorption chromatography, normal phase partition chromatography and size exclusion chromatography. It consists of silicon atoms bridged by oxygen atoms as seen in the figure 2.4. Silica gel can be produced by complete hydrolysis of sodium silicate, tetraalkoxysilane or silicon tetrachloride giving polysilicic acid then by dehydration we get pure silica gel. This type of silica gel is irregular in shape. Spherical silica gel can also be produced by using tetraethoxysilane.

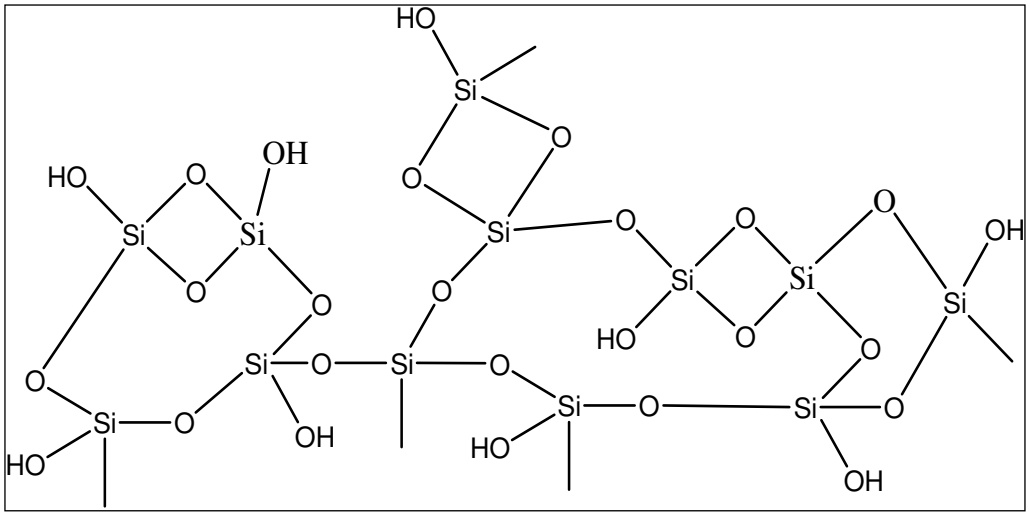

Figure 2.4. Chemical structure of silica gel.

Chemically Modified Silica Gel (Bonded Phase)

Silica gel can easily be modified at the surface by bonding desired functional groups to silanol groups existing on the surface of silica to give stationary phases with specific properties. This type of columns can be used for normal phase partition chromatography, for reversed-phase partition chromatography or for ion exchange chromatography depending on the functional group bonded to the silica surface. Functional groups used in chemically modified silica gel are shown in the table 2.2.

Table 2.2. Functional groups in chemically modified silica gel.

Group	Formula	Group	Formula
Octadecyl	$-(CH_2)_{17}CH_3$	Dimethylamino	$-N(CH_3)_2$
Octyl	$-(CH_2)_7CH_3$	Amino	$-NH_2$
Hexyl	$-(CH_2)_5CH_3$	Aminopropyl	$-CH_2CH_2CH_2NH_2$
Dimethyl	$-Si(CH_3)_2$	Alkylamino	$-(CH_2)_nNH_2$
Trimethyl	$-Si(CH_3)_3$	Nitro	$-NO_2$
Cyclohexyl	$-C_6H_{11}$	Nitrile	-CN
Phenyl	$-C_6H_5$	Alkylnitrile	$-(CH_2)_nCN$
Diphenyl	$(-C_6H_5)_2$	Propionitrile	$-CH_2CH_2CN$

Octadecylsilane, known as C18, is the most widely used of these chemically modified products. It is extremely non-polar and is the preferred choice for use in reversed-phase chromatography. These chemically modified bonded phase stationary phases can be produced using three different methods as summarized in the figure 2.5. Depending on the production method, surface chemistry of bonded phase silica changes and so does their stability. In order to improve their stability, other modifications are also applied. Silanol groups present at the surface are very open to hydrolysis. To reduce the amount of silanol groups at the surface, the most widely applied procedure is “end capping”. In this modification, surface silanol groups are end-capped by using trimethylchlorosilane. Another method involves binding a bulky group to surface Si molecules where functional group is bonded preventing

protons to reach the oxygen atoms of siloxane groups on the surface as seen in the figure 2.6.

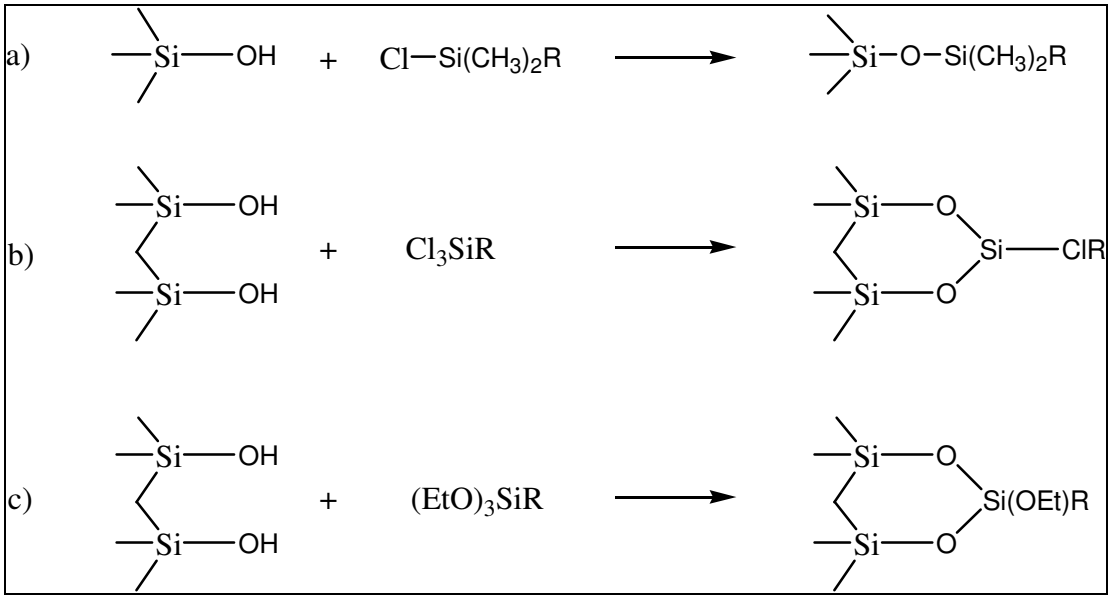

Figure 2.5. Production of bonded phase silica gel (32).

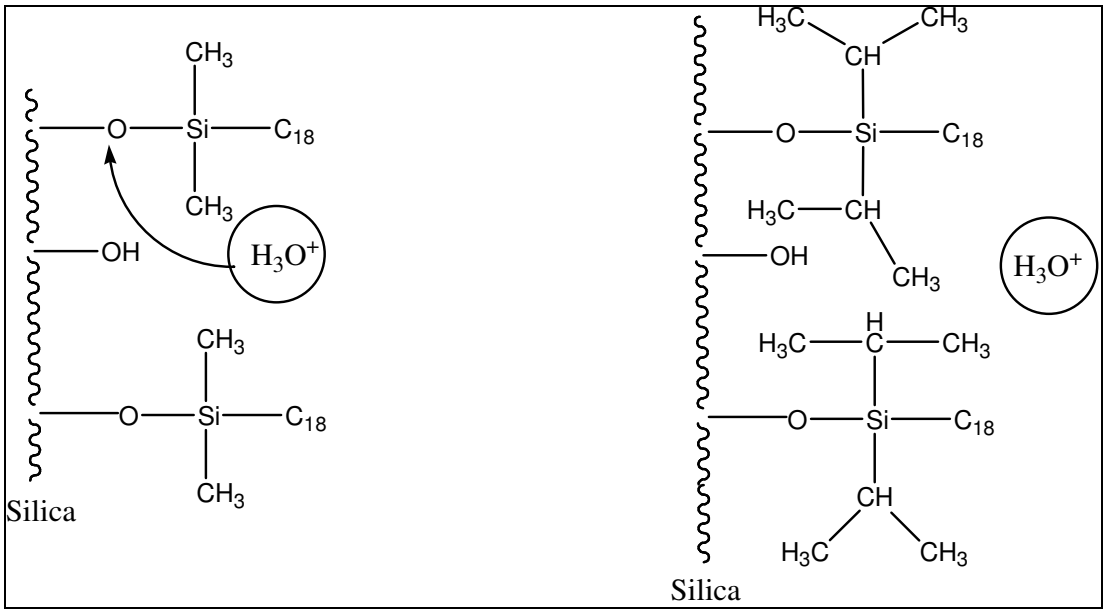

Figure 2.6. Protection from hydrolysis.

2.3.2. Polymeric Stationary Phases

Polymeric stationary phases have some advantages and disadvantages over silica based HPLC columns. Their main advantages are that they are chemically and thermally much more stable than silica based columns. Their most important disadvantage is swelling. They can swell in some solvents. And their mechanical stability is lower than silica based columns. Besides, their monodispersity is low and surface modification of polymeric stationary phases is not as easy as those of silica based stationary phases. However, polystyrene-divinylbenzene based stationary phase (Figure 2.7), which is the only commercial polymeric HPLC stationary phase, has overcome most of these disadvantages.

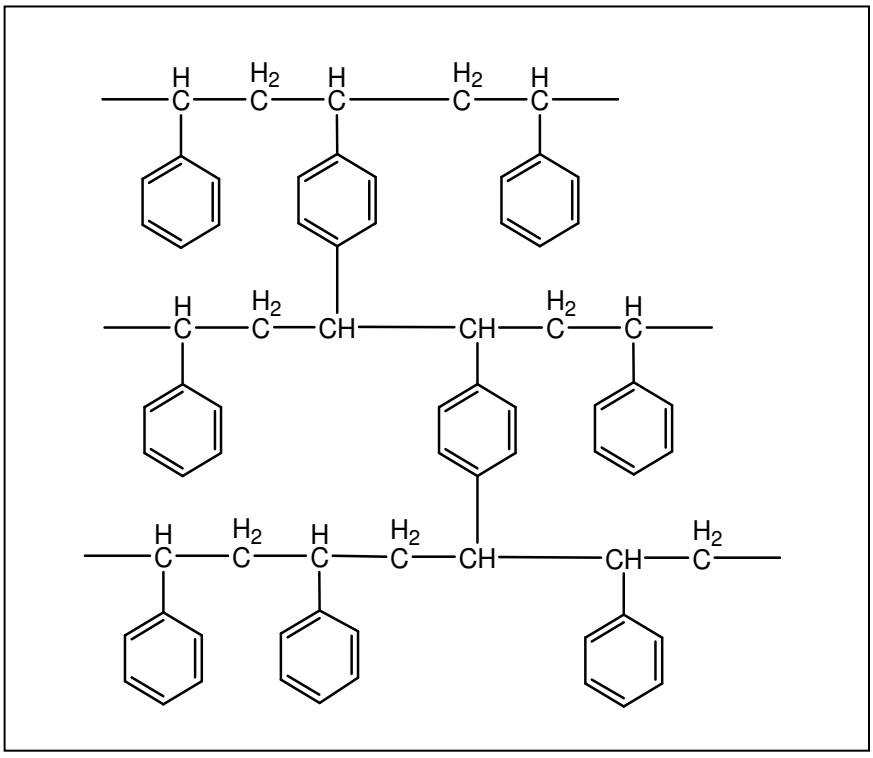

Figure 2.7. Structure of polystyrene-divinylbenzene.

2.3.3. Metal Oxide Stationary Phases

Zirconia (ZrO_2), titania (TiO_2) and alumina (Al_2O_3) based columns are the most widely applied stationary phases among metal oxide based columns. Metal

oxide based HPLC stationary phases show superiority at some points over silica based columns. For instance, their thermal and chemical stability, especially those of zirconia, are much higher than silica based columns and this eliminates some limitations coming from the silica based stationary phases, such as limited pH range and mobile phase composition. Since reversed phase applications are far predominant in today's chromatographic separations, surface chemistry of the stationary phases is also very important. The surface chemistry of the metal oxides is not as well understood as silica counterparts and therefore their surface chemistry is not as controllable as silica based ones are. For this reason, reproducible synthesis of this type stationary phases is more difficult which cause a need for much more detailed process. Besides, it also makes the surface modification much more complicated.

Comparison of various properties of different HPLC column substrates and their reverse phase (RP) bonded phase stationary phases are given in the table 2.3 and 2.4.

Table 2.3. Comparison of HPLC column substrates.

Property	**SiO_2**	**PSTY-DVB**	**Al_2O_3**	**ZrO_2**
Monodispersity	+++	++	++	++
Pore Structure	++	++++	++	++
Surface Area/Pore Diameter	++++	+++	+++	++
Controlable Surface Chemistry	++++	+++	++	++
Mechanical Strength	++	+++	+++	+++
Chemical Stability	++	++++	+++	++++
Thermal Stability	++	+++	+++	+++
Column Efficiency	++++	++	+++	+++
Homogeneity	+++	++	+	+

Table 2.4. Comparison of chemical and thermal stabilities of substrates and RP bonded phase stationary phases (33).

Phase	**Chemical Stability**		**Thermal Stability**
	Low pH	Intermediate and high pH	
SiO_2	+	-	++
RP Bonded Phase	+++	+++	++
PSTY-DVB	++++	++++	+++
RP Bonded Phase	++++	++++	+++
Al_2O_3	+++	+++	+++
RP Bonded Phase	+	+	+
ZrO_2	++++	++++	++++
RP Bonded Phase	+	+	+

2.4. HPLC Column Packing Morphology and Design

In this part, developments on packing morphology and particle design are explained. Although there are several improvements achieved in column packing so far, it can be said that the most remarkable development on this field is the introduction of monolithic columns showing remarkable advantages over conventional packed columns. Therefore, it would be convenient to divide HPLC column packing into two main parts: packed columns and monolithic columns.

2.4.1 Packed Columns

Prior to the advent of HPLC, low-pressure, gravity-fed columns with large (~100 μm), irregularly shaped porous particles such as silica gel or alumina were in use. These particles were sieved into suitable size ranges. Although their sample capacity is very high due to their high surface area, large particle size causes poor column efficiency. Increasing the flow rate even gives broader peaks with loss of resolution because of slow solute mass transfer in and out of these large particles.

In 1967 Horvath and Lipsky (34) introduced another packing design (pellicular packing) with 50 μm particles. These particles had a thin porous film of stationary phase, with 1-3 μm thickness, deposited on a spherical glass bead. So that the efficiency of the column was increased due to thin stationary phase, that is; solute molecules do not spend much time to get in and out of these particles. However, due to the small amount of stationary phase in the column, column capacity was very low.

At the end of 1960s, the use of small porous particles overcame the limitation of porous layer beads but it could not be commercialized till 1972. Since such particles with diameters smaller than 20 μm have a static charge built-up, it was very difficult to pack them into a column with the conventional packing method of those days which is dry packing. In 1972 Majors (35) introduced slurry packing technique which made it possible to pack smaller particles into a column.

After the introduction of slurry packing technique, the particle size used in the columns starts to get smaller and smaller in order to achieve better and faster separations. Surface area and therefore efficiency increases with decreasing particle size. However, it causes another problem, higher back-pressures with decreasing particle size. Then the tendency of using smaller particles continued simultaneously with the development of better pumps being able to produce higher pressures and also with the development of column packing materials showing higher resistances to high pressures. Today most of the commercial HPLC columns include porous particles with an average diameter of 3 μm.

One of the major developments occurred in column packing was the introduction of perfusion chromatography by Afeyan in the early 1990s (36). Compared to porous packing, the perfusion packing consists of two different types of pores; diffusive pores and through-pores. The diffusive pores were the same with those present in porous packing. The through-pores allowed a portion of the mobile phase to pass through the packing itself, so that the rate of mass transfer in the stationary phase was increased, giving narrower peaks.

Through these years, pellicular packing was also revisited, but these newer versions had reduced particle sizes (1,5-2,5 μm) compared to former ones and they used non-porous solid silica or resin instead of glass beads. However, the

backpressure was very high for these columns due to small particle size and sample capacity was again low. These columns were used especially for large bio-molecules. Later, Kirkland (37) described poroshell packing commercialized by Agilent Technologies, in which a thicker porous layer than those of pellicular packing is formed on a solid silica core material with a diameter of 5 µm. Due to its larger particle size, the back-pressure is much lower, which makes separations of bio-molecules faster at relatively lower pressures.

General structures of conventional packing materials and their historical development are given in the Figure 2.8 and Table 2.5, respectively.

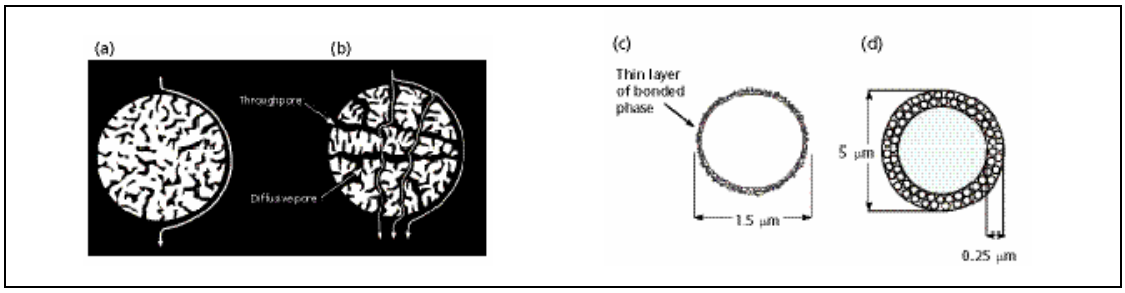

Figure 2.8. Schematic representation of a) porous packing b) perfusion packing c) pellicular packing and d) poroshell packing.

Table 2.5. History of HPLC product development.

Year	**Packing**	**Particle Size (µm)**
1950s	Porous (Irregular Shape)	100
1967	Pellicular (Glass Bead)	50
1972	Porous	10
1986	Porous	5
1992	Porous	3-3.5
1998	Pelllicular (non-porous silica)	1.5
1999	Poroshell (non-porous silica)	5
2000	Porous	2.6
2003	Porous	1.8

2.4.2. Monolithic Columns

The most exiting HPLC column innovations in the last several years has been the commercial development of monoliths. Monoliths are columns that have the stationary phase cast as a continuous homogeneous phase rather than packed as individual particles. Today both silica and polymeric monoliths are present in the market. Monolith phases show some similarities to perfusion packing, that is, monolithic columns have macro and meso-pores similar to through and diffusive pores in perfusion packing respectively. Monoliths have two important properties, one is that they have a chromatographic efficiency equal to 3-5 μm sized packed columns and the other one is that they show this efficiency with much more pressure drop compared to packed columns. Therefore, speeding up the analysis by increasing the flow rate is much easier without over-pressuring the pump. Beside, their efficiency changes less with increasing flow rate than those of micro-particulate packed columns. Thus, monolithic columns let use of longer columns or use of columns in a series for challenging separations.

Although monolith concept can be traced back to late 1960s and early 1970s with the works to make a single-piece stationary phase from highly swollen polymer gel (38) and open pore polyurethane foams (39), the first paper describing the application of porous silica rods for HPLC was published by Tanaka et al. in 1996 (40). While synthesizing this silica rod, they used sol-gel process which is also the method of making monolithic columns today with some improvements. Sol-Gel process can be summarized as in the Figure 2.9. In this process below polymerization reactions occur at 40 °C:

$$Si(OCH_3)_4 + x\ H_2O \rightarrow Si(OH)_x(OCH_3)_{4-x} + x\ CH_3OH$$

$$\text{-Si-OH} + CH_3O\text{-Si-} \rightarrow \text{-Si-O-Si-} + CH_3OH$$

$$\text{-Si-OH} + \text{HO-Si-} \rightarrow \text{-Si-O-Si-} + H_2O$$

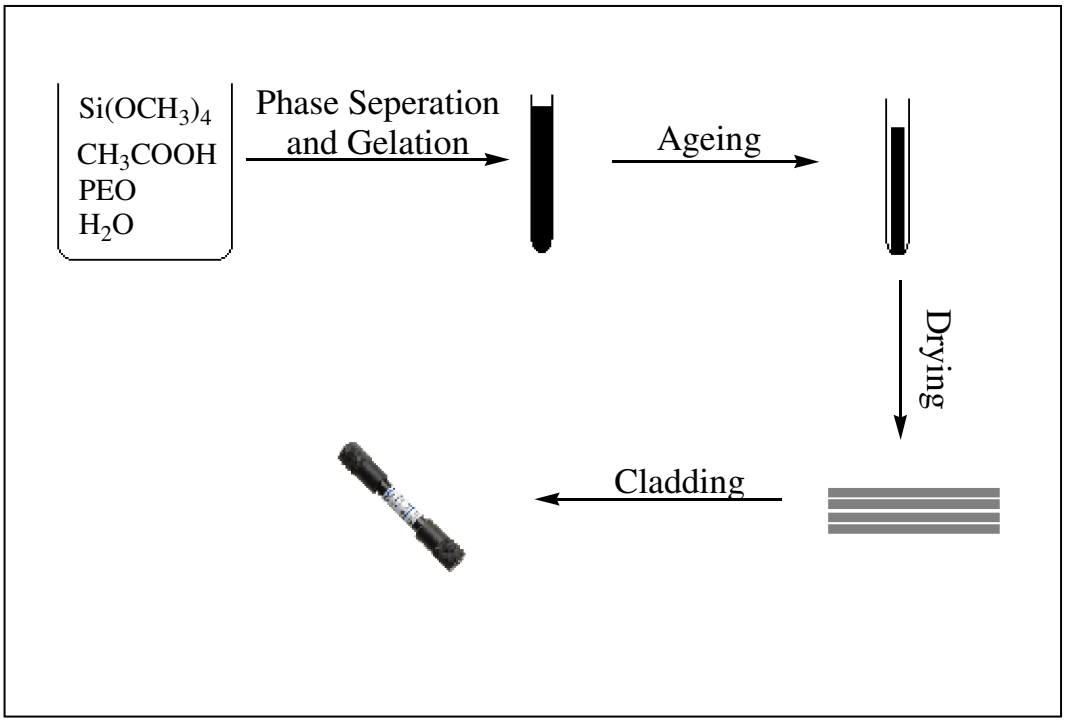

Figure 2.9. Representation of Sol-Gel Process.

The first monolithic HPLC column, which is silica based, is commercialized by Merck Industry with the name of Chromolith in the year 2000 and they have a strong, well-written patent. Immediately Chromolith column attracted significant attention. However certain steps of this production method, particularly drying and cladding, are very tough to handle, especially in academic laboratories. Merck Industry offers only three different models (Si, RP8 and RP18) with five different diameters but only short lengths (10 and 15 cm.). Due to the difficult preparation method of silica monoliths and the strong patent hold by Merck Industry, researchers have been focused on the synthesis of polymeric monoliths. Although a few commercial polymeric monolithic columns exist in the market, polymeric monolith researches have been mostly conducted on academic laboratories to provide the need of easily producible monoliths. The pioneering works for the preparation of polymeric monoliths was performed by Hjerten (41) and contributed much by the work of Svec reported on in-situ polymerization technique (42). Recently, so many studies conducted on the preparation of polymeric monoliths for HPLC applications have been performed, including MIPs (43-46).

Although the use of monolithic columns among chromatographers is not wide-spread yet, due to the lack of variety of commercial monolithic columns, it is obvious that they will dominate the chromatographic separations in the near future with their special abilities. As mentioned before, monolithic columns are able to achieve chromatographic separations as successfully as micro-particulate packed columns but with much lower pressures that eliminate the need for very powerful pumps and therefore in the near future it will reduce the cost of HPLC devices remarkably.

The feature that makes monolithic HPLC columns so advantageous is their skeleton structure with total porosity reaching up to % 90. High porosity means high permeability, so that it is possible to do analysis by using monolithic HPLC columns with a flow rate of 10 mL/min applying a pressure less than 100 bar.

3. MATERIALS AND METHODS

3.1. Chemicals

MAA, AAM, isobornyl methacrylate (IBMA) and EDMA was purchased from Aldrich. 2,2'-Azobis(2-methylpropionitrile) (AIBN) was supplied by Merck (Germany). MAA, IBMA and EDMA were purified by vacuum distillation to remove stabilizers prior to use.

4-AMP and P were purchased from SIGMA (USA). All solvents, 1-dodecanol, benzyl alcohol, dimethylformamide, tetrahydrofuran, acetonitrile (ACN), methanol, acetic acid, were analytical or HPLC grade and obtained from commercial sources.

3.2. Thermal Stability Studies

Thermal stability studies of 4-AMP at 50 °C and 60 °C in different solvent mixtures were conducted as follow; 0.6 mg 4-AMP was dissolved in 12 mL of a mixture of different solvent-porogen pairs. While tetrahydrofuran and dimethylformamide were used as potential solvents, 1-dodecanol and benzyl alcohol were used as potential porogens. 4-AMP was dissolved in a mixture of one solvent and one porogen with different compositions. Then its uv-spectra were continuously taken by a Shimatzu UV-1700 spectrophotometer coupled with a temperature control unit (Shimatzu, CPS-240A) for 24 hours at desired temperature with 1 hour time intervals.

3.3. Preparation of 4-AMP Imprinted Polymeric Monoliths

4-AMP imprinted polymers were prepared by in-situ polymerization technique as shown in the figure 3.1. General procedure was as follow: known amount of template molecule, 4-AMP, was dissolved in 2.1 mL of dodecanol/dimethyl formamide solvent mixture then monomer(s) (0.75 mmol), crosslinker (EDMA) and 12.3 mg initiator, AIBN, were added to the mixture. The

mixture was ultrasonicated for 5 minutes and deoxygenated by a stream of nitrogen for 10 minutes. After the solution was filled in an empty stainless steel HPLC column (150x3.9 mm i.d.) and sealed from both ends, it was placed in a vacuum oven and polymerized in-situ by thermal initiation at 50 °C for 24 hours. After polymerization was completed, the column was connected to an HPLC pump and washed exhaustively with acetic acid/methanol (1/4) (v/v) to remove the template molecule, porogenic solvent and unreacted monomers. Then acetic acid was washed away by flowing methanol through the column. The non-imprinted monoliths for control experiments were also prepared by the same procedure described above but in the absence of 4-AMP. In order to optimize polymerization conditions, some parameters such as polymer composition, solvent-porogen ratio, template-monomer ratio and monomer-crosslinker ratio were adjusted as listed in the table 3.1.

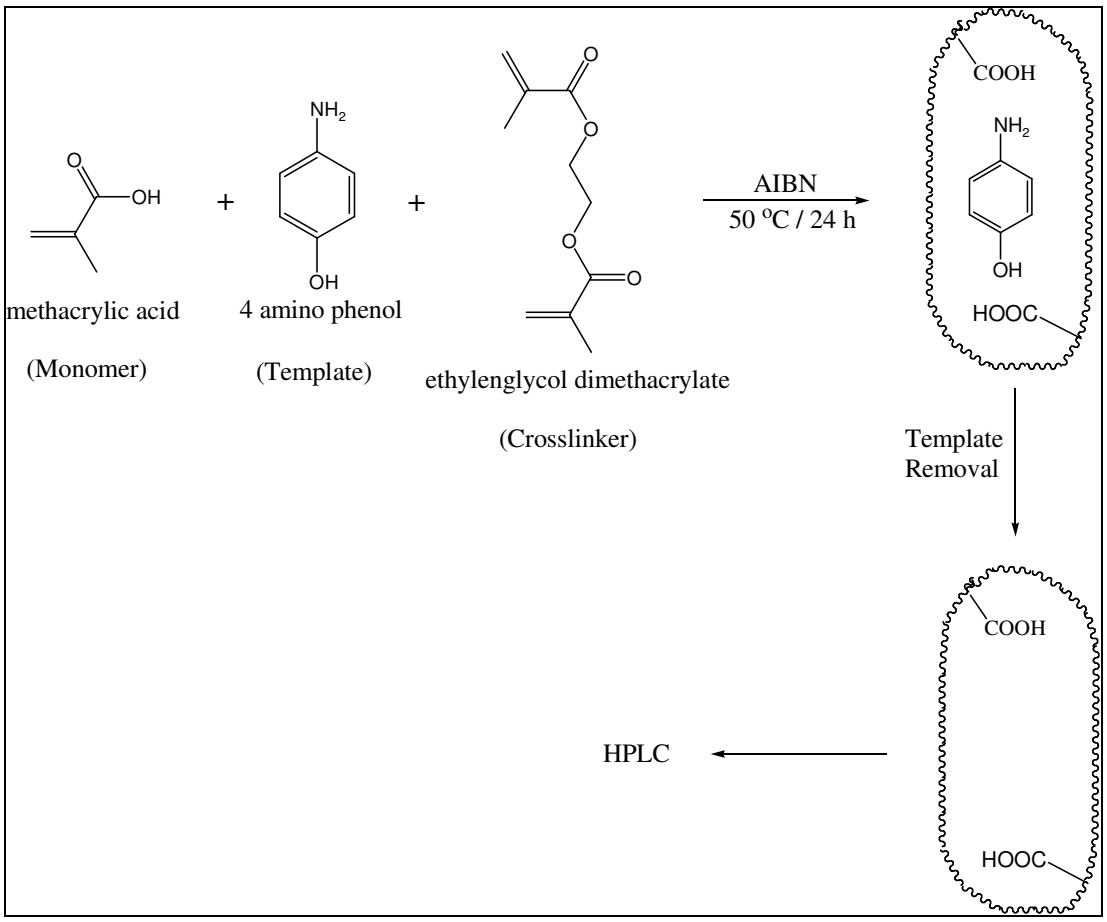

Figure 3.1. Schematic representation of general molecular imprinting procedure.

Table 3.1. List of prepared polymeric monoliths.

Polymer	MAA:AAM Ratio (mole:mole)	Template-Monomer Ratio (mole:mole)	Monomer-Crosslinker Ratio (mole:mole)	Solvent-Porogen Ratio (v:v)
Optimization of Polymer Composition				
MIP_1	1:0	1:4	1:5	1:3
MIP_2	3:1	1:4	1:5	1:3
MIP_3	1:1	1:4	1:5	1:3
Optimization of Solvent-Porogen Ratio				
MIP_1	1:0	1:4	1:5	1:3
MIP_4	1:0	1:4	1:5	1:4
Optimization of Template-Monomer Ratio				
MIP_5	1:1	1:2	1:5	1:3
MIP_3	1:1	1:4	1:5	1:3
MIP_6	1:1	1:6	1:5	1:3
MIP_7	1:0	1:2	1:5	1:3
MIP_1	1:0	1:4	1:5	1:3
MIP_8	1:0	1:6	1:5	1:3
Optimization of Monomer-Crosslinker Ratio				
MIP_9	1:0	1:6	1:4	1:3
MIP_8	1:0	1:6	1:5	1:3
MIP_{10}	1:0	1:6	1:6	1:3
Control Experiment				
NIP_6	1:1	Non-Imprinted	1:5	1:3
NIP_9	1:0	Non-Imprinted	1:4	1:3

3.4. Morphological Analysis

The topographic morphologies of monolith samples were examined by scanning electron microscopy (SEM) (JEOL 6400). Micrographs of monoliths were obtained after being coated with gold.

The surface area and meso-pore properties were determined by a Quantachrome**,** Autosorb-1 surface area and pore size analyzer by applying nitrogen as adsorbant. BET and DFT-Monte-Carlo models were used for the evaluations of surface area and meso-pore properties respectively.

3.5. HPLC Analyses

3.5.1. Chromatographic Conditions

HPLC (Dionex Ultimate 3000 system) analyses of 4-AMP and P were carried out in ACN with a flow rate of 1 mL/min at 25 °C. Detection was via diode array UV detector at 300 nm. Void volume was determined by injection of acetone diluted by ACN (1:9). Injection volume was 20 μL.

3.5.2. Preparation of Standard Samples

A series of mixture of 4-AMP and P (0.1, 0.2, 0.5, 0.75 and 1.0 mmol/L) having the same concentration for both of the compounds in each standard were prepared in ACN. Solutions having a concentration of less than 1 mmol/L were prepared by diluting the solution of 1.0 mmol/L with ACN.

3.5.3. Calculations of Chromatographic Parameters

Capacity Factor

Capacity factors (k') were directly calculated from the chromatograms of mixture (0.1 mM) by using the equation:

$$k' = (t_R - t_0) / t_0 \quad (3.1)$$

where t_R and t_0 are the retention times of the analyte and the void marker, respectively. t_R values were calculated by average of six injections.

Resolution

Resolution (R_S) of 4-AMP and P peaks was calculated from the chromatograms of mixture (0.1 mM) by using the equation:

$$R_S = 1.18 \times [(t_2 - t_1)/(W_{\%50\,(2)} + W_{\%50\,(1)}) \quad (3.2)$$

Where t_2 and t_1 are the retention times of 4-AMP and P, respectively and $W_{\%50\,(2)}$ and $W_{\%50\,(1)}$ are the peak widths at % 50 peak height of P and 4-AMP, respectively.

Selectivity

Selectivity (α) of the molecularly imprinted monoliths for 4-AMP against P was directly calculated from the chromatograms by using the following equation:

$$\alpha = (t_2 - t_0) / (t_1 - t_0) \quad (3.3)$$

where t_2 and t_1 are the retention times of 4-AMP and P, respectively and t_0 is the retention time of the void marker (acetone).

Peak Asymetry

Peak asymmetry (A) values was calculated by using the equation:

$$A = (RW_{\%5} + LW_{\%5}) / 2 \times LW_{\%5} \quad (3.4)$$

where $RW_{\%5}$ and $LW_{\%5}$ are the right and left peak widths of a given peak at % 5 peak height.

3.6. Frontal Chromatography

The binding properties of imprinted and non-imprinted monoliths were investigated by frontal chromatography method introduced into MIPs by Mosbach in 1991 (47). This method enables the determination of the dissociation constant (K_D) and the effective number of binding sites (L_t) for the interaction between the template and the polymer monolith.

$$1/([A]_0 (V-V_0)) = (K_D / ([A]_0 L_t)) + 1 / L_t \quad (3.5)$$

Using the equation above, effective number of binding sides and dissociation constant were calculated from the plot of $1/[A]_0 (V-V_0)$ versus $1/[A]_0$, where $[A]_0$ is the concentration of the analyte, V and V_0 are elution volumes of the analyte and

void volume, respectively. The number of effective binding sites was calculated from the intercept on the ordinate ($1/L_t$) and the dissociation constant was calculated from the intercept on the abscissa ($-1/K_D$).

In frontal analysis, different concentrations of 4-AMP and P (0.1, 0.2, 0.5 and 1.0 mmol/L) prepared in mobile phase were loaded to the column at 25 °C as follow: the column was connected to the HPLC and equilibrated with mobile phase at 300 nm. Then the column was disconnected from the system, the tube from the reservoir to the inlet of the monolithic column was filled with the mobile phase solution containing the lowest concentration of the analyte and the column was re-connected to the system. After that, the solution was pumped to the column with a flow rate of 1 mL/min and simultaneously recording the signal at 300 nm, the breakthrough curve was obtained. In the following, the column was re-equilibrated and the solution containing the next higher 4-AMP concentration was pumped to the column. By this way a breakthrough curve was obtained for each 4-AMP solution. The same procedure was also applied to acetone/ACN (5/95) solution which was used as a void marker. Binding isotherms can also be obtained from break-through curves by using the equation 3.6.

$$\text{Amount of adsorbed analyte} = [A]_0 (V-V_0) \quad (3.6)$$

3.7. Imprinting Factor

Imprinting factor (IF) is used to determine whether or how much the imprinting process makes difference in MIPs. It was calculated from the equation:

$$IF = k'_{\text{imprinted}} / k'_{\text{non-imprinted}} \quad (3.7)$$

where $k'_{\text{imprinted}}$ and $k'_{\text{non-imprinted}}$ are the capacity factors of 4-AMP imprinted and non-imprinted monolithic columns, respectively.

4. RESULTS AND DISCUSSION

In this study, a series of molecularly imprinted polymeric monoliths which are selective for 4-AMP were prepared by in-situ polymerization technique in a stainless steel HPLC column (150 x 3.9 mm i.d.). The prepared MIP monoliths have been used as stationary phase in HPLC to separate structurally similar compounds; 4-AMP and P.

Figure 4.1. An example photograph of MIP monoliths which were pushed out of the column by the help of an HPLC pump.

The preparation process of molecularly imprinted monolithic stationary phases is quite simple. However the affinity and selectivity of the MIP monolith towards the template molecule are dependent on several factors such as choice of monomer, crosslinker, solvent and porogen, concentration of functional monomer-template and monomer-crosslinker, methodology and polymerization temperature etc. (6). Preparation conditions affecting the performance of molecularly imprinted monolithic stationary phases will be discussed in the following section.

4.1. Optimization of Polymerization Conditions

4.1.1. Selection of Solvent-Porogen Pair

For a successful imprinting by non-covalent interactions, it is crucial to use solvents which have no or low hydrogen bonding capacity. Therefore the solubility and the thermal stability of 4-AMP in various proper solvent and porogen mixtures was studied. It was seen that 4-AMP is soluble in pure dimethyl formamide, in pure tetrahydrofuran, in pure ACN and insoluble in pure toluene. Benzyl alcohol and 1-dodecanol was studied as potential porogens. While dimethylformamide and tetrahydrofuran were miscible with both of the porogens for solvent/porogen compositions starting from 1/1 to 1/5 and showed no solubility problem, ACN is completely immiscible with these porogens. Thus thermal stability studies were carried on at 50 and 60 ºC in mixtures of dimethylformamide/dodecanol, dimethylformamide/benzyl alcohol, tetrahydrofuran/dodecanol and tetrahydrofuran/benzyl alcohol mixtures with different compositions. It was observed that 4-AMP is much more stable in mixtures involving dimethylformamide and 1-dodecanol at 50 ºC (Figure 4.2) than it is in other pairs. Therefore dimethylformamide and 1-dodecanol were chosen as the solvent and as the porogen, respectively. It was also observed that the stability of 4-AMP is decreasing with increasing 1-dodecanol amount in the mixture and with increasing temperature.

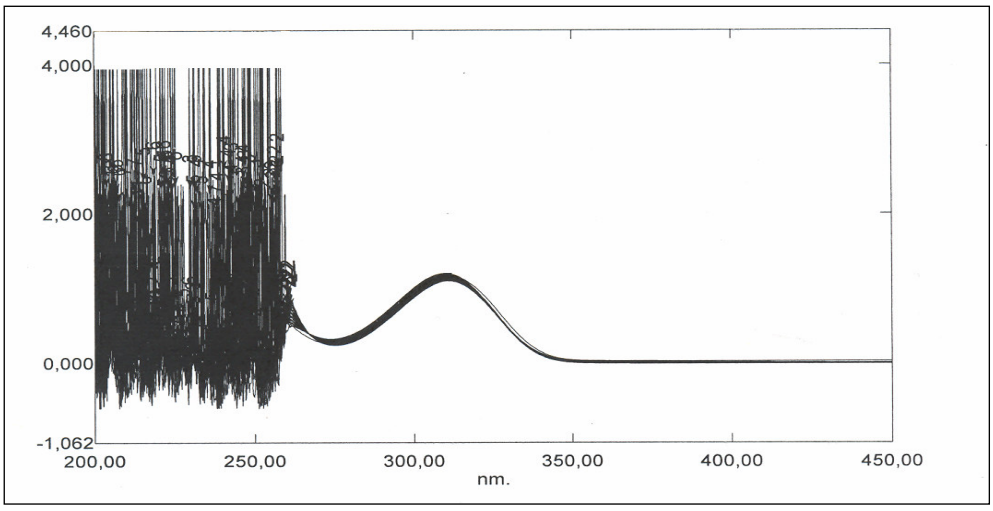

Figure 4.2. UV spectrum of 4-AMP in dimethylformamide/dodecanol (1/3) at 50 °C.

4.1.2. Selection of Functional Monomer

Selective recognition properties of MIPs are critically based on the strength and positioning of monomer-template interactions (6,8). Therefore the selection of functional monomer to obtain MIP monolith with good molecular recognition properties is a crucial step. For these reason, a polar-acidic monomer, MAA, a polar-neutral monomer, AAM and an apolar monomer, IBMA, were tried individually by employing ethylenglycol dimethacrylate as crosslinker in MIP monolith synthesis. Polymerization conditions were the same as in MIP_1 (see table 3.1).

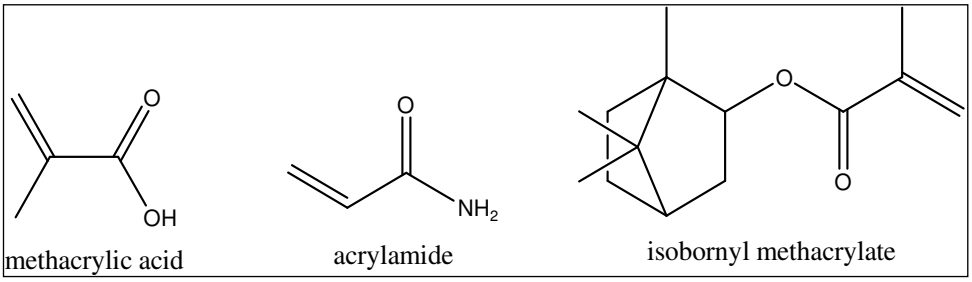

Figure 4.3. Structures of functional monomers.

When MAA was used as the monomer MIP monolith exhibited good retention, while the other MIP monoliths had almost no retention to the template molecule. This result indicates that the stable complex formed between MAA and 4-AMP, probably due to the strong hydrogen bonding interactions as suggested in the figure 4.4. Since IBMA is an apolar monomer, it cannot interact with the polar template molecule so no retention to 4-AMP ($k' = 0.07$) occurred as expected. However, it was very surprising that MIP monolith showed almost no retention ($k' = 0.3$) to the template molecule, when AAM was used as the functional monomer. The reason may come from that the amide group on the AAM monomer forms weak hydrogen bonds with the template molecule so that formed monomer-template complex does not have enough stability during imprinting process. Besides, for the basic template molecule (pKa=10.46), the acidic MAA would be the best imprinting monomer. For this reason MAA was chosen as the functional monomer, on the other hand AAM was also used as a co-monomer in order to see the co-monomer effect in MIP monoliths.

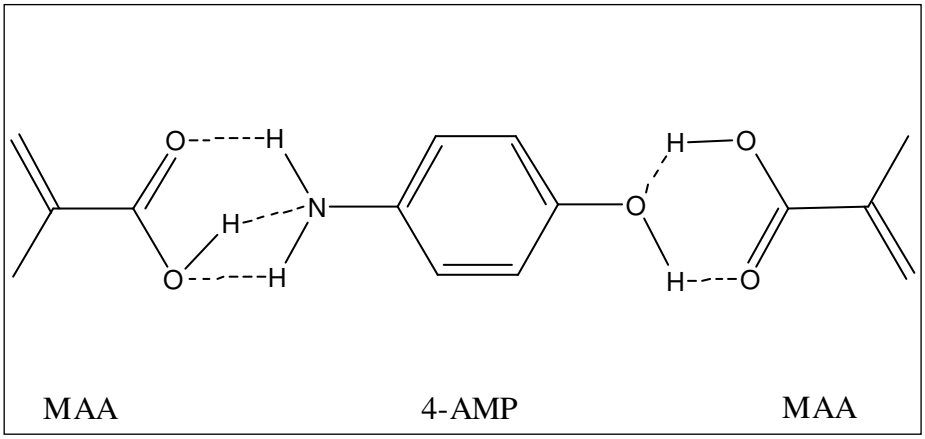

Figure 4.4. Suggested interactions between MAA and 4-AMP.

MIP_2 and MIP_3 monoliths were also prepared as copolymers of MAA and AAM involving 25 % and 50 % AAM monomer in total moles of monomer, respectively, by keeping all other conditions constant. When chromatograms of MIP_1 and MIP_3 columns were compared, it was observed that both resolution and selectivity decreased from 1.51 (MIP_1) to 1.29 (MIP_3) and from 3.86 (MIP_1) to 2.15 (MIP_3), respectively, with increasing AAM amount. The weak interactions between the template molecule and AAM and the competition of the monomer-monomer interaction with the monomer-template interactions in the pre-polymerization step might cause a decrease in both resolution and selectivity of MIP_3 monolith. In addition, the peak symmetry and capacity factor of 4-AMP also decreased by the existence of AAM in MIP monoliths as seen in the table 4.1.

Table 4.1. Some chromatographic parameters of MIP_1, MIP_2 and MIP_3 monoliths.

Polymer	**t_R (min.)**		**k'**		**A**		**R_S**	**α**
	4-AMP	P	4-AMP	P	4-AMP	P		
MIP_1	4.834	2.475	1.93	0.50	1.64	0.87	1.51	3.86
MIP_2	3.567	2.392	1.10	0.41	2.05	0.78	1.32	2.70
MIP_3	3.417	2.475	1.06	0.49	1.93	0.81	1.29	2.15

4.1.3. Optimization of Solvent-Porogen Ratio

The most advantageous property of monolithic columns is their low back pressure they show against the flow of mobile phase. In this study, the back pressure of MIP_1 column was about 22 bars using ACN as mobile phase with the flow rate of 1 mL/min. Although this pressure seems to be very low when compared to packed columns, it is not such a low pressure for a monolithic column. Therefore it was tried to reduce the column pressure to lower values by increasing the amount of porogen; 1-dodecanol, from 1:3 to 1:4 (v:v) (MIP_4) in the polymerization mixture. The column pressure of MIP_4 monolith was reduced to 4 bars for the same chromatographic conditions mentioned above, due to the bigger lumps (porons) and higher external porosity formed in the MIP_4 monolith (figure 4.5.).

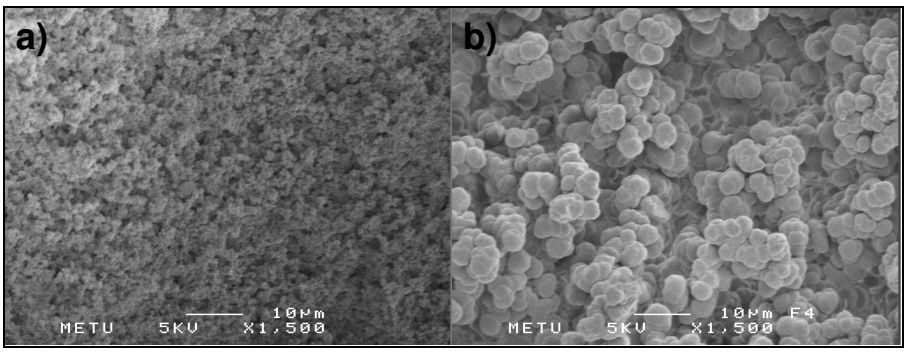

Figure 4.5. SEM images of a) MIP_1 and b) MIP_4 monoliths.

However, the resolution of MIP_4 column reduced to 1.27 as seen in the table 4.2. The resolution decreases despite the fact that while capacity factor of 4-AMP was increased significantly; there was a small increase in capacity factor of P. This decrease in resolution comes from the broadening of 4-AMP peak as seen in figure 4.6. According to Van Deemter's equation, three reasons might cause this band broadening; multiple pathways in the column (i.e. inhomogeneity of the column), longitudinal diffusion of the analyte molecules and low mass transfer rate of the analyte between mobile and stationary phase. For such porous monolithic columns, the first reason can not cause that band broadening and considering the flow rate of 1 mL/min the second one can not either. So it can be said that the reason for this band

broadening is that the equilibration time of the analyte molecules between the mobile phase and stationary phase gets longer with increasing poron size.

Table 4.2. Comparison of chromatographic properties of MIP_1 and MIP_4 monoliths.

Polymer	**t_R (min.)**		**k'**		**A**		**R_S**	**α**
	4-AMP	P	4-AMP	P	4-AMP	P		
MIP_1	4.834	2.475	1.93	0.5	1.64	0.87	1.51	3.86
MIP_4	4.858	2.0	2.91	0.61	1.39	1.26	1.27	4.81

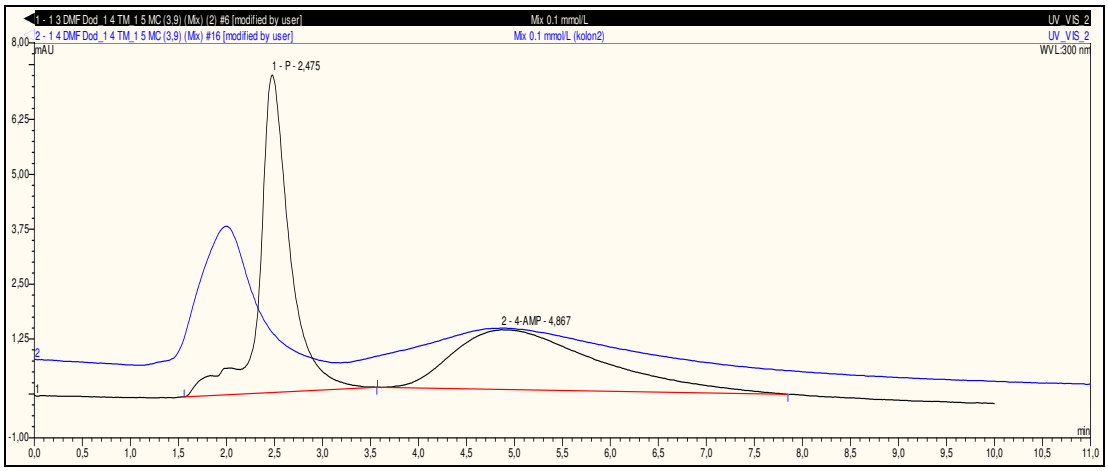

Figure 4.6. Chromatograms of MIP_1 (black) and MIP_4 (blue). Chromatographic conditions: injection of 4-AMP and P mixture (0.1 mM in each), 1 ml/min mobile phase flow of ACN, detection at 300 nm.

Due to the lost of resolution in MIP_4 monolith, using solvent:porogen ratio as 1:3 (v:v) for further investigations was preferred, in spite of the fact that all other parameters (k', α and A) are better than those of MIP_1 column.

4.1.4. Optimization of Template-Monomer Ratio

Template-monomer ratio is an important factor for molecular imprinting. In general, functional monomer is added in large excess to increase the possibility of surrounding the template molecules with functional monomers in non-covalent

approach. Yet, increasing the functional monomer amount may also increase the non-specific binding sites. On the other hand low monomer-template ratio affords less than optimal complexation on account of insufficient functional monomer (6, 8). Therefore a proper template-monomer ratio has to be set. In this study, the influence of template monomer ratio (1:2, 1:4 and 1:6) on the chromatographic parameters, particularly on resolution, was evaluated for MIP_1 and MIP_3. For MIP monolith with MAA:AAM ratio of 1:1, it was tried to improve the resolution by altering the template monomer ratio. However, the resolution could not be improved further than 1.30 (MIP_6) (Figure 4.7.). Thus no further research was done for the copolymer of MAA-AAM monolith.

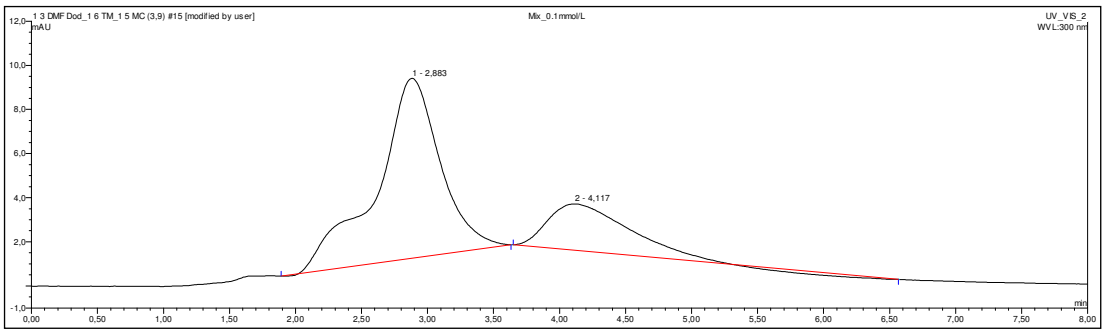

Figure 4.7. Chromatogram of MIP_6. Chromatographic conditions: injection of 4-AMP and P mixture (0.1 mM in each), 1 ml/min mobile phase flow of ACN, detection at 300 nm.

As seen in the table 4.3., there is a tendency of increasing resolution but decreasing selectivity with decreasing template amount in the polymerization mixture. MIP_7 monolithic column, with template monomer ratio of 1:2, has the highest selectivity (5.59) but its resolution was very low (1.18). On the other hand, MIP_8 monolith, with the template:monomer ratio of 1:6, has the highest resolution (1.61). By also taking into account the sharpness of peaks, template: monomer ratio of 1:6 (MIP_8) was chosen as the optimum ratio (Figure 4.8).

Table 4.3. Influence of template:monomer ratio on chromatographic parameters.

Polymer	**t_R (min.)**		**k'**		**A**		**R_S**	**α**
	4-AMP	P	4-AMP	P	4-AMP	P		
MIP_5	3.158	2.150	1.11	0.43	2.52	1.26	1.15	2.55
MIP_3	3.417	2.475	1.06	0.49	1.93	0.81	1.29	2.15
MIP_6	4.092	2.883	1.27	0.60	1.84	0.84	1.30	2.12
MIP_7	5.82	2.396	2.53	0.45	1.55	1.32	1.18	5.59
MIP_1	4.834	2.475	1.93	0.50	1.64	0.87	1.51	3.86
MIP_8	4.667	2.562	1.72	0.49	2.17	0.89	1.61	3.49

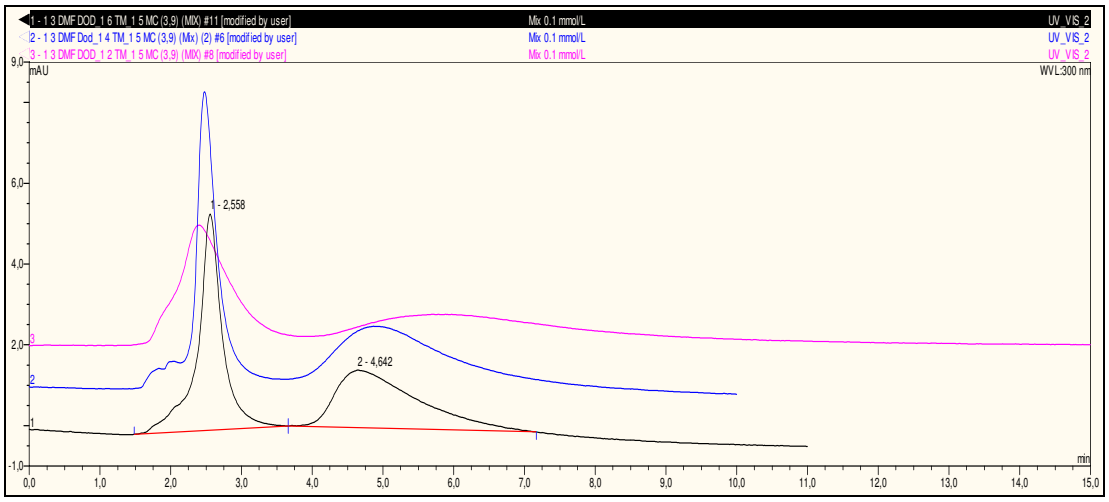

Figure 4.8. Chromatograms of MIP_7 (pink), MIP_1 (blue) and MIP_8 (black). Chromatographic conditions: injection of 4-AMP and P mixture (0.1 mM in each), 1 ml/min mobile phase flow of ACN, detection at 300 nm.

4.1.5. Optimization of Monomer-Crosslinker Ratio

In molecular imprinting technology, the amount of crosslinker has a crucial role which is not only to maintain the stability of recognition sites by forming rigid specific cavities for the template molecule but also to have some extent of flexibility in order to make the template molecules enter the cavities easily (48). Therefore an appropriate monomer-crosslinker ratio should be maintained. In this study, monomer-crosslinker ratios of 1:4 (MIP_9), 1:5 (MIP_8) and 1:6 (MIP_{10}) were examined. When the monomer-crosslinker ratio was 1:4, the retention time of 4-AMP was significantly increased to 9.1 (table 4.4.). This significant increase means

that the required flexibility for template molecules to be able to enter the cavities easily was achieved. Thus, capacity factor, resolution and selectivity values were also increased significantly to 4.1, 1.91 and 5.68 respectively. At higher crosslinker concentrations (MIP_8 and MIP_{10}) the recognition for the template molecule 4-AMP decreased due to the difficulty of entering the cavities of rigid MIPs for the template molecule (Figure 4.9).

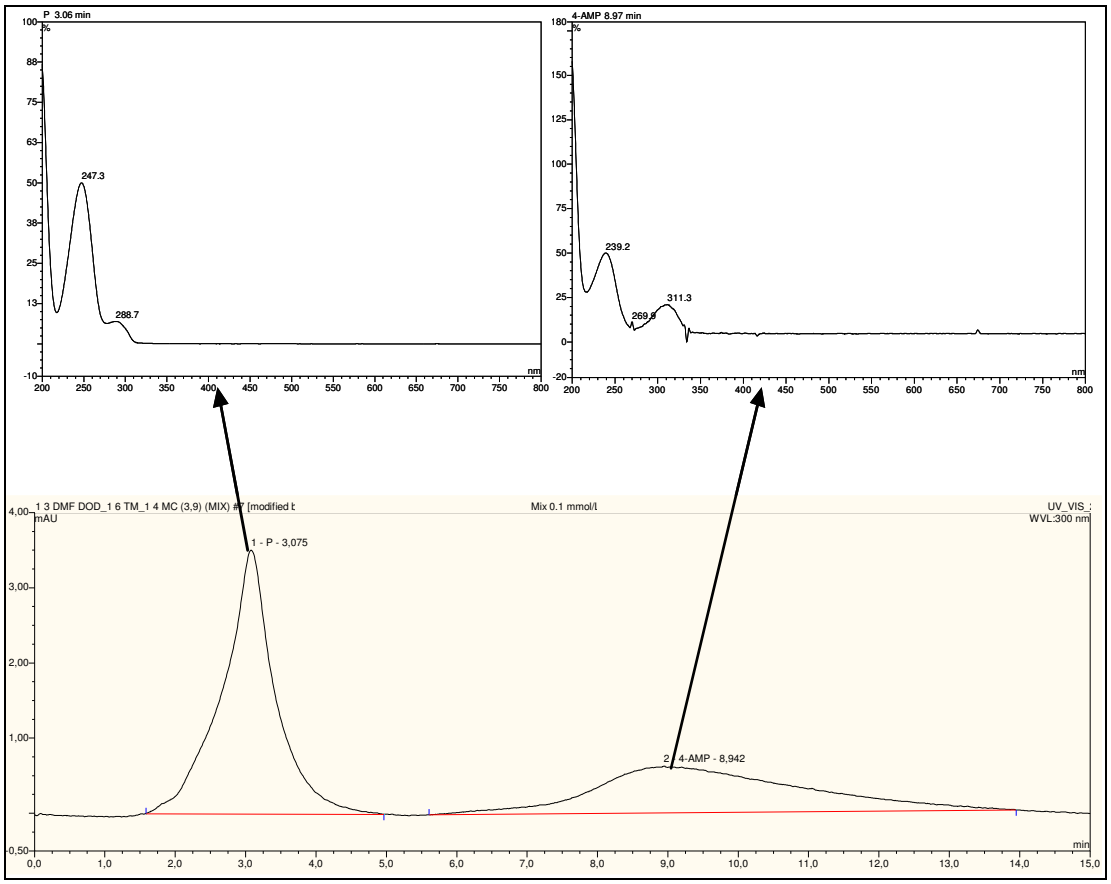

Figure 4.9. Chromatogram of MIP_9 with UV-spectra of 4-AMP (upper right) and P (upper left) peaks. Chromatographic conditions: injection of 4-AMP and P mixture (0.1 mM in each), 1 ml/min mobile phase flow of ACN, detection at 300 nm.

Table 4.4. Effect of crosslinker concentrations on chromatographic parameters.

Polymer	**t_R (min.)**		**k'**		**A**		**R_S**	**α**
	4-AMP	P	4-AMP	P	4-AMP	P		
MIP_8	4.667	2.562	1.72	0.49	2.17	0.89	1.61	3.49
MIP_9	9.10	3.072	4.10	0.72	1.33	0.96	1.91	5.68
MIP_{10}	4.39	2.170	2.03	0.50	1.68	1.44	1.49	4.08

4.2. Overall Chromatographic Evaluation of MIP Monolith

In order to decide which MIP monolith has given the best chromatographic results, it would be beneficial to look at their resolution, selectivity and peak asymmetries. When the resolution is considered, a lower limit of 1.4 was set in this study (in general, values higher than 1.25 is acceptable and the resolution of 1.5 is accepted as ideal among chromatographers). As seen in the figure 4.10., MIPs whose resolution is higher than 1.4 are MIP_1 (1.51), MIP_8 (1.61), MIP_9 (1.91) and MIP_{10} (1.49). For selectivity, monoliths showing the highest values are MIP_1 (3.86), MIP_4 (4.81), MIP_7 (5.59), MIP_8 (3.49), MIP_9 (5.68) and MIP_{10} (4.08) as shown in the figure 4.11.

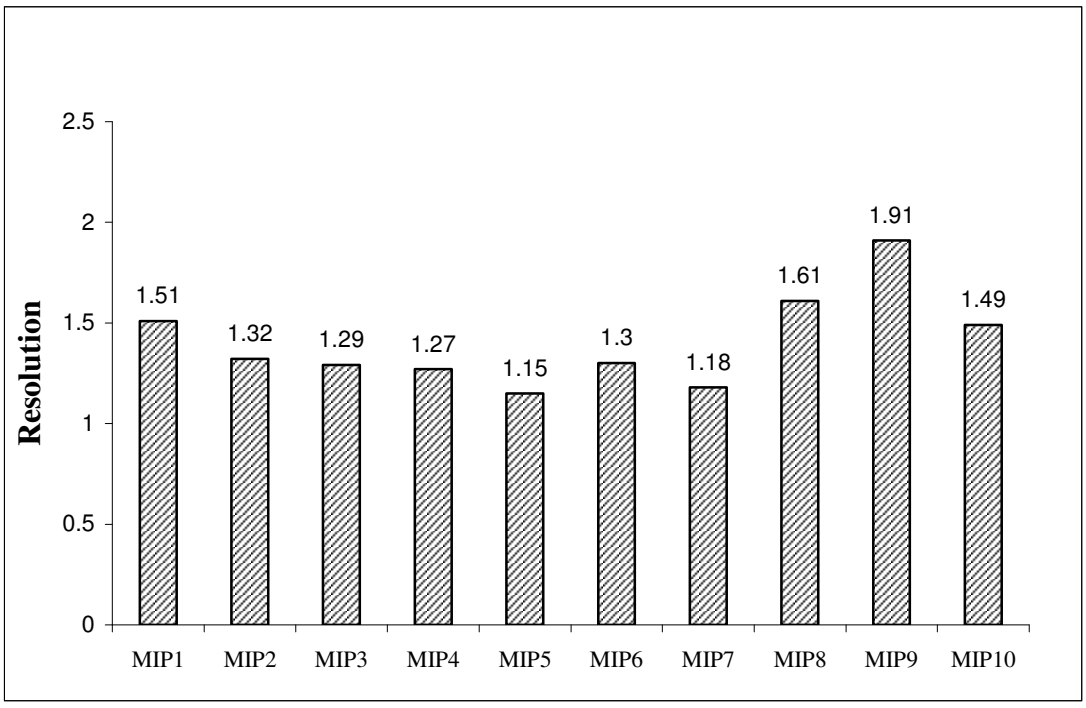

Figure 4.10. Resolution diagram of MIP monoliths.

Peak asymmetry is another parameter that helped us decide the column efficiency of MIP monoliths. The ideal value of peak asymmetry (A_S) is 1. The monolith which has the nearest A_S values to ideal case both for 4-AMP and P is MIP_9 with A_S of 1.33 for 4-AMP and 0.96 for P (figure 4.12.). These values are much better than those of other MIP monoliths. In the view of these findings, it can

easily be said that MIP_9 monolith, with no doubt, has the best column efficiency when compared to other MIP monoliths.

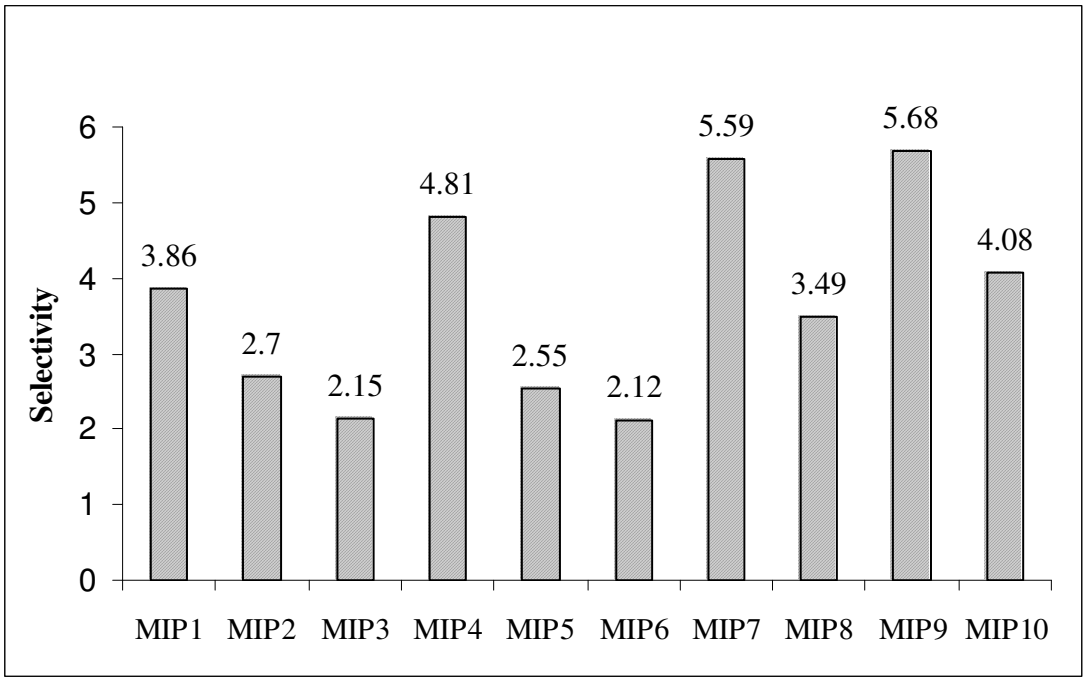

Figure 4.11. Selevtivity diagram of MIP monoliths.

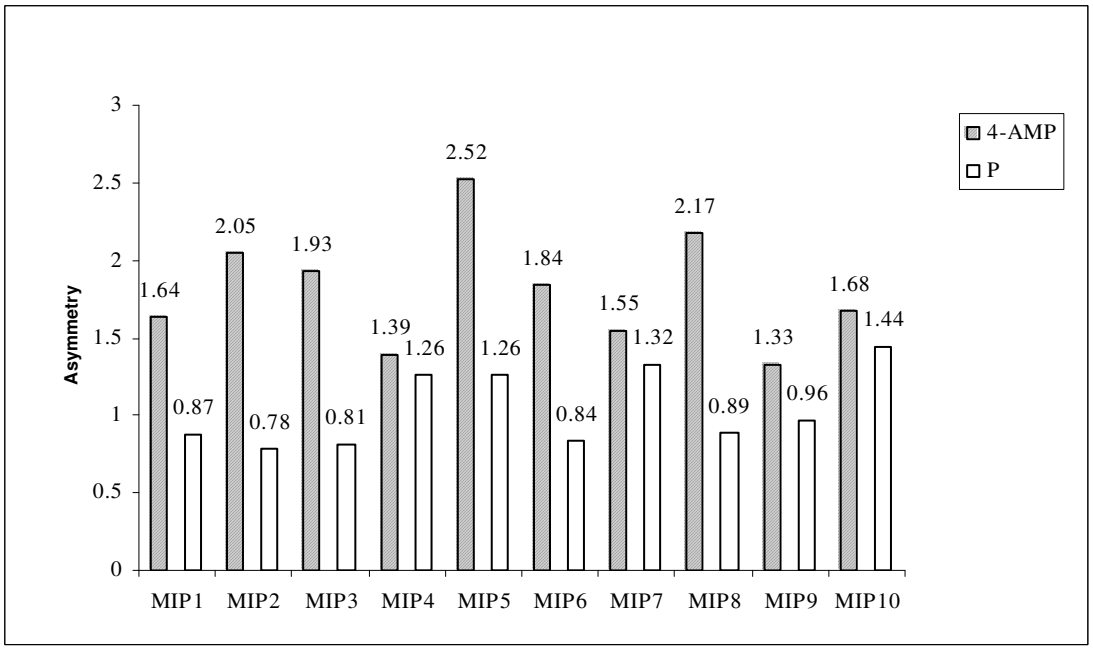

Figure 4.12. Peak asymmetry diagram of MIP monoliths.

In order to see if there is any leakage in the column, column pressure of MIP_9 column against increasing flow rate was also examined by applying ACN as mobile phase (figure 4.13.). The column pressure increased linearly (R^2 = 0.9982) with increasing flow rate. The SEM image of a MIP column taken from its wall was also an evidence of good adsorption between polymer and the inner wall of HPLC column (figure 4.14.).

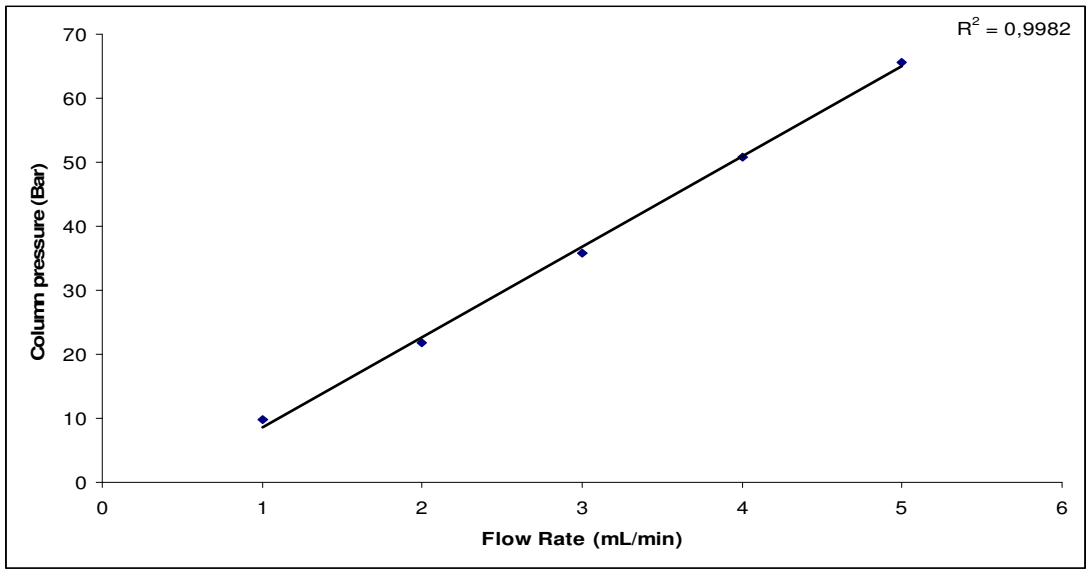

Figure 4.13. Plot of column pressure vs. flow rate for MIP_9 column.

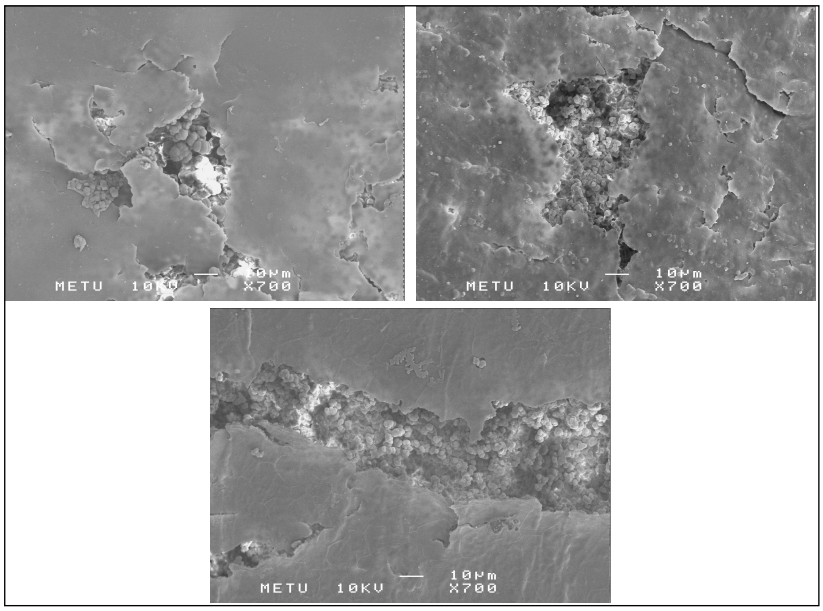

Figure 4.14. SEM images of walls of some MIPs.

4.3. Morphological Characteristics of Monoliths

The morphological characteristics of MIPs were evaluated by SEM and surface area and pore size analyzer. The SEM analysis showed that the morphological structures of MIPs showed some differences with changing polymerization conditions. As mentioned before, the poron size of MIP_4 was significially greater than those of MIP_1 as a result of higher porogen amount and therefore the surface area was also decreased from 49.77 m^2/ g (MIP_1) to 17.80 m^2/g. It was observed that changing the template: monomer ratio does not have any significant effect on surface area of MIPs. SEM images of MIP_9 and MIP_8 monoliths (figure 4.15) show that the poron size of the monolith increased with decreasing crosslinker amount which leads to bigger flow through pores and so that the column pressure could be reduced to 10 Bars from 22 Bars. This also results in a lower surface area of 23.48 m^2/g. The size of porons in MIPs involving AAM increased significantly, therefore the skeleton structure and flow through pores of monoliths can be better seen in the SEM images of MIP_3 (figure 4.16.).

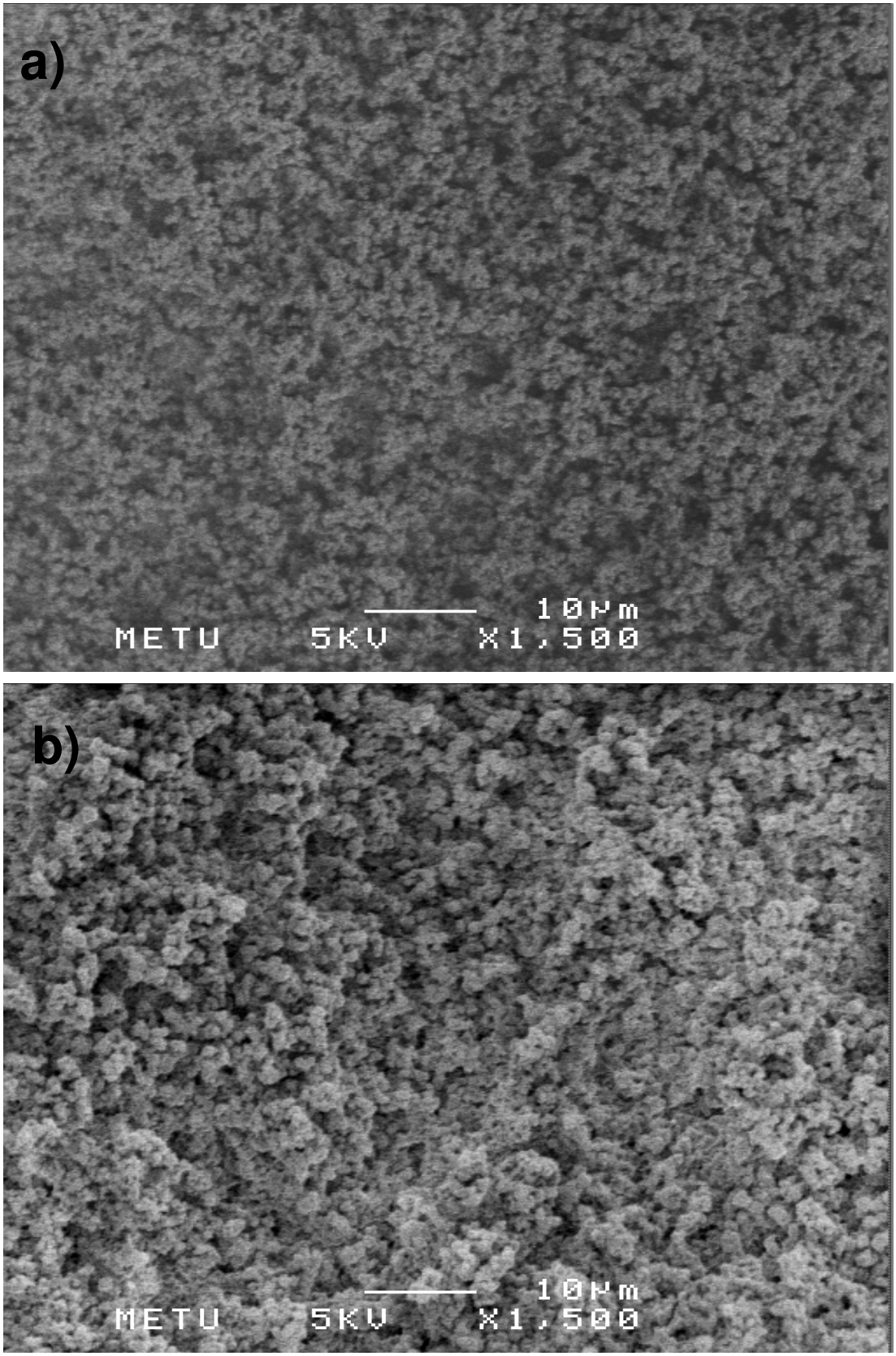

Figure 4.15 SEM images of a) MIP_8 and b) MIP_9 monoliths.

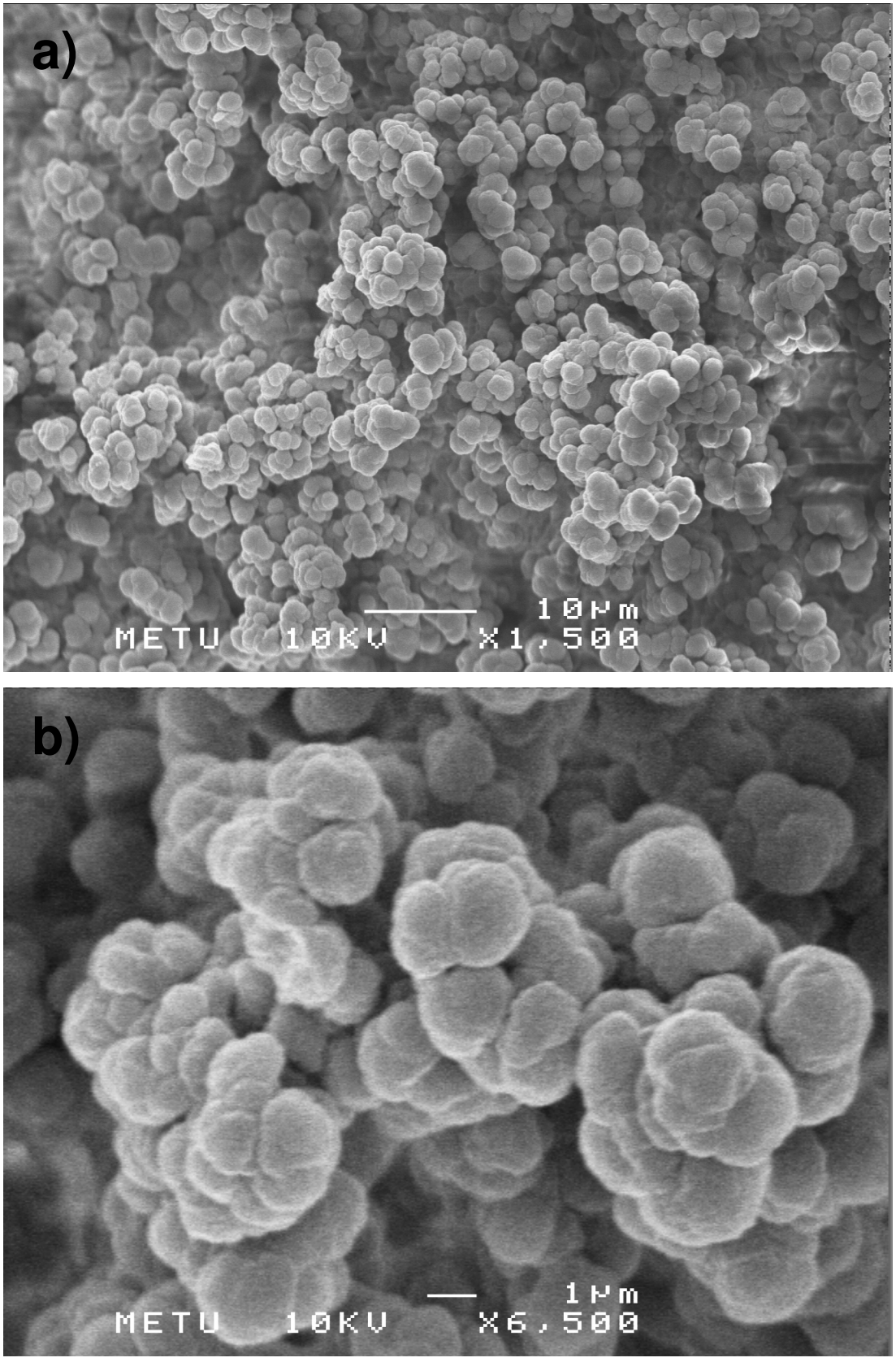

Figure 4.16. SEM images of MIP_3 monolith a) x 1500 fold b) x 6500 fold.

When surface areas of MIP_9 and its non-imprinted monolith (NIP_9) were compared, it was seen that the surface area of NIP_9 monolith (105 m^2/g) was about 4 times more than those of MIP_9 (23.48 m^2/g). Since the existence of template does not change the macro-pore structure of polymer significantly, higher surface area of NIP_9 should have come from the different meso-pore structures. Meso-pore size distributions of both monoliths were given in the figure 4.17 and 4.18. As seen, the main meso-pore size was the same which is 5 nm for MIP_9 and NIP_9. However, total pore volume of this pore increased significantly in NIP_9. In order to show this more precisely, total pore volume of meso-pores smaller than 43 nm was calculated for MIP_9 and NIP_9 and found as 2.99 x 10^{-2} cc/g and 9.24 x 10^{-2} cc/g respectively. Therefore, it can be said the existence of template molecule in the polymerization mixture changes the meso-pore structure of monolith in the direction of decreasing the amount of meso-pores so that the surface area of MIP_9 monolith decreases. It was also observed that the meso-pores of NIP_9 monolith are much more well-defined than meso-pores of MIP_9. While NIP_9 monolith has almost no meso-pore bigger than 5 nm, MIP_9 monolith has some other meso-pores reaching up to 30 nm as seen in the Figure 4.17 and Figure 4.18. From this point, it can be suggested that size of specific cavities created by molecular imprinting technique were between 5 and 30 nm, mostly around 10 nm (Figure 4.17).

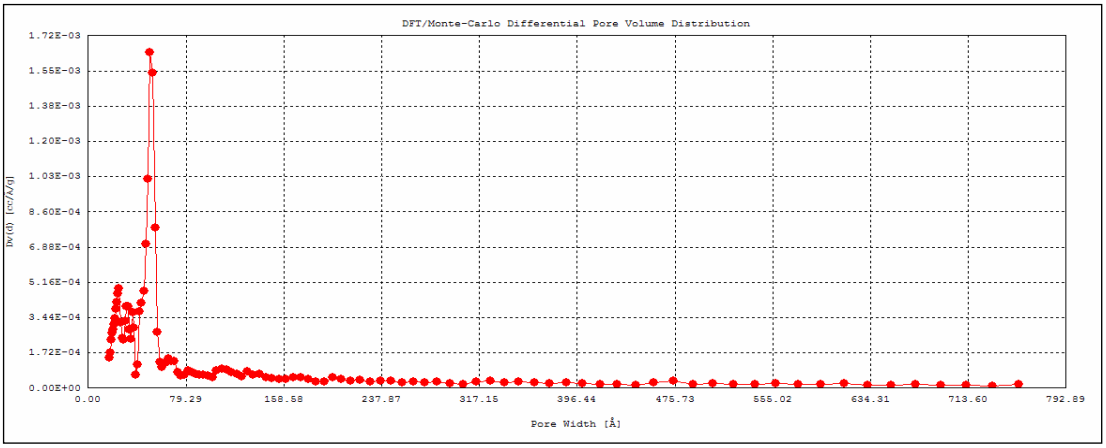

Figure 4.17. Meso-pore size distribution of MIP_9 monolith.

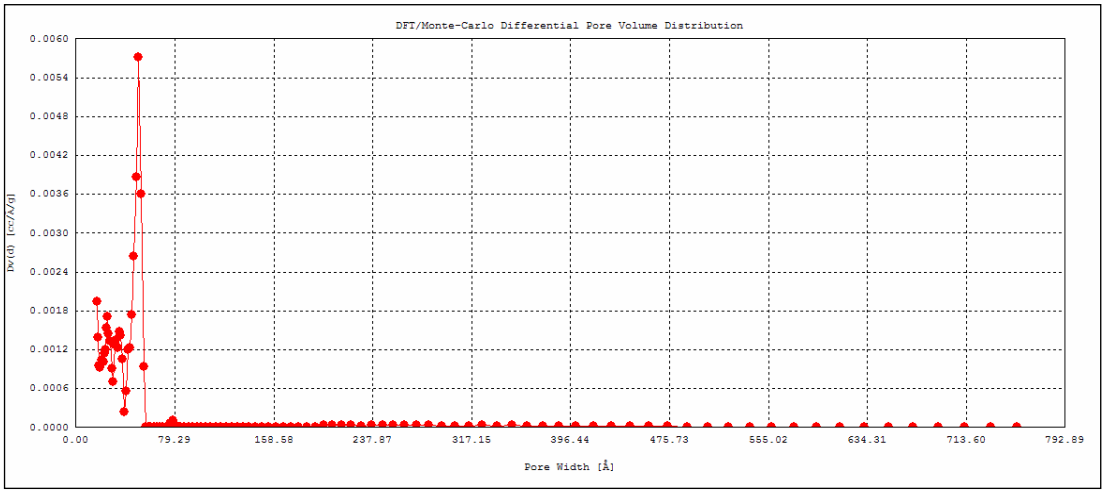

Figure 4.18. Meso-pore size distribution of NIP_9 monolith.

4.4. Effect of Molecular Imprinting

In order to evaluate the effect of molecular imprinting, MIP_9 and its non-imprinted monolith were compared. HPLC analysis showed that monolithic column of MIP_9 could successfully separate our two analytes (figure 4.19), 4-AMP and P, with a resolution of 1.91 and with a selectivity of 5.68 (table 4.5). While the retention time of P was only 3.072 on MIP_9 column, retention time of 4-AMP was 9.10 and the capacity factors of P and 4-AMP were 0.72 and 4.10, respectively. However, it was very surprising that the non-imprinted column could also separate, but not with an acceptable resolution, such two molecules with similar structures and polarities showing some selectivity, even if not as much as MIP_9. Probably, the difference between the elution times of 4-AMP and P comes from the strong hydrogen bonding interactions between 4-AMP and functional groups on the surface of polymer backbone of NIP_9 monolith as suggested in the (figure 4.20a). As figured out from the HPLC analysis, P molecules could not form such hydrogen bonding interactions with the functional groups on NIP_9 surface. Since 4-AMP and P differs structurally only on amide group, the difference should have come from this side. Intra-molecular H-bonding of P molecule prevents the formation of hydrogen bonding (figure 4.20b) with the functional groups on polymer monolith during the elution.

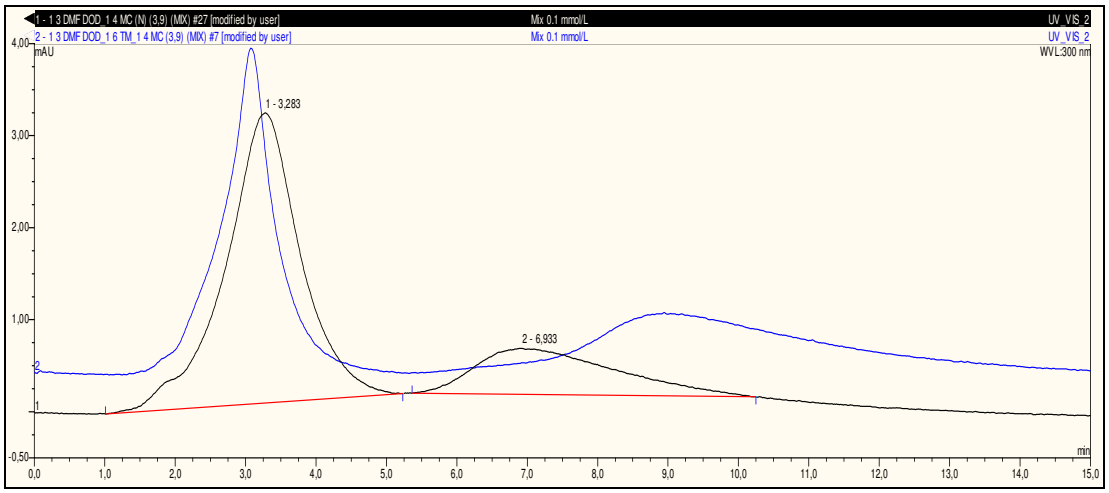

Figure 4.19. Chromatograms of MIP_9 (blue) and NIP_9 (black). Injection of 4-AMP and P mixture (0.1 mM in each), 1 ml/min flow of ACN, detection at 300 nm.

Table 4.5. Comparison of chromatographic properties of MIP_9 and NIP_9 monoliths.

Polymer	**t_R (min.)**		**k'**		**A_S**		**R_s**	**α**
	4-AMP	P	4-AMP	P	4-AMP	P		
MIP_9	9.10	3.072	4.10	0.72	1.33	0.96	1.91	5.68
NIP_9	6.931	3.267	2.89	0.83	1.68	1.09	1.30	3.47

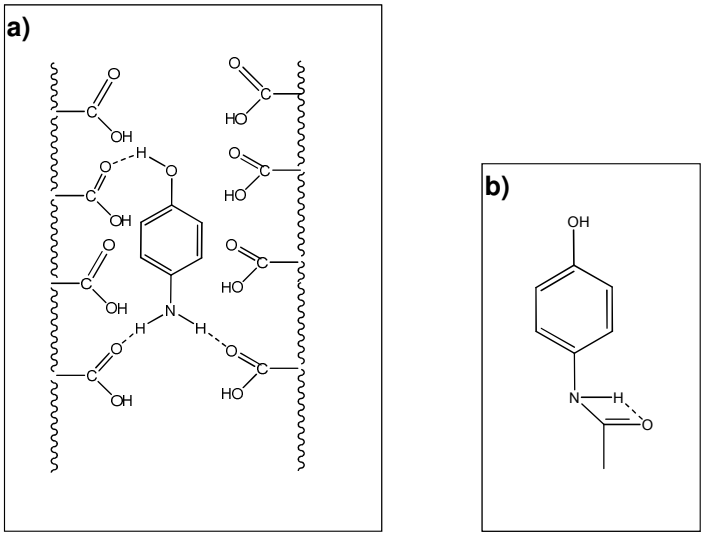

Figure 4.20 a) Suggested interactions of 4-AMP in the column, b) intra-molecular H-bonding of P.

HPLC analysis carried on different column temperatures also support the idea mentioned above. As seen in the figure 4.21, the capacity factors of 4-AMP and P decreased with increasing column temperature. While the decrease in capacity factor of 4-AMP was very significant, the capacity factor of P decreased slightly. By increasing the column temperature, the formation of hydrogen bonds was prevented. Therefore, it is expected that the effect of temperature on the capacity factor of the analyte having more functional groups capable of forming hydrogen bonds with the polymer would be more significant. In the lights of these findings, it can be suggested that 4-AMP forms hydrogen bonds with the functional groups of polymer via both its amine and hydroxyl group, while P can form hydrogen bonds with the polymer only by its hydroxyl group due to intra-molecular hydrogen bonding between the hydrogen on amine and the carbonyl group.

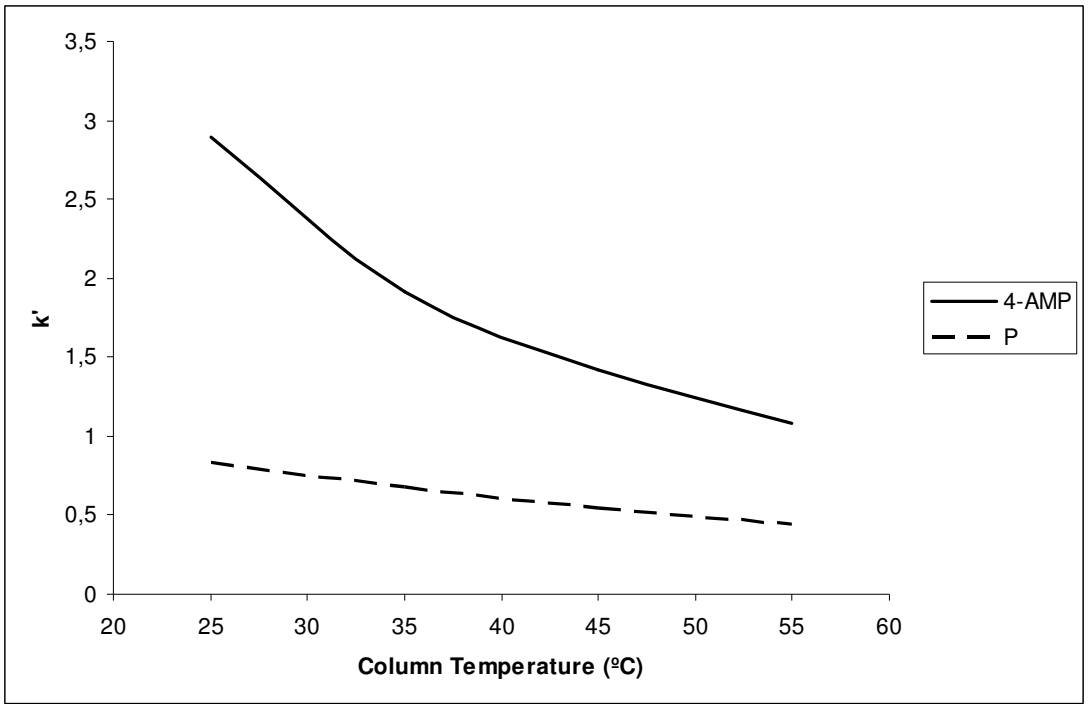

Figure 4.21. Effect of column temperature on retention times of 4-AMP and P on NIP_9 column.

Despite non-imprinted column was achieved to separate 4-AMP and P, imprinted column shows a higher selectivity to 4-AMP as expected and the imprinting factor (IF) was calculated as 1.42. The imprinting effect was also

investigated on monolith with MAA:AAM ratio of 1:1. It was seen that while imprinted column was able to separate 4-AMP and P with a resolution of 1.3 (MIP_6), non-imprinted column could not achieve the separation (NIP_6) (Figure 4.22), because of having no specific binding site.

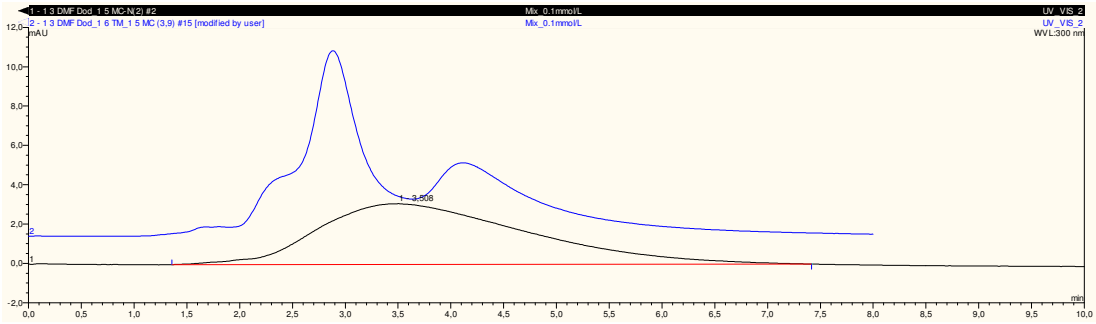

Figure 4.22. Chromatograms of MIP_6 (blue) and NIP_6 (black). Injection of 4-AMP and P mixture (0.1 mM in each), 1 ml/min flow of ACN, detection at 300 nm.

4.5. Frontal Analysis Results

Frontal analysis, also known as frontal chromatography, is commonly used to investigate specific interactions. The dissociation constant, binding density for the interaction between the analyte and polymer can be determined by this method. Binding isotherms can also be generated from an adequate number of experimental break-through curves. A typical example of break through curves for 4-AMP on MIP_9 monolith was shown in figure 4.23. Binding isotherms of 4-AMP on both MIP_9 and NIP_9 columns was generated from their break-through curves obtained by loading different concentrations (0.1, 0.2, 0.5 and 1.0 mM) of 4-AMP prepared in ACN and given in the figure 4.24. It is seen that the binding isotherms of 4-AMP on MIP_9 and NIP_9 was almost identical. In order to see the binding behavior of 4-AMP on both imprinted and non-imprinted columns, effective binding site densities of MIP_9 and NIP_9 for 4-AMP were calculated from the plot of $1 / ([A]_0 (V-V_0))$ vs. $1 / [A]_0$ (figure 4.25 and 4.26) and found as 7.95 µmol/g for MIP_9 and 8.82 µmol/g for NIP_9. The reason for that NIP_9 monolith has higher binding site density than MIP_9 is surface area of NIP_9 is much more than the surface area of MIP_9.

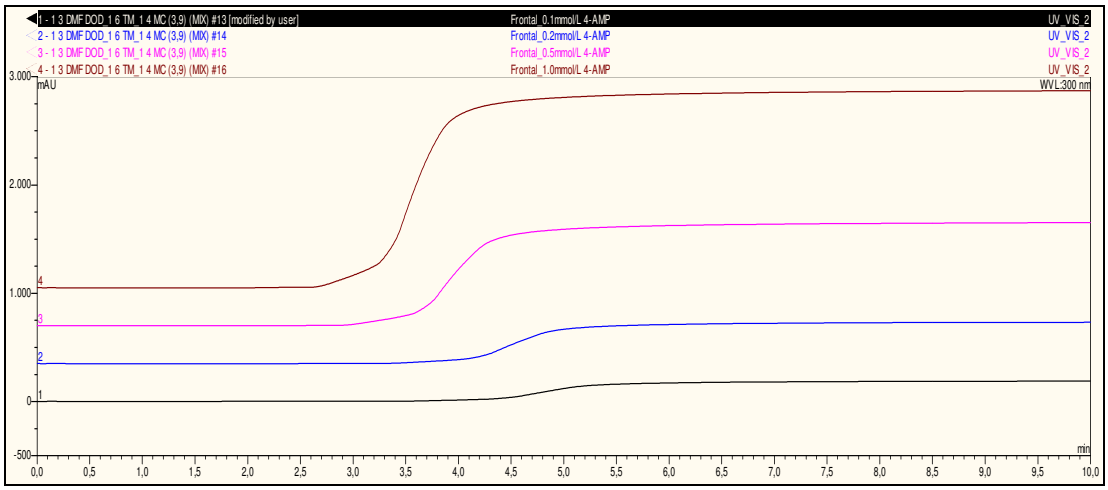

Figure 4.23. Break-through curves of 4-AMP on MIP_9 monolith. Loading concentrations were 0.1 mM (black), 0.2 mM (blue), 0.5 mM (pink) and 1.0 mM (brown) in ACN. Flow rate was 1 ml/min and detection was at 300 nm.

Although the effective binding site density of non-imprinted column for 4-AMP is slightly higher than those of imprinted column, retention time of 4-AMP on MIP_9 column was higher than it was on NIP_9 column, while the retention time of P was almost the same on both columns, which means that specific recognition sites were successfully created by molecular imprinting of 4-AMP leading to binding sites with higher affinity. When surface areas of these two monoliths are compared, as mentioned in the section 4.3, it was observed that while the surface area of NIP_9 monolith is much higher than those of MIP_9 monolith, the binding site densities of these two molecules are almost the same. Therefore, it is clearly seen that molecular imprinting resulted in creation of specific recognition sites for 4-AMP. The dissociation constants (K_D) of imprinted and non-imprinted columns for 4-AMP, calculated from the plot of $1/([A]_0\ (V-V_0))$ vs $1/[A]_0$, were 1.06 and 1.28 mM respectively. These values also prove the formation of specific binding sites showing higher affinity to the template molecule.

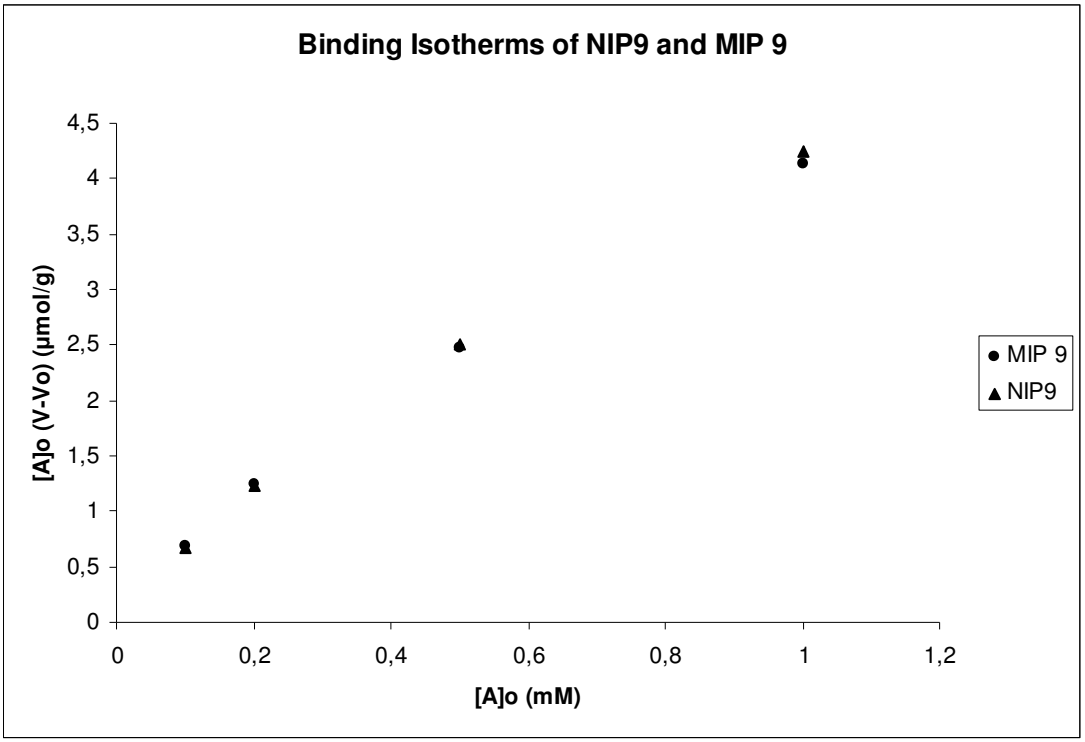

Figure 4.24. Binding isotherms of 4-AMP on MIP_9 and NIP_9.

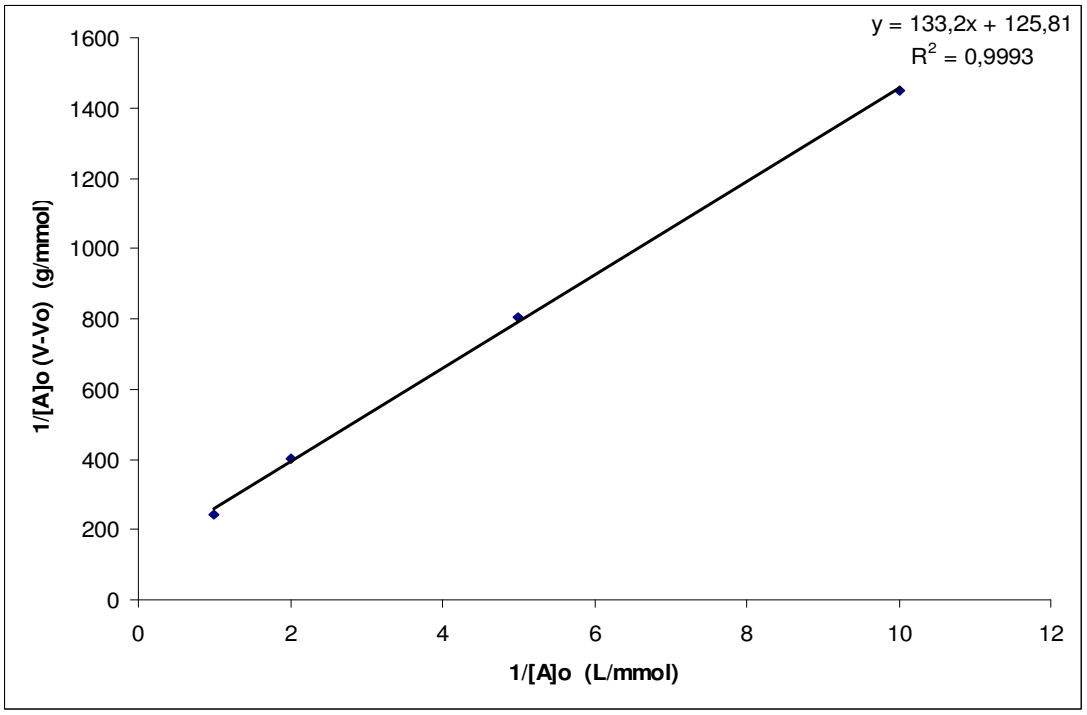

Figure 4.25 Plot of $1/([A]_0\ (V\text{-}V_0))$ vs $1/[A]_0$ obtained from the frontal analysis of 4-AMP on MIP_9.

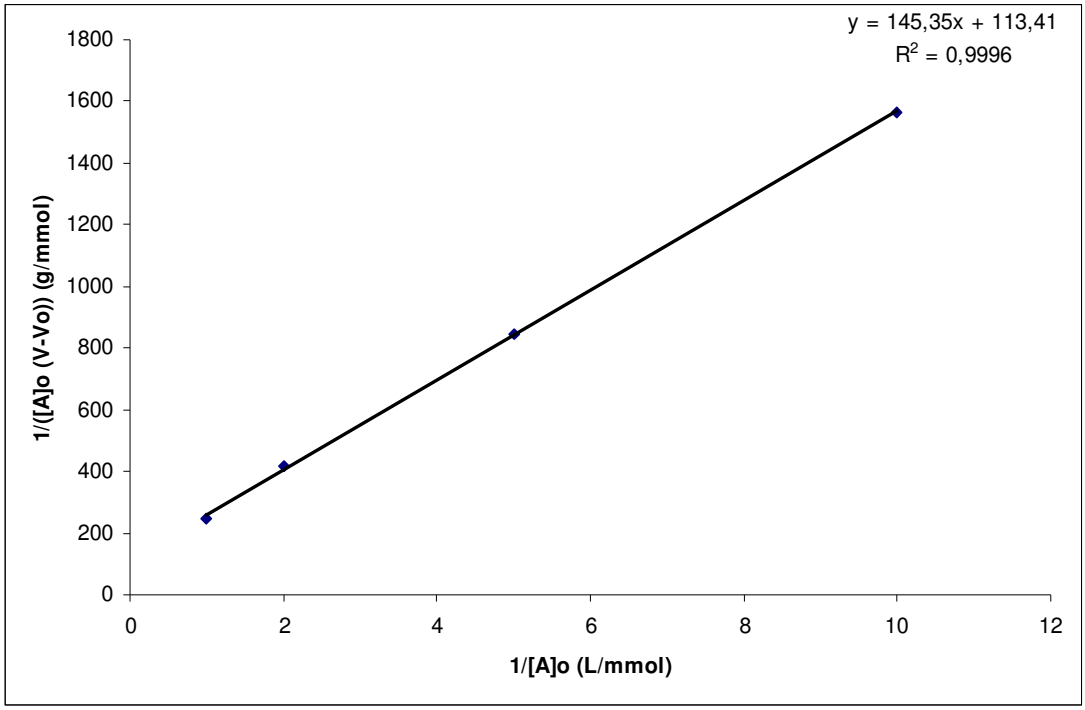

Figure 4.26. Plot of $1/([A]_0 (V-V_0))$ vs $1/[A]_0$ obtained from the frontal analysis of 4-AMP on NIP_9.

Binding capacities of 4-AMP and P on NIP_9 column with different methanol content in the mobile phase was also investigated (figure 4.27). It was observed that, as expected, binding capacities of both 4-AMP and P decreased with increasing methanol content. In organic media, adsorptions of both molecules to the polymeric monolith occur by the H-bonding interactions. When small portion of methanol was included in the mobile phase, methanol molecules as competetive ligand forms H-bonds with the binding sites on the monolith firstly resulting in the retention times of both 4-AMP and P decreased. As seen in the figure 4.27, the binding capacity of NIP_9 for 4-AMP was always higher than it was for P regardless of the methanol content. The reason for this is that the amine group in 4-AMP, which is capable of forming hydrogen bonds, increase the ability to reach the free functional groups on the polymer. For P molecule, it is more difficult to reach those free functional groups due to intra-molecular hydrogen bonding.

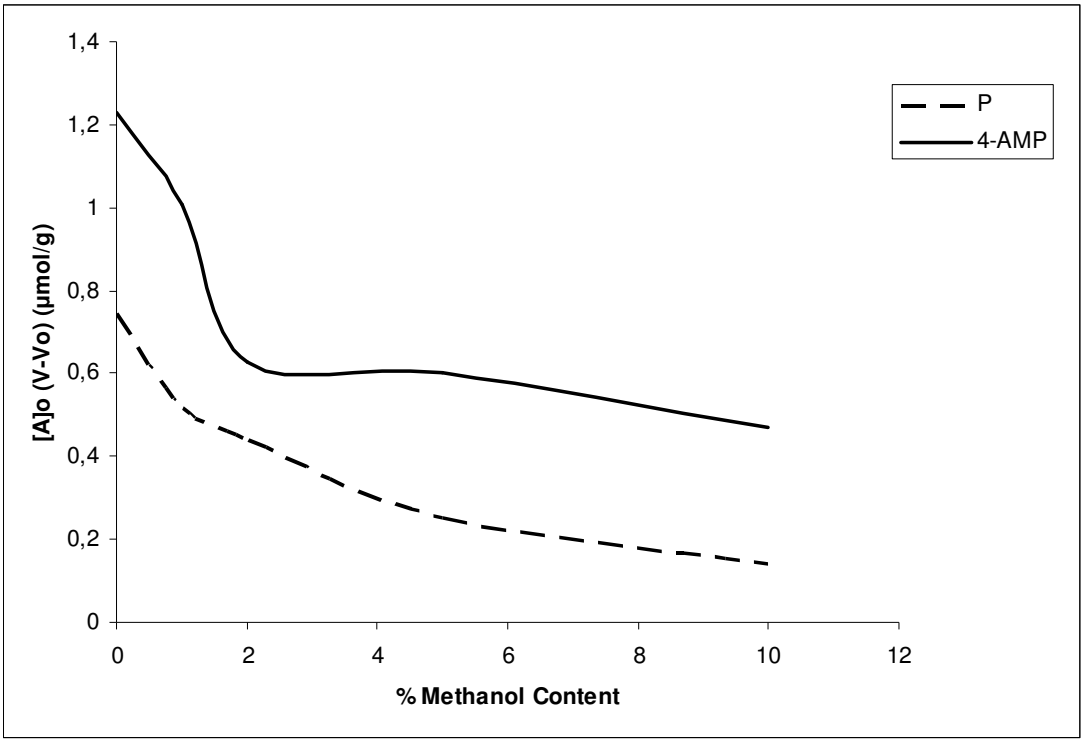

Figure 4.27. Effect of methanol content in mobile phase on binding capacity of NIP_9 column for 4-AMP and P.

5. CONCLUSION AND FUTURE DIRECTIONS

In this study, polymerization conditions and binding characteristics of MIPs were optimized and recognitive polymeric monolith not only with good flow-through properties but also high resolution and selectivity for chromatographic separation of structurally similar compounds was obtained. 4-AMP and P were completely resolved on prepared MIP monolith directly without a need of method development process. It is obvious that there is a lack of commercial monolithic columns in the market due to the very difficult preparation processes of monolithic silica columns. Therefore, this study may contribute much to the monolithic columns field by its very easy preparation steps and it can be an alternative to silica-based monolithic columns. Prepared monolithic column can also be used as a pre-column or enrichment column by further researches. It is also highly possible to get better performance from this molecularly imprinted monolith when applied as a stationary phase for capillary columns which is also planned as a post-study.

Although the use of monolithic columns in the chromatographic systems is not wide-spread yet, it is indispensable that monolithic columns will dominate the market when the variety of commercial monolithic columns increased, which can only be speeded up by such works.

We are currently engaged in the extension of this work to the development of molecularly imprinted monolithic HPLC columns with high selective binding capacity and low back pressure against mobile phase flow.

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