SYNTHESIS OF MOLECULARLY IMPRINTED POLYMERS AS SELECTIVE SOLID PHASE EXTRACTION SORBENTS FOR ANTICANCER DRUGS PRIOR TO CHROMATOGRAPHIC DETERMINATION

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by
Anıl ÖZDEMİR

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İZMİR

We approve the thesis of **Anıl ÖZDEMİR**

Examining Committee Members:

Prof. Dr. Ahmet E. EROĞLU
Department of Chemistry, İzmir Institute of Technology

Prof. Dr. Mustafa M. DEMİR
Department of Materials Science and Engineering, İzmir Institute of Technology

---

Assoc. Prof. Dr. Yaşar AKDOĞAN
Department of Materials Science and Engineering, İzmir Institute of Technology

---

Assoc. Prof. Dr. Nesrin HORZUM POLAT
Department of Engineering Sciences, İzmir Katip Çelebi University

---

Assoc. Prof. Dr. Levent PELİT
Department of Chemistry, Ege University

09 October 2018

Prof. Dr. Ahmet E. EROĞLU
Supervisor, Department of Chemistry
İzmir Institute of Technology

Prof. Dr. Mustafa M. DEMİR
Head of Department of Materials Science and Engineering, İzmir Institute of Technology

---

Prof. Dr. Aysun SOFUOĞLU
Dean of the Graduate School of Engineering and Sciences

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I dedicate this work to my best friend's little cutest 6 months old baby girl Alya Akbal.

// ABSTRACT

SYNTHESIS OF MOLECULARLY IMPRINTED POLYMERS AS SELECTIVE SOLID PHASE EXTRACTION SORBENTS FOR ANTI CANCER DRUGS PRIOR TO CHROMATOGRAPHIC DETERMINATION

In recent years, the number of usage of chemotherapic drugs has considerably increased. It is necessary to develop analytical methods to analyse these compounds for quality control of formulations, quality control of diluted formulations before patient usage and understanding compatibility and stability. In this study, novel analytical methods were developed for mostly used anti cancer drugs, namely 5-fluorouracil (5-FU) and 5-azacytidine (5AC).

Firstly, two analytical methodologies using capillary electrophoresis (CE) and liquid chromatography (LC) were developed for the determination of 5-FU and 5AC. The CE analysis was performed in a bare fused-silica capillary with 75 µm i.d. and total length of 50.0 cm with a buffer solution of 10.0 mM borate buffer, pH 11.5. The applied voltage was 20.0 kV. The LC analysis was performed with a YMC 30 column in reversed phase and a mobile phase of water–acetonitrile (90:10) at a flow rate of 0.8 mL/min. In both analyses, detection was by ultraviolet absorption.

Also, molecularly imprinted polymers (MIPs) with different formulations (different functional monomers, porogens and monomer:crosslinker ratios) and morphologies (monolith and micro/nanosphericalbeads) were synthesized by using bulk and precipitation polymerization strategies. The adsorption capacity of imprinted polymers were compared to their corresponding non-imprinted polymer. MIP prepared by using bulk polymerization strategy with the formulation of 1:1:20 AA was chosen as selective solid phase extraction (SPE) sorbent due to its sorption capacity prior to determination of HPLC-DAD analysis. Selectivity of this imprinted polymer were examined by using 5-FU and 5AC molecules. To improve method, parameters were tested such as pH shaking time and amount of sorbent. So, optimization parameters of method were determined to be pH 5.0 of solution, 100.0 mg of sorbent, 10.0 mL 100.0 mg/L working solution, 60.0 min. sorption time.

ÖZET

ANTİKANSER İLAÇ ETKEN MADDELERİNİN KROMATOGRAFİK TAYİNİ ÖNCESİ MOLEKÜLER BASKILANMIŞ POLİMERLERİN SEÇİCİ KATI FAZ EKSTRAKSİYON SORBENTİ OLARAK SENTEZİ

Son yıllarda, kemoterapik ilaçların kullanım sayısı önemli ölçüde artış göstermektedir. Bu ilaçların formülasyonlarının kalite kontrolü, hasta kullanımları öncesi seyreltik hallerinin kalite kontrolü ve uyumluluk ve kararlılıklarının anlaşılması için analitik metotların geliştilmesine ihtiyaç vardır. Bu çalışmada çoğunlukla kullanılan antikanser ilaçları, 5-florourasil (5-FU) ve 5-azasitidin (5AC) için yeni analitik yöntemler geliştirilmiştir.

Öncelikle 5-FU ve 5AC tayini için kılcal elektroforez (CE) ve sıvı kromatografi (LC) kullanan iki analitik metodoloji geliştirilmiştir. CE analizi, 10.0 mM borat tamponu, pH 11.5 tampon çözeltisi ile 75 μm i.d. ve toplam uzunluğu 50.0 cm olan silika kapiler içinde gerçekleştirilmiştir. Uygulanan voltaj 20.0 kV oldu. LC analizi, ters fazda bir YMC 30 kolonu ve 0.8 mL / dakikalık bir akış hızında su-asetonitrilin (90:10) hareketli fazı ile gerçekleştirildi. Her iki analizde de ultraviyole bölgede çalışma gerçekleştirilmiştir.

Ayrıca, farklı formülasyonlar (farklı fonksiyonel monomerler, porojenler ve monomer: çapraz bağlayıcı oranları) ve morfolojileri (monolit ve mikro / nanosferik şişeler) ile moleküler olarak baskılı polimerler (MIPs) toplu ve çökeltme polimerizasyon stratejileri kullanılarak sentezlenmiştir. Baskılı polimerlerin adsorpsiyon kapasitesi, ilgili baskılanmamış polimerleriyle karşılaştırıldı. Toplu polimerizasyon stratejisi kullanılarak hazırlanan 1:1:20 AA formülasyonu ile hazırlanan MIP, HPLC-DAD analizinin belirlenmesinden önce sorpsiyon kapasitesine bağlı olarak selektif katı faz ekstraksiyonu (SPE) sorbenti olarak seçilmiştir. Bu baskılı polimerin seçiciliği, 5-FU ve 5AC molekülleri kullanılarak incelenmiştir. Metot geliştirmek için örnek çözelti pH'sı, çalkalama zamanı, sorbent miktarı, gibi parametreler test edilmiştir. SPE de kullanılan parametrelerin optimum değerleri 5.0 solüsyon pH' sı, 100.0 mg sorbent, 10.0 mL 100.0 mg/L çalışma solüsyonu, 60.0 dakika sorpsiyon zamanı olarak belirlenmiştir.

TABLE OF CONTENTS

ABSTRACT iv

ÖZET v

TABLE OF CONTENTS vi

LIST OF FIGURES viii

LIST OF TABLES x

CHAPTER 1 INTRODUCTION 1

1.1. Anticancer Drugs 1

1.1.1. 5-Fluorouracil (5-FU) 2

1.1.2. 5-Azacytidine (5AC) 2

1.2. Determination of Anticancer Drugs 3

1.3. Capillary Electrophoresis (CE) 4

1.3.1. Theoretical Background 4

1.3.2. Capillary Electrophoresis Modes 6

1.4. Solid Phase Extraction (SPE) 8

1.5. Molecular Imprinted Polymers (MIPs) 10

1.6. Aim of This Study 13

CHAPTER 2 EXPERIMENTAL 14

2.1. Chemicals 14

2.2. Instrumentation and Apparatus 14

2.3. Synthesis of MIP/NIP 16

2.3.1. Synthesis of MIP/NIP Monoliths using MAA as Monomer 16

2.3.2. Synthesis of MIP/NIP Monoliths using AA as Monomer 17

2.3.3. Synthesis of MIP/NIP Micro/Nanospheres using MAA as Monomer 18

2.4. Rebinding Experiments........ 18

2.5. Cross Selectivity........ 19

2.6. SPE Optimization Parameters........ 19

CHAPTER 3 RESULTS AND DISCUSSION........ 21

3.1. Anticancer drug analysis by HPLC-DAD system........ 21

3.2. Anticancer drug analysis by CE-DAD system........ 22

3.3. Preparation and Characterization of monolith micro/nanosphere printed polymers........ 28

3.4. Rebinding Experiments........ 31

3.5. Cross Selectivity........ 34

3.6. SPE Optimization Parameters........ 35

CHAPTER 4 CONCLUSION........ 40

REFERENCES........ 41

LIST OF FIGURES

Figure **Page**

Figure 1.1. Molecular structure of 5-fluorouracil. 2
Figure 1.2. Molecular structure of 5-azacytidine. 3
Figure 1.3. Schematic representation of the fractional solute migration that overall effect of electroosmotic flow and electrophoretic flow in capillary. 5
Figure 1.4. Schematic representation of capillary electrophoresis device……… 6
Figure 1.5. Schematic illustration of the SPE modes 9
Figure 1.6. Schematic illustration of 5-FU imprinting process 11
Figure 3.1. Chromatogram of 10.0 mg/L 5-FU 21
Figure 3.2. Calibration plot of 5-FU 22
Figure 3.3. Electropherogram of 20.0 mg/L 5AC.. 23
Figure 3.4. Electropherogram of 20.0 mg/L 5-FU 23
Figure 3.5. Electropherogram of 20.0 mg/L 5AC and 5-FU mixture 24
Figure 3.6. Electropherogram of 20.0 mg/L 5AC and 5-FU mixture................ 24
Figure 3.7. Electropherogram of 20.0 mg/L 5AC and 5-FU mixture................ 25
Figure 3.8. Electropherogram of 20.0 mg/L 5AC and 5-FU mixture................ 26
Figure 3.9. Electropherogram of 20.0 mg/L 5AC and 5-FU 26
Figure 3.10. Electropherogram of 20.0 mg/L 5AC and 5-FU mixture.............. 27
Figure 3.11. Calibration plots of 5AC and 5-FU mixture................................. 27
Figure 3.12. Possible synthesis mechanism of 5-FU imprinted polymer 28
Figure 3.13. SEM images of NIP/MIP monoliths 29
Figure 3.14. Possible synthesis mechanism of 5-FU imprinted polymer. 30
Figure 3.15. SEM images of NIP/MIP monoliths 31
Figure 3.16. Rebinding characteristics assay of MIP/NIP monoliths. 32
Figure 3.17. Rebinding characteristics assay of MIP/NIP monoliths. 32
Figure 3.18. Rebinding characteristics assay of MIP/NIP monoliths 33
Figure 3.19. Rebinding characteristics assay of MIP/NIP monoliths. 33
Figure 3.20. Rebinding characteristics assay of MIP/NIP. 34
Figure 3.21. Cross Selectivity of MIP/NIP monoliths...................................... 35
Figure 3.22. Effect of solution pH on the sorption of 5-FU. 36
Figure 3.23. Effect of solution pH on the sorption of 5-FU. 36

Figure 3.24. Effect of solution pH on the sorption of 5-FU 37
Figure 3.25. Effect of solution pH on the sorption of 5-FU. 37
Figure 3.26. Effect of solution pH on the sorption of 5-FU. 38
Figure 3.27. Effect of amount of sorbent on the sorption of 5-FU. 38
Figure 3.28. Effect of shaking time on the sorption of 5-FU.............................. 39

LIST OF TABLES

Table **Page**

Table 2.1. Operation conditions for HPLC analysis. 15

Table 2.2. Operation conditions for CE analysis. 16

Table 2.3. All studied parameters 20

CHAPTER 1

INTRODUCTION

1.1. Anticancer Drugs

Cancer occurs as a disease in which the control of growth is lost in one or more cells, leading either to a solid mass of cells known as a tumour or to a liquid cancer (i.e. blood or bone marrow-related cancer). It is one of the leading causes of death throughout the world, which has treatments like surgery, chemotherapy, and/or radiotherapy (Shewach et al., 2009). Chemotherapy involves the use of low-molecular-weight drugs to selectively destroy tumour cells or at least limit their proliferation. There are some disadvantages of many cytotoxic agents such as bone marrow suppression, gastrointestinal tract lesions, hair loss, nausea, and the development of clinical resistance. These side effects occur because cytotoxic agents act on both tumour cells and healthy cells (Thurston et al., 2007). In 1940s, the use of chemotherapy began with nitrogen mustards, which are extremely powerful alkylating agents and antimetabolites. Since the early success of these initial treatments, a large number of additional anticancer drugs have been developed (Shewach et al., 2009). Anticancer drugs can be classified according to their mechanism of action, such as DNA-interactive agents, antimetabolites, antitubulin agents, molecular targeting agents, hormones, monoclonal antibodies and other biological agents (Thurston et al., 2007).

Today, with the increase in cancer incidence, treatments containing cytotoxic drugs are widely used. Due to the aging population and the arrival of new treatments, the demand for pharmacy cancer services is expected to more than double over the next 10 years (Hoppe-Tichy et al., 2010). Even if more selective therapies are developed, treatment schemes will continue to be associated with classical cytotoxic agents.

Consequently, the need for analytical methods to determine anticancer drugs is of outmost importance.

1.1.1. 5-Fluorouracil (5-FU)

Since the synthesis of 5-fluorouracil (5-FU) in 1957, it has shown an important role for the treatment of solid cancer tumors such as, gastrointestinal tract, neck, head and breast tumors (Santos et al., 2015). At first 5-FU was designed by the replacement of hydrogen atom in position 5 of uracil with similarly sized fluorine atom. It interacts with active sites of enzyme targets and blocking metabolism in malignant cells.

Although 5-FU is a small molecule with a molecular mass of 130.08 g/mol molecular weight, it does not show superior properties about bioavailability and absorption. It has basic properties with a pKa of 8.0. Plasma levels of 5-FU are not known precisely because of its irregular absorption. As all other anti cancer drugs, 5-FU is a quite toxic molecule due to its phosphorylation in the digestive tract (Martino et al., 2002). Besides its toxic effects, short half life period and fast metabolism are other significant disadvantages of 5-FU (Zheng et al., 2016). Rapid degradation of up to 85% of the 5-FU into the blood stream causes leaving of very low amount of drug for cancer action (Santos et al., 2015).

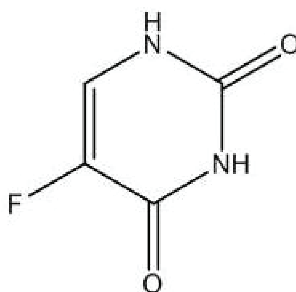


Figure 1.1.Molecular structure of 5-fluorouracil

1.1.2. 5-Azacytidine (5AC)

According to records, 5-azacytidine (5AC) was firstly synthesized in 1964. Molecular weight of 5AC is 244.20 g/mol and it shows strong basic properties with its pKa of 12.55. The compound has antimicrobial and antitumor activities. It is an analogue of nucleoside and hypomethylating agent that has anticancer properties. The United States of America has approved the use of 5AC in the treatment of five myelodysplastic syndromes. It is an unstable molecule and rapidly hydrolyzed to

several byproducts such as 5-azacytosine and 5-azauracil in aqueous solutions (Zhao et. al., 2004).

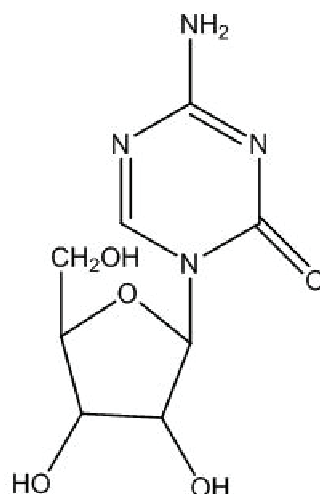


Figure 1.2. Molecular structure of 5-azacytidine.

1.2. Determination of Anticancer Drugs

A high number of analytical methods for the determination of 5-FU, related prodrugs and their metabolites in biological matrices have been developed in the last 30 years. For stability studies of 5-FU in pharmaceutical dosage forms, stability-indicating LC-UV methods have been developed with good quantitative performance in terms of accuracy and precision (Sinha et al., 2009). Drug combinations of 5-FU were also successfully determined by LC-UV in injection solutions and biological samples (Fahmy et al., 2004). Published methods for the analysis of tegafur and capecitabine allowed a simultaneous separation and quantification of 5-FU (Badea et al., 2002). Zero-crossing first-derivative spectrometry and CE-UV were used for the determination of 5-FU and tegafur in pharmaceutical formulations (Yang et al., 2007). In the presence of formaldehyde, sensitised chemiluminescence based on potassium permanganate oxidation has also been used for the determination of 5-FU in pharmaceutical fluids (Sun et al., 2007).

It is important to analyse 5-FU in environmental samples because it is one of the most used cytotoxic agents at high doses and therefore an ideal marker compound for other potential contaminants. Surface contamination monitoring using GC–MS (Schmaus et al., 2002) or LC (Rubino et al., 1999) was successfully performed. Due to

the high polarity of 5-FU, low retention times were recorded when reversed phase LC columns were used, and separation from different antimetabolites was difficult to obtain. For this reason, the use of hydrophilic interaction liquid chromatography (HILIC) coupled to MS/MS appears to be an attractive approach for the analysis of antimetabolites in wastewater (Kovalova et al., 2009). In the described conditions, baseline separation was obtained for 5-FU, cytarabine, gemcitabine and their metabolites (uracil 1--d-arabinofuranoside and 2 ,2 - difluorodeoxyuridine). In addition, CE-UV allowed the determination of 5-FU in hospital effluents after enrichment by solid-phase extraction (SPE) (concentration factor 500) (Mahnik et al., 2004).

Liquid chromatograghic methods have also been used for the analysis of bulk drugs and pharmaceutical formulations containing cytarabine and azacitidine (Kissinger et al., 1986). Spectrophotometry and LC-UV were used for degradation studies (Argemi et al., 2007) and for the development of drug formulations containing azacitidine (Argemi et al., 2009). LC-UV (Rustum et al., 1987) and LC–MS/MS (Zhao et al., 2004) were reported for azacytidine determination in plasma.

1.3. Capillary Electrophoresis (CE)

1.3.1. Theoretical Background

For almost three decades, capillary electrophoresis (CE) has taken increasing popularity as a separation method regarding to its high separation power, fast and cost-effective analysis, ease of operation and environmental-friendly approach (Siren et al., 2008). The term of CE refers to a set of techniques that operate in liquid media and use capillary columns where solvated ions, ionized and neutral species migrate with different velocities and therefore can be separated under the action of an electric field (Geiger et al., 2012). Another important feature of CE is the instrumental simplicity, which enables the performance of several modes of analysis on the same equipment. Thus, different electrophoresis techniques have appeared, allowing the analysis of various types of analytes in complex matrices (Baker et al., 1995).

Electroosmotic flow (EOF) and electrophoretic flow (EPF) are the two transport mechanisms that are fundamental to CE. Electroosmotic flow is the bulk flow of the background electrolyte. In a bare fused silica capillary EOF moves from the anode toward the cathode due to the negative surface charge of the capillary and the presence

of the electric field. Electrophoretic flow is a function of charge-to-size ratio. Analyte is attracted to either the anode or cathode and the size of the molecule determines the speed of transport. These two modes of transport are superimposed in most CE separations. In a CE separation with the anode at the site of injection in a bare fused silica capillary, when EOF is present the order of analyte migration is small positively charged molecules, larger positively charged molecules, comigrating neutral molecules, large negatively charged molecules, and smaller negatively charged molecules (Grace et al., 2018). The velocity of an ion is the sum of its electrophoretic flow (EPF) velocity and its electroosmotic velocity which are shown in Figure 1.3 (Agilent Technologies 2014) basicly.

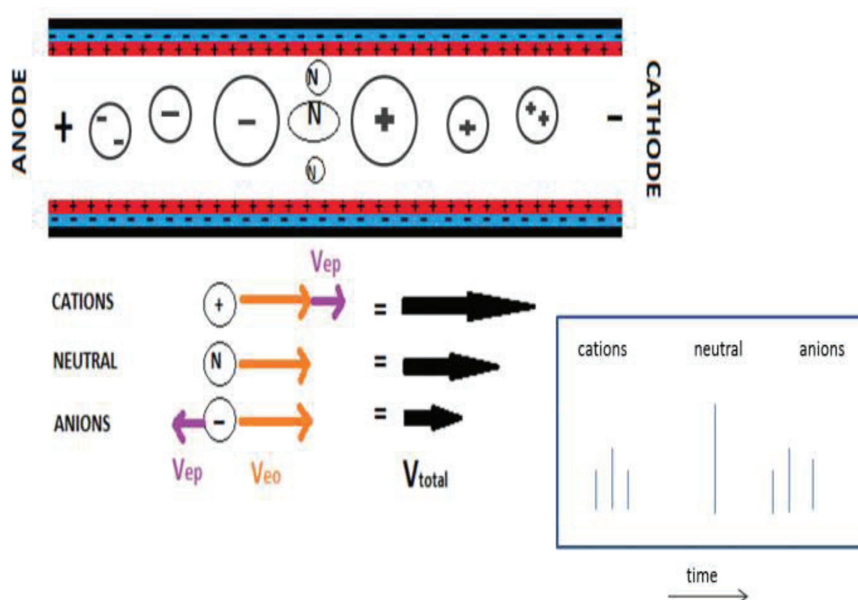


Figure 1.3. Schematic representation of the fractional solute migration that overall effect of electroosmotic flow and electrophoretic flow in capillary.

A simple CE system consist of a sample vial, inlet and outlet vial which contain background electrolyte (buffer solution), capillary, detector (UV-Vis, DAD, Laser Induced Fluorescence, Contactless Conductivity Detection, etc.), high voltage supplier and computer (Figure 1.4) (Agilent Technologies 2014).

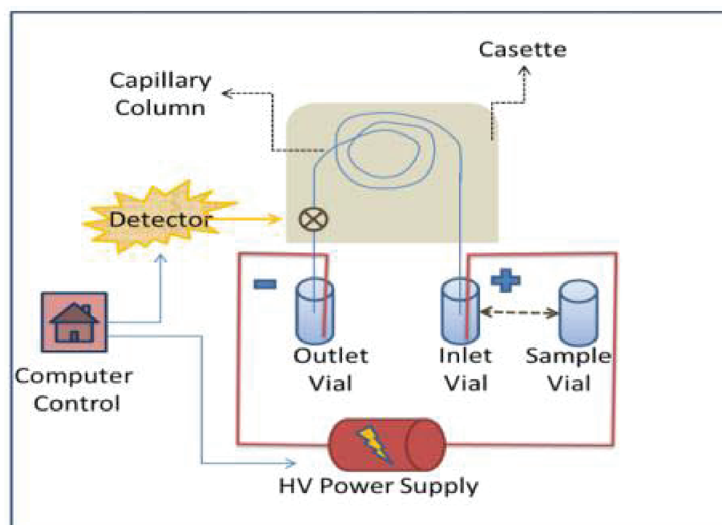


Figure 1.4. Schematic representation of capillary electrophoresis device

1.3.2.Capillary Electrophoresis Modes

Capillary zone electrophoresis (CZE) is certainly the most common mode among the electrophoresis techniques in which the separation is based only on charged species mobility differences (Blatny et al., 1999). The capillary which is usually made on fused silica with a suitable polymeric external coating filled with an electrolyte solution as buffered. According to this condition, the solution is also influenced by the action of the applied electric field, generating an electroosmotic flow (EOF), which may influence the quality of separation.

Another widely used technique is micellar electrokinetic chromatography (MEKC). It is especially for analysis of neutral species (Silva et al., 2013). The capillary is filled with an electrolyte containing ionic surfactants able to form charged micelles in this mode. It can be observed that the aqueous and micellar phases have different affinities for analytes and distinct migration speeds. The differentiated solute distribution between these two phases associated with the differences between migration speeds allows even neutral species to be separated.

Capillary electrochromatography (CEC), (Miksik et al., 2007) can be considered a hybrid between CE and liquid chromatography (LC). It also employs two distinct phases, with the advantage of blending the high separation efficiency of LC with low solvent consumption and flat flow profile of CE. In this technique, a capillary containing a stationary phase is filled with an electrolytic mobile phase able to provide

an EOF. The flow of capillary mobile phase is induced by application of voltage with or without assisted pressure. CEC can be used for separating neutral species or ions. The speed of migration is a result of the combination of chromatographic and electrophoretic mechanisms. Three types of stationary phase are commonly found: the packed-particulate material, the open tubular and monolithic (Pyell et al., 2000).

In capillary gel electrophoresis (CGE), (Zhu et al., 2012) the capillary is filled with a gel, which behaves as a sieving system. The separation is based on differences in size and shape of the analytes. Molecules with higher mass-to-charge ratios moving at lower speeds in crossing the polymer network gel. It has wide application in the separation of proteins in various matrices.

The capillary isotachophoresis (CITP), (Mala et al., 2013) employs a system where the sample aliquot is inserted in the capillary between a leading electrolyte, with high mobility and a terminating electrolyte. During the run, the sample plug remains between the two electrolytes and as the electric field is applied. The analytes are focused on different and adjacent zones, migrating with the same isotachophoretic speed, in just one set. If the aqueous electrolyte system is replaced by a mixture of organic solvents, the separation mode is referred to as non-aqueous capillary electrophoresis (Kenndler et al., 2014). The viscosity, relative permittivity, apparent pH, electrostatic interactions, among other characteristics provide to this method entirely different selectivity, solubility, separation efficiency and electric current ranges in relation to other CE modes.

Capillary isoelectric focusing (CIEF), (Silvertand et al., 2008) consists of a separation mode for amphoteric compounds according to their isoelectric points (pI) in a pH gradient along the capillary. At first, a large sample volume is introduced in the capillary together with a solution containing ampholytes with different pI´s. Then, the capillary is immersed in an acid solution at inlet and in a basic solution at the outlet. When the voltage is applied, under the action of electric field, a pH gradient is generated along the capillary, as the ampholytes and different analytes migrate to distinct regions. When a component reaches a region whose pH is equal to their pI, its charge becomes null and therefore does not migrate anymore.

When the electrophoretic separation is conducted in devices containing microchannels instead of capillaries, the term microfluidic chip electrophoresis (MCE) is employed. In this technique, the sample is inserted, separated and detected in a microchip, easily generated in the laboratory with material (glass, silica, quartz and

several organic polymers). Electrophoresis can be achieved when voltage is applied on both ends of a microchannel. Microfluidic chip electrophoresis is characterized by miniaturization, automation, integration, low cost, portability and fast and efficient analysis (Cong et al., 2015). Isoelectric focusing, isotachophoresis, electrochromatography, MEKC and gel electrophoresis, can be performed on MCE (Castro et al., 2015), after selection of appropriate mobile and stationary components.

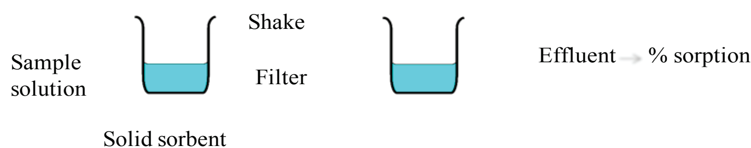
1.4. Solid-Phase Extraction (SPE)

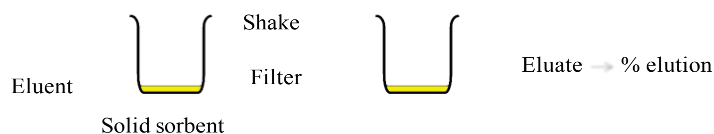
Many separation and pre-concentration methods can be found in literature for various purposes. Liquid-liquid extraction (solvent extraction), electro-deposition, ion exchange and membrane filtration can be found in literature.

One of the most common techniques is solid-phase extraction (SPE) and is used for extraction and pre-concentration of analytes in liquid samples due to the advantages over other methods such as high enrichment factor, high recovery, rapid phase separation, low cost, low consumption of solvents. Solid-phase extraction (SPE) is a non-equilibrium process and is based on the extraction of the desired species on a sorbent and then elution of the retained species using a suitable solvents.

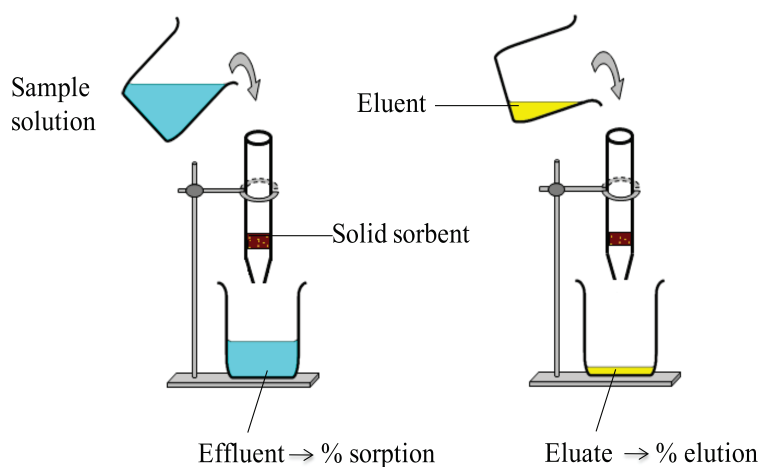
There are basicly two types of SPE which are called as the batch and the column modes. In batch type SPE, solid sorbent is weighed and put into the sample liquid solution. Large surface area is provided by the particles in solid phase to obtain efficient mass transfer. At the end of the shaking process, two phases can be separated by filtration (Figure 1.5 (a)). In column type SPE, the column is loaded with the sample solution for the sorption of the analyte by the solid phase (Figure 1.5 (b)). A higher percentage of extraction is expected in column type SPE than batch type extractions. The concentration of analyte in the effluent (non-sorbed fraction) is determined and used for the calculation of the percentage sorption. The analyte (retained by the sorbent) is eluted by using, generally, a smaller volume of a proper eluent. The analyte concentration in the elute gives the percentage elution. Finally, total recovery calculations can be made according to Equation 1.1.

$$Total\ Recovery = \frac{[analyte]_{eluate}}{[analyte]_{sample}} \times 100 \qquad (1.1)$$





(a)



(b)

Figure 1.5. Schematic illustration of the SPE modes (a) batch type, (b) column type.

As mentioned above, SPE has both advantages and disadvantages over the other methods. In addition, the target species can be fixed in a more stable chemical form on the solid surface. A special care must be given to the possible plugging problem that can be caused by the amount, type, size of the particulates in the sample, pore size of the sorbent and surface area of the sorbent bed. There is also a potential for the association of analyte with particulate and colloidal matter contamination in the sample. To avoid these problems sample particulate matter should be removed by filtration prior to SPE analysis.

1.5. Molecular Imprinted Polymers (MIPs)

Complexes of molecules have relatively small lifetimes in gases or solutions when they have very low concentration in solutions. Some of the molecules can recognize only one specific substance among other substances which are called receptors. Receptors can easily distinguish their own partner molecule in crowd solutions. They can make stable complexes with quite high concentrations.

There are many receptors which are naturally found in body. These receptors have important roles in many processes which are essential for existence of life. The artificial receptors have some advantages according to natural receptors. These human-made molecules do not only deal with proteins. A specific molecule can be designed for a variety of compounds. Also flexibility, stability and activity in different conditions can be determined. In addition, artificially produced substance that has the sites, completely suits to an analyte by designing. In general, this can be called by 'molecular recognition', the key idea of 'molecular imprinted polymers'.

Molecularly imprinted polymer is generated by the polymerization of monomer(s) and template molecule (analyte or its analog) surrounded by crosslinking agent with use of an initiator. Template molecule is necessary for the synthesis of MIP. Any functional group of the template stops or retards the inhibition of polymerization. So, it should not have functional groups and it should be stable in a wide range of UV-radiation and temperature.

Figure 1.6 basicly shows molecular imprinting process in three steps: pre-polymerization, polymerization and removal of template. In pre-polymerization step, monomer and template molecule connect to each other via covalent or non-covalent interaction (Puoci et al., 2007).

Total process gets its specific name by these interactions: semi-covalent imprinting or non-covalent imprinting. H-bonding and electrostatic interactions are polar interactions which occur in non-covalent imprinting whereas semi-covalent imprinting is based on covalent bonding. Generally, non-covalent imprinting is preferred even if the covalent imprinting has the clearer cavity structure and conditions of free polymerization.

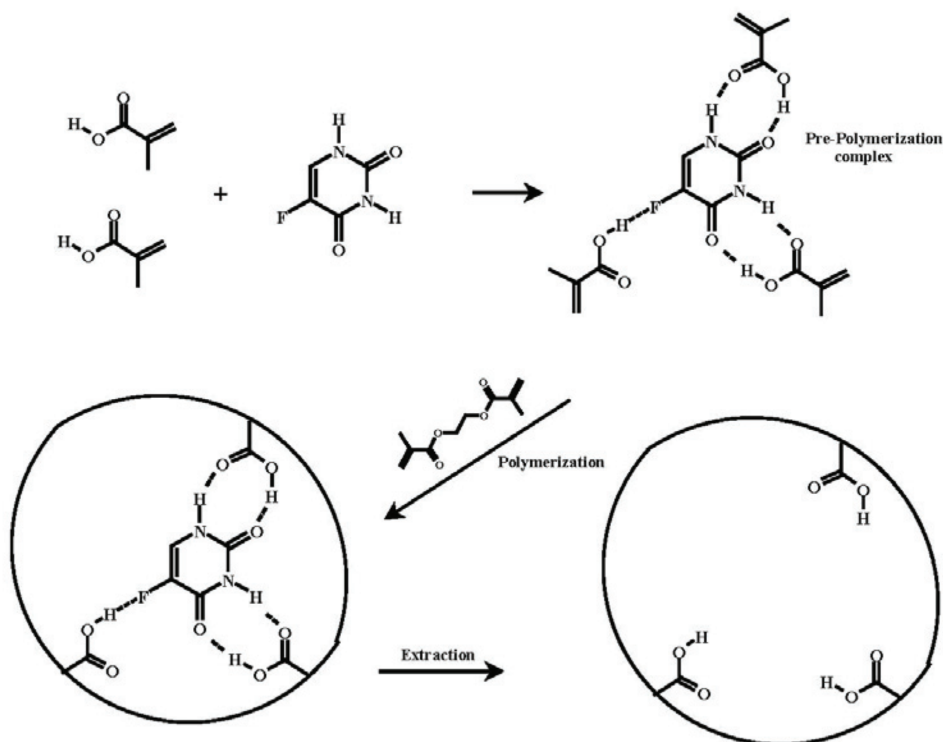


Figure 1.6. Schematic illustration of 5-FU imprinting process.

In polymerization process, reaction mixture polymerizes with a fixed ratio of crosslinking agent and initiator. The morphology of MIP is controlled by crosslinking agent. It stabilizes the specific binding sites (imprinted region) and supply robustness to the polymer. If radical copolymerization is chosen as the method of preparation, the addition of the initiator into the reaction mixture under specific conditions effects the polymerization step. Oxygen is removed by degassing with nitrogen or argon gas or freeze-and-thaw cycle. The non-covalent interaction between monomer and template can be destructed by high temperatures applied for initiation. For this reason, photo-initiation can be applied under low temperatures by UV-light. In addition, in the absence of initiator, UV-absorbable monomers can be used for the initiation (Komiyama et al., 2003).

The solid particles after polymerization are called as MIP's. MIP's are firstly filtered, than washed until any template molecule is not observed. During template removal, the most significant problem is template bleeding in trace analysis. Analyte molecule which is used as template can be observed at real samples at the higher amount than expected because of having been not removed completely before the usage of MIP as sorbent. Dummy molecule can be used as template to overcome this problem.

Even if dummy molecules have similarities with the target analyte in terms of shape, size and functionality, they have different chromatographic separations. So, the analyte molecule and the bleeding molecule can be distinguished during chromatographic processes (Fontanals et al., 2010).

The polymerization techniques that are generally used in MIP synthesis are one-step or multi-step swelling, suspension, grafting, bulk and precipitation polymerization. Bulk and precipitation techniques, that were used in this study, will be briefly explained.

Bulk polymerization is a simple technique which is widely used in MIP synthesis. The resulting polymer monolith is needed to be crushed, grounded and then sieved to have usable particles before placing into disposable cartridges for SPE process or for other applications. These processes require extra time. In addition to that, due to the separation of only definite sized particles during sieving process, there are lots of wastes. Precipitation polymerization is used to have micro and nano-sized spherical particles. Crosslink ratio and solvent amount must be carefully determined to avoid agglomerated polymer particles. The usage of these particles does not require any crushing, grounding and sieving processes. Compared to monoliths the spheres supply higher surface area. However, for SPE process, these micro and nano-spheres are not large enough to be used.

Adding the template to the synthesis results in a polymer that is specific for an analyte or selective to a group of analytes by creating imprinted sites. To understand the existence of imprinted sites, an extra polymer is synthesized under same conditions with MIP. However, this time template or dummy molecule is not added into the reaction medium. Thereby, imprinted sites are not created. This second substance is called Non Imprinted Polymer (NIP). By comparison of the results of two polymers, selectivity can be clarified.

1.6. Aim of This Study

The general purpose of this thesis is to develop novel SPE sorbents for determination of anti cancer drug agent 5-FU by HPLC-DAD. The thesis includes the preparation of molecularly imprinted polymers as a solid phase extraction material. This deals with the synthesis and characterization of the sorbents for 5-FU. The sorption performances of MIPs prepared with different matrices were investigated in terms of binding capacities. According to sorption performances of MIPs, one of them was chosen and optimized for improving the binding characteristics.

CHAPTER 2

EXPERIMENTAL

2.1. Chemicals

All reagents and chemicals were of analytical grade unless stated, otherwise ultrapure water (18.2 MΩ, Millipore) was used in all parts of the study. Plastics and glassware were washed with acetone, detergent and ultrapure water.

Anticancer drugs, 5-fluorouracil (5-FU) (99%) and 5-azacytidine (5AC) were obtained from Sigma-Aldrich. All studied solutions were prepared daily. Methanol was HPLC grade (Sigma–Aldrich, St. Louis, MO, USA). Before HPLC analysis all filtered solutions were filtered through 0.25 µm polyamide or cellulose acetate filter depending on the solvent system and degassed for 10.0 min. in ultrasonic bath. For the MIP/NIP synthesis, the functional monomers methacrylic acid (MAA) or acrylamide (AA), and crosslinker trimethylolpropane trimethacrylate (TRIM) were purchased from Sigma–Aldrich and the initiator azobisisovaleronitrile (AIVN) were obtained from Alfa Aesar.

2.2. Instrumentation and Apparatus

Ino Lab Level 1 pH meter (Weilheim, Germany) was used for pH adjustments. IKA yellow line OS 5 basic orbital shaker (Staufen, Germany) was used for effective mixing. The determination of 5-fluorouracil was performed by using Agilent 1200 Series HPLC system with a C30 (YMC, 250 mm x 4.6 mm) column applying isocratic elution with 90% ultrapure water – 10% acetonitrile as mobile phase at a flow rate of 0.8 mL/min. Agilent 7100 Series CE equipped with DAD was used in capillary electrophoresis measurements. Agilent Technologies undeactivated fused capillaries were used (i.d. 50 µm or 75 µm). Quanta 250FEG Scanning Electron Microscope (SEM) were used for morphological examination of the surface. For HPLC analysis, the operation conditions were optimized and listed in Table 2.1. Before the determination of optimum parameters and rebinding experiments, the calibration curve was obtained by measuring 5-FU standard solutions using eigth different concentrations (1.0-500.0 mg/L). Limit of detection (LOD) was calculated by analyzing the least concentrated standard 10 times with HPLC-DAD.

Table 2.1. Operation conditions for HPLC analysis.

HPLC	Agilent 1200
Analytical column	C30 (250 mm x 4.6 mm, 5 µm)
Mobile phase	90% UPW, 10% ACN
Flow rate	0.8 mL/min
Column temperature	30.0 ºC
Sample volume	20.0 µL
Selected λ	220 nm
Standard solutions	1.0, 5.0, 10.0, 25.0, 50.0, 100.0 mg/L

For CE-DAD analysis, the operation conditions were optimized and listed in Table 2.2. Before each run a conditioning step was applied to the capillary column. Conditioning steps are;

1. Wash with MeOH for 5.0 min.
2. Wash with UPW for 5.0 min.
3. Wash with 1M NaOH for 5.0 min.
4. Wash with 0.1M NaOH for 5.0 min.
5. Wash with UPW for 5.0 min.
6. Wash with buffer solution for 5.0 min.

Also, the pre-conditioning steps were repeated after every 4 injections to ensure the repeatability.

Table 2.2. Operation conditions for CE analysis.

CE	Agilent 7100
Analytical column	Agilent Technologies, FS, Undeactivated (75 µm i.d. column)
Casette temperature	30.0 ºC
Injection pressure	50.0, 60.0 mbar
Injection time interval	5.0, 10.0 sec.
Power and Current	6.0 W, 300.0 µA
Voltage	20.0 kV
Selected λ	234 nm, 270 nm
Electrolyte solution	25.0 mM borate buffer (pH 9.5, 10.5, 11.5), 10.0 mM borate buffer (pH 9.5, 10.5, 11.5)
Standard solutions	1.0, 5.0, 10.0, 25.0, 50.0, 100.0 mg/L

2.3. Synthesis of MIP/NIP

Five different types of MIP monoliths were prepared by using bulk polymerization method. Molecularly imprinted polymers and their corresponding non-imprentid polymers were synthesized by using different functional monomers and different ratios of reagents. Also synthesis of uniform sized MIP microspheres as well as NIP polymers were performed by precipitation polymerization

2.3.1. Synthesis of MIP/NIP Monoliths using MAA as Monomer

Pre-polymer solution for MIP was synthesized at ratio 1:8:20 (template molecule: functional monomer: crosslinker). Firstly, 1.0 mmol 5-FU (template molecule) dissolved in 5.0 mL DMSO (porogen), 8.0 mmol MAA (functional monomer) added to the solution at room temperature for 1.0 hour. Then, 20.0 mmol

TRIM (crosslinker) and 2.0 % mole AIVN (initiator) were added to the reaction system under nitrogen gas. To obtain the monolith polymer, reaction system was sealed and kept at 60.0 ˚C. The resultant rigid bulk polymer was incubated for 24 hour at 60 °C at oven. After drying procedure, obtained polymers were crushed, powdered and then siewed through 150.0 µm stainless steel siew. Soxhlet system was used in order to extract template molecule from synthesized polymers. Acetic acid-methanol mixture (1:1, 200.0 mL) were chosen as the solvent for at least 48 hours, followed by methanol (200.0 mL) for another 48 hours. After complete removal of template molecule, monoliths were dried in oven at 60.0 °C. The NIP monoliths were prepared following the same procedure except the addition of 5-FU.

2.3.2. Synthesis of MIP/NIP Monoliths using AA as Monomer

There different MIP/NIP monolith matrices at the ratios (1:1:20), (1:4:20) and (1:8:20) prepared by using MIP/NIP synthesis procedure. Firstly, MIP monolith pre-polymer solution was prepared at ratio 1:1:20. After that, 1.0 mmol 5-FU dissolved in 5.0 mL DMSO, 1.0 mmol AA added to the solution at room temperature for 1 hour to obtain non-covalent interactions between template molecule and the functional monomer. Then, 20.0 mmol TRIM and 2.0 % mole AIVN were added to the reaction system under nitrogen gas. For polymerization, all reaction system was sealed at 60.0 ˚C. The resultant rigid bulk polymer was incubated for 24 hour at 60.0 °C. Dried materials were crushed, powdered and then siewed through 150.0 µm stainless steel siew. The resultant materials extracted with soxhlet system. Acetic acid-methanol mixture (1:1, 200.0 mL) was used to remove the template molecule at least 48 hours, followed by methanol (200.0 mL) for another 48 hours. After complete removal of the template molecule, monoliths were dried at oven at 60 °C. The NIP monoliths were prepared following the same procedure except the addition of 5-FU.

In the study of (Puoci et al., 2007), MIP/NIP monoliths were prepared with ratio (2:16:20) in the presence of DMF as porogen. With some modifications, 2.0 mmol 5-FU dissolved in 4.0 mL DMF and 16.0 mmol AA were added to the solution at room temperature for 1 hour. Then, 20.0 mmol TRIM and 2.0 % mole AIVN were added to reaction system under nitrogen gas. For polymerization, the reaction system was sealed and kept at 60.0 ˚C. The resultant rigid bulk polymer was incubated for 24 hour at 60.0 °C. After drying, the solid was crushed, were powdered and then siewed through 150.0

μm stainless steel siew. The resultant materials extracted with soxhlet system. Acetic acid-methanol mixture (1:1, 200.0 mL) was used for removing the template molecule from MIP at least 48 hours, followed by methanol (200.0 mL) for another 48 hours. After complete removal of the template molecule, monoliths were dried in oven at 60.0 °C. The NIP monoliths were prepared following the same procedure except the addition of 5-FU.

2.3.3. Synthesis of MIP/NIP Micro/Nanospheres using MAA as Monomer

The procedure utilized in the synthesis of MIP/NIP micro/nanospheres was compiled from literature (Cirillo et. al., 2018) with some modifications. Pre-polymer solution for MIP synthesis was prepared at ratio 1:8:10 (template molecule: functional monomer: crosslinker). Firstly, 1.0 mmol 5-FU dissolved in 20.0 mL methanol. After dissolvation, 20.0 mL of acetonitrile were added to the solution. Then, 1.0 mmol MAA was added to the solution at room temperature and were mixed for 1 hour to obtain non-covalent interactions between template molecule and the functional monomer. Furthermore, 20.0 mmol TRIM and 50.0 mg AIVN were added to the reaction system under nitrogen gas. For polymerization, all reaction system was sealed and kept at 60.0 ˚C. The resultant polymer was incubated for 24 hour at 60.0 °C. Dried materials extracted with soxhlet system. Acetic acid-methanol mixture (1:1, 200.0 mL) was used to remove the template molecule from MIP at least 48 hours, followed by methanol (200.0 mL) for another 48 hours. After complete removal of the template molecule, polymer microspheres were dried in oven at 60.0 °C. The NIP microspheres were prepared following the same procedure except the addition of 5-FU.

In addition, MIP nanospheres were prepared at the same ratios (1:8:10) by using different porogen amounts. In this synthesis, acetonitrile amount was increased from 20.0 mL to 180.0 mL. NIP nanospheres were obtained by following the same procedure except the addition of 5-FU.

2.4. Rebinding Experiments

The binding capacities of all synthesized MIP/NIP monoliths were investigated. At the same time the highest amount of extraction was determined by using binding

efficiencies of MIP and NIP. Ten milliliters of 5-FU solutions with different concentrations (1.0-500.0 mg/L) were separately added onto 5.0 mg MIP/NIP monoliths in 15.0 mL centrifuge tubes and then were shaken at 560.0 rpm for 60.0 min. At the end of the shaking period, the mixtures were filtered and free 5-FU concentrations in supernatants were determined by HPLC-DAD.

2.5. Cross Selectivity

The anticancer drug 5-FU and its structuraly related anti cancer drug agent 5AC were used for selective recognition studies at concentration of 100.0 mg/L in 10.0 mL. Selected concentrations of 5-FU and 5AC were added onto 100.0 mg of MIP/NIP monoliths. Sorption was achieved on an orbital shaker at 560.0 rpm and 60.0 min. Cellulose acetate syringe filters were used for the separation of sorbents from the mixtures. All effluents were analyzed by HPLC-DAD and UV detection at 220 nm.

2.6. SPE Optimization Parameters

For the determination of 5-FU, the extraction efficiency was optimized by using parameters such as pH of sample solution, amount of sorbent and shaking time.

First of all, the influence of pH change on the adsorption efficiency was determined. For this purpose, pH of the 5-FU solutions at a concentration of 100.0 mg/L (10.0 mL) were adjusted to 1.0, 3.0, 5.0, 7.0 and 10.0 and added to 5.0 mg of each MIP/NIP (1:1:20 AA, 1:4:20 AA, 1:8:20 MAA, 1:8:20 AA, 1:8:10 AA) sorbent pairs. Sorption process was realized using orbital shaker at 560.0 rpm for 60.0 min. Effluents were analyzed by HPLC-DAD and UV detection at 220 nm.

The amount of MIP was optimized by changing amount from 5.0 to 100.0 mg for again 10.0 mL of 100.0 mg/L 5-FU solutions.

Effect of shaking time was investigated by changing time interval from 1.0 to 120.0 min with the use of 100.0 mg MIP monolith. Other parameters were kept constant and the effluents were analyzed by HPLC-DAD.

Reusability of the synthesized sorbent was checked via sorption/desorption cycle for 100.0 mg/L 5-FU and 100.0 mg MIP. After each cycle the sorbent was dried in an oven. This protocol was applied 10 times for the same sorbent. Summary of optimization parameters are given in Table 2.3.

Table 2.3. All studied parameters

standard concentration	100.0 mg/L
pH	1.0, 3.0, 5.0, 7.0, 10.0
amount of sorbent	5.0, 10.0, 25.0, 50.0, 100.0 mg
adsorption time	1.0, 5.0, 15.0, 30.0, 60.0, 120.0 min.
desorption solvents	acetic acid, methanol

CHAPTER 3

RESULTS AND DISCUSSION

3.1. Anticancer drug analysis by HPLC-DAD system

In HPLC analysis, C30 column was preferred for 5-FU analysis for its good resolution in reverse phase mode. An isocratic program has lead 5-FU to be eluted at retention times of approximately 4.5 min. The mobile phase used was the mixture of 10 % ACN – 90 % ultrapure water. Different percentages of ACN-UPW mixtures and various flow rates were tried to obtain the best resolution in the shortest time. Finally, it was decided to use a 0.8 mL/min flow rate at 30.0 °C column temperature. Diode array detector was used to detect the analytes at three different wavelengths (220, 230 and 280 nm). Results were calculated based on the chromatogram of 5-FU given Figure 3.1.

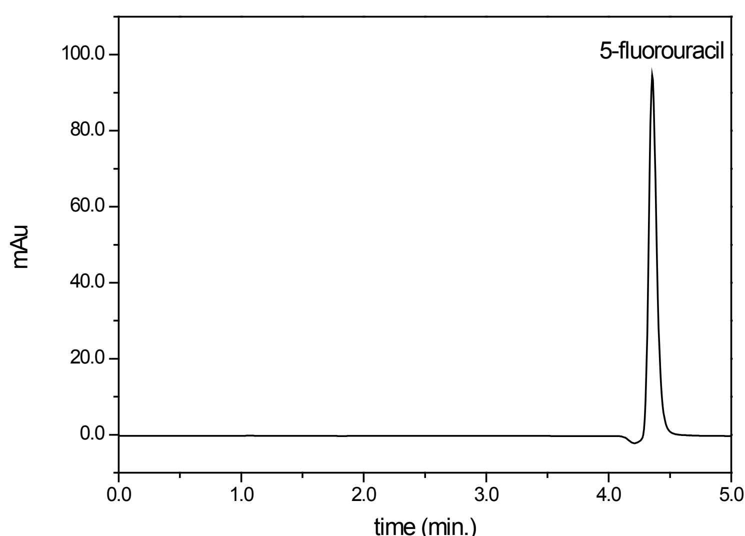


Figure 3.1. Chromatogram of 10.0 mg/L 5-FU (Agilent 1200 Series HPLC-DAD (YMC C30 (250 mm x 4.6 mm)), 90:10 water:acetonitrile (pH 5.0) mobile phase 0.8 mL/min flow rate, 220 nm)

Instrumental parameters are given in Table 2.1. Plot of signal versus 5-FU concentration can be seen in Figure 3.2. LOD and LOQ values were calculated as 0.73 and 2.4, respectively.

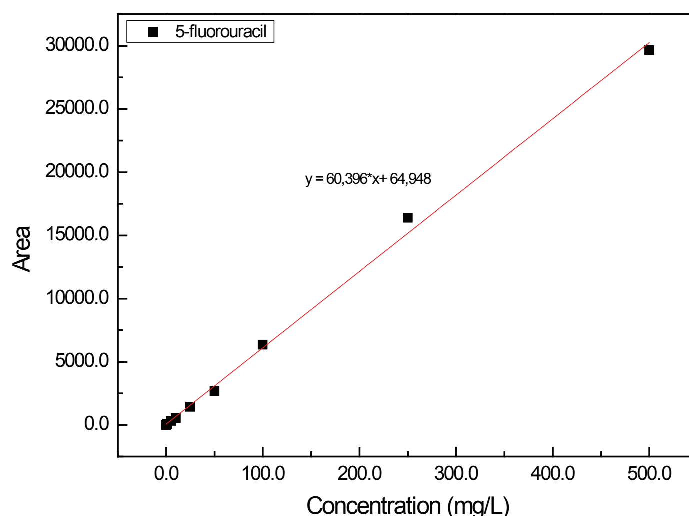


Figure 3.2. Calibration plot of 5-FU (Agilent 1200 Series HPLC-DAD (YMC C30 (250 mm x 4.6 mm)), 90:10 water:acetonitrile (pH 5.0) mobile phase 0.8 mL/min flow rate, 220 nm), (n=3).

3.2. Anticancer drug analysis by CE-DAD system

In the capillary electrophoresis (CE) analysis, several factors effect the electroosmotic flow and should be optimized; such as voltage, type of buffer system pH of buffer, temperature. For this purpose, working parameters have been systematically changed and optimal conditions were determined for the analysis of anticancer drugs. Instrumental parameters were given in Table 2.2. 5-azacytidine (5AC) and 5-fluorouracil (5-FU) solutions were prepared as 20.0 mg/L. Borate buffer (25.0 mM) was used as an electrolyte solution that is suitable for initial trial for the separation of these anticancer drugs. Analytes were given to the CE system first separately and then as a mixture. Electropherograms of 5AC, 5-FU and their mixture are given in Figure 3.3, 3.4 and 3.5, respectively. As seen in Figure 3.3, 5AC shows undesirable shouldered peak. Between the two, 5AC comes first and 5-FU the second. The reason must be stemming from the pKa values of analytes. 5-fluorouracil is completely ionized at pH 9.5 (pKa 8.0) and interact with silonol groups of silica capillary column more strongly compared to 5AC.

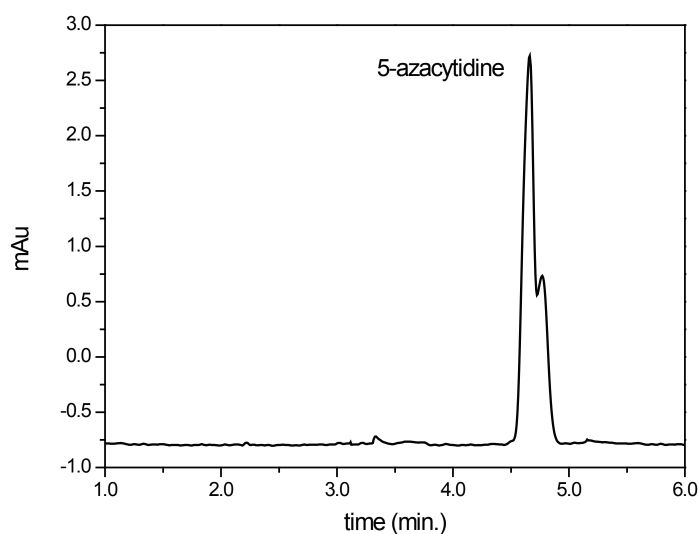


Figure 3.3. Electropherogram of 20.0 mg/L 5AC (Agilent 7100 Series CE-DAD, FS, Undeactivated (75 µm i.d. column), 25.0 mM borate buffer, pH 9.5, 234 nm)

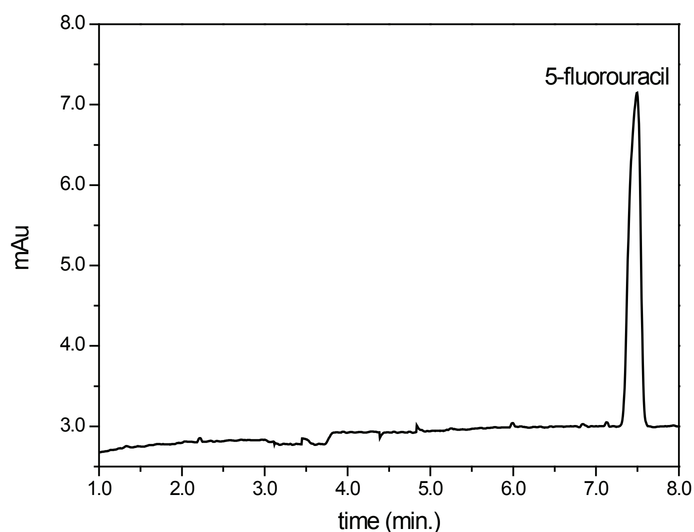


Figure 3.4. Electropherogram of 20.0 mg/L 5-FU (Agilent 7100 Series CE-DAD, FS, Undeactivated (75 µm i.d. column), 25.0 mM borate buffer, pH 9.5, 270 nm)

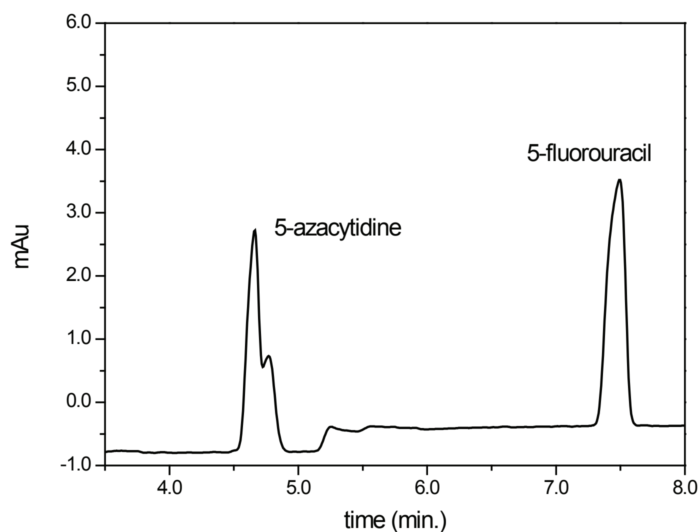


Figure 3.5. Electropherogram of 20.0 mg/L 5AC and 5-FU mixture (Agilent 7100 Series CE-DAD, FS, Undeactivated (75 µm i.d. column), 25.0 mM borate buffer, pH 9.5, 234 nm)

In order to obtain better 5AC peak, pH of the buffer system was changed to first 10.5 and then to 11.5. As seen in Figures 3.6 and 3.7 by increasing the pH, mobilities of each analyte reach the optimum and better results were obtained.

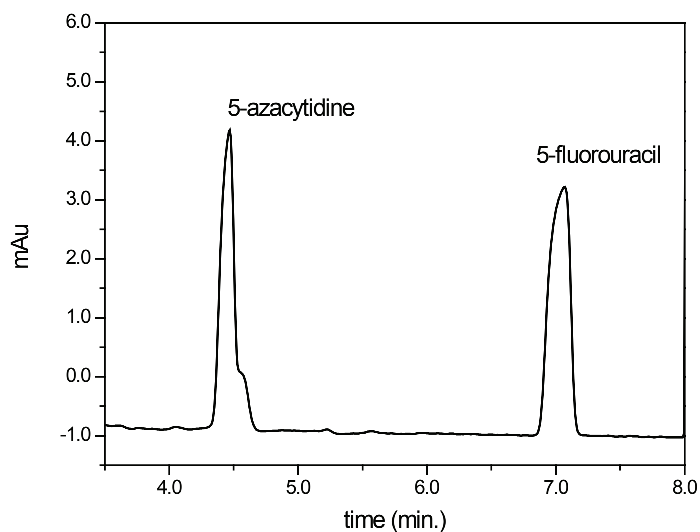


Figure 3.6. Electropherogram of 20.0 mg/L 5AC and 5-FU mixture (Agilent 7100 Series CE-DAD, FS, Undeactivated (75 µm i.d. column), 25.0 mM borate buffer, pH 10.5, 234 nm)

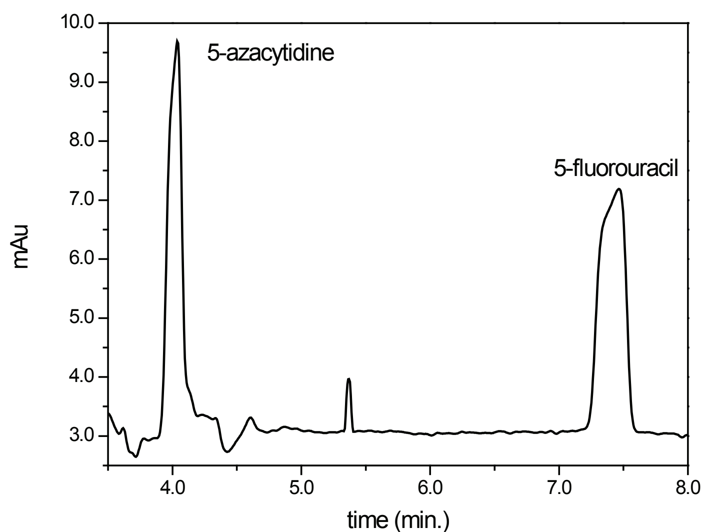


Figure 3.7. Electropherogram of 20.0 mg/L 5AC and 5-FU mixture (Agilent 7100 Series CE-DAD, FS, Undeactivated (75 µm i.d. column), 25.0 mM borate buffer, pH 11.5, 234 nm)

Second important parameter is the concentration of buffer solution because it highly affects the electroosmotic flow by changing the thickness of the diffuse double layer; therefore, reducing the buffer concentration to 10.0 mM shortens the retention times. Figure 3.8, 3.9 and 3.10 show the electropherograms of 20.0 mg/L mixture of 5AC and 5-FU at three different pH values with 10.0 mM borate buffer. Also the other parameters such as the applied voltage, injection pressure, injection time, peak width, cassette temperature were changed with no improvement. The optimized parameters were as; 20.0 kV voltage (with 6.0 W, 300.0 µA), 10.0 mM borate buffer (pH 11.5), 50.0 mbar injection for 10.0 sec., 30.0 ˚C cassette temperature, 2.5 Hz peak width.

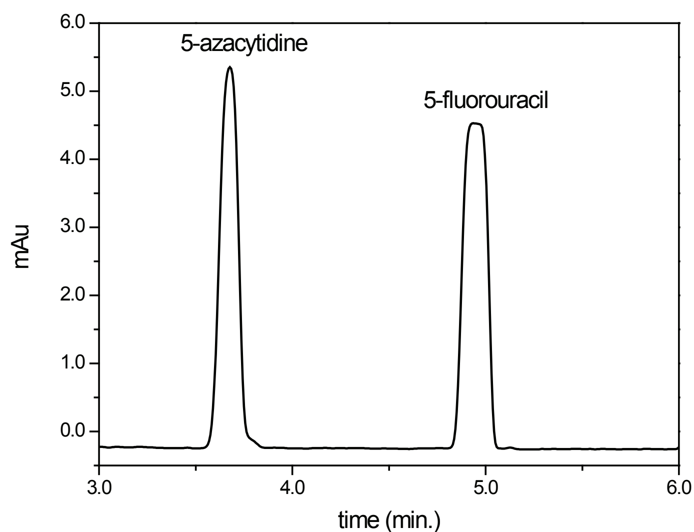


Figure 3.8. Electropherogram of 20.0 mg/L 5AC and 5-FU mixture (Agilent 7100 Series CE-DAD, FS, Undeactivated (75 µm i.d. column), 10.0 mM borate buffer, pH 9.5, 234 nm)

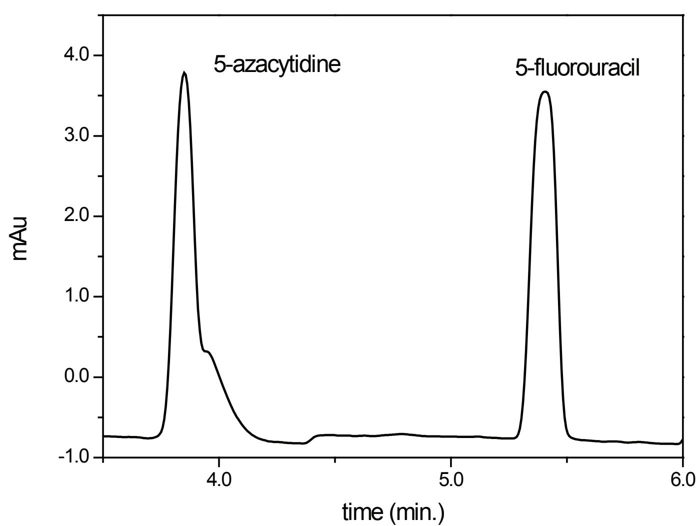


Figure 3.9. Electropherogram of 20.0 mg/L 5AC and 5-FU (Agilent 7100 Series CE-DAD, FS, Undeactivated (75 µm i.d. column), 10.0 mM borate buffer, pH 10.5, 234 nm)

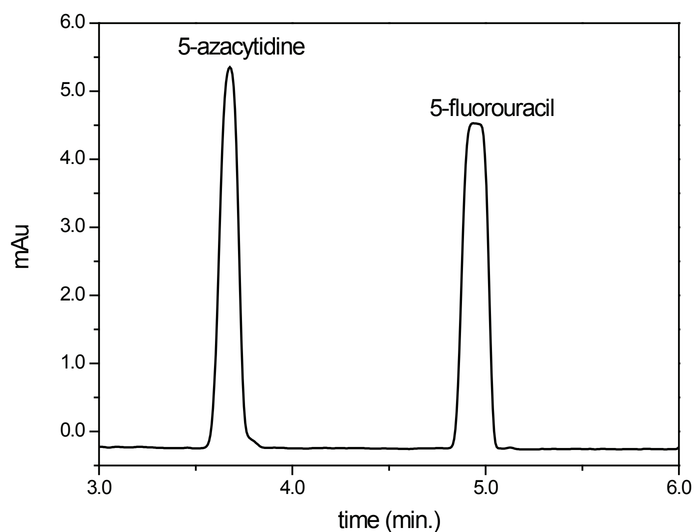


Figure 3.10. Electropherogram of 20.0 mg/L 5AC and 5-FU mixture (Agilent 7100 Series CE-DAD, FS, Undeactivated (75 µm i.d. column), 10.0 mM borate buffer, pH 11.5, 234 nm)

With the optimized conditions, limit of detection (LOD) values were calculated as 0.40 mg/L and, 0.28 mg/L, and limit of quantification (LOQ) values are found as 1.33 mg/L, 0.92 mg/L for 5-FU and 5AC respectively. Calibration plots are shown in Figure 3.11.

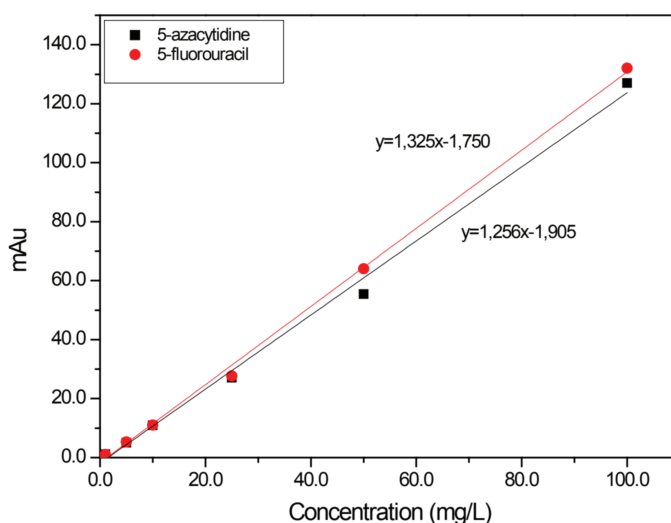


Figure 3.11. Calibration plots of 5AC and 5-FU mixture (Agilent 7100 Series CE-DAD, FS, Undeactivated (75 µm i.d. column), 10.0 mM borate buffer, pH 11.5, 234 nm), (n=3)

3.3. Preparation and Characterization of Monolith and Micro/nanosphere Printed Polymers

In the first part of the study, AA-TRIM-DMSO polymer formulation with the different template:monomer:cross-linker molar ratios (1:1:20, 1:4:20, 1:8:20 and 1:8:10) were used for the creation of the recognition sites of template molecule, 5-FU. Figure 3.12 shows the possible synthesis mechanism of 5-FU-MIP.

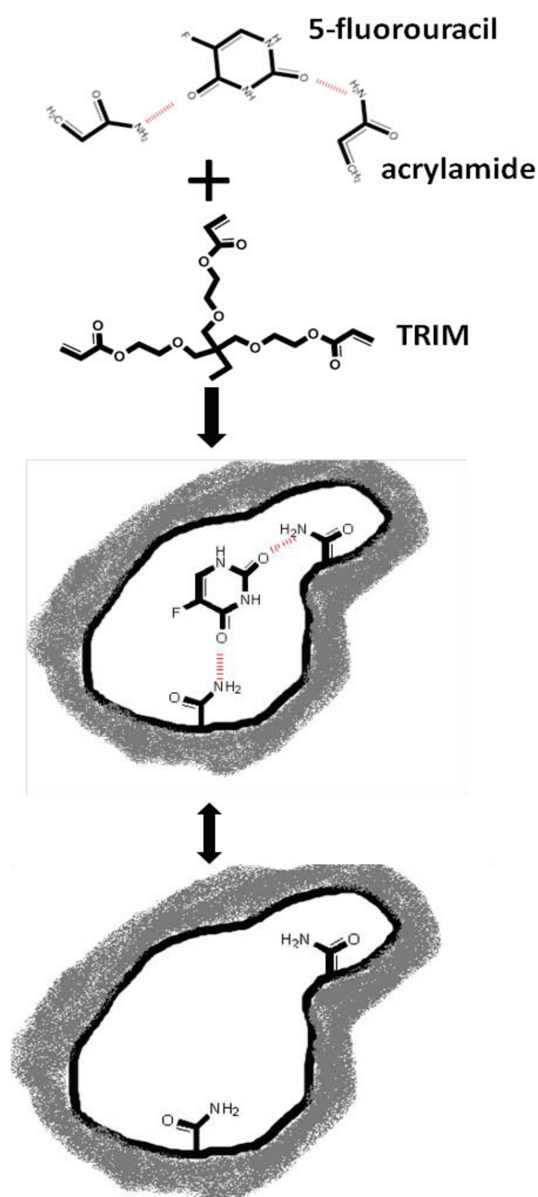


Figure 3.12. Possible synthesis mechanism of 5-FU imprinted polymer

Functional monomer, AA, is responsible for the binding interactions in the imprinted binding sites. The basic resaon of chosing AA as functional monomer is the interaction between the basic amine moiety in AA and the template molecule. The SEM images of synthesized 5-FU printed and nonimprinted polymers are given in Figure 3.13. Monolithic polymers were obtained by bulk polymerization method. It is clearly seen that there are no significant differences between morphology of NIP and MIP particles.

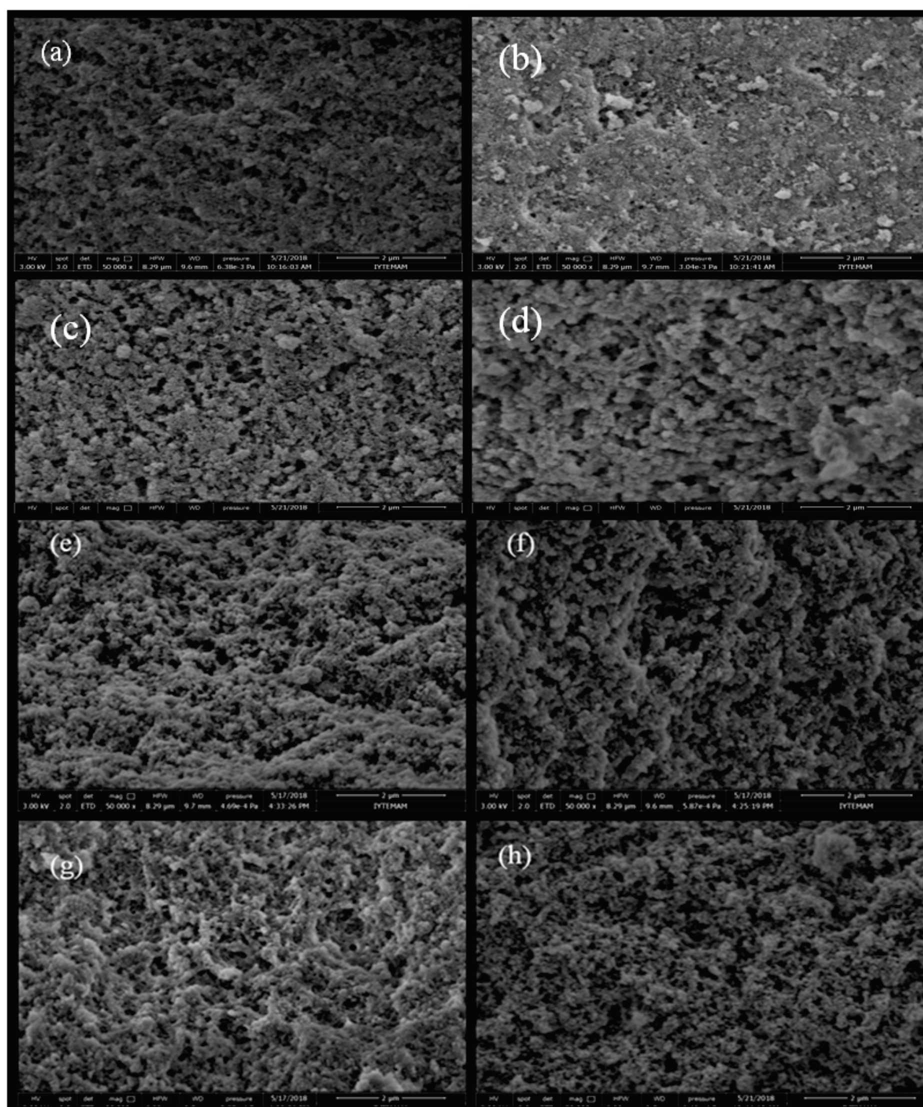


Figure 3.13. SEM images of NIP/MIP monoliths (a) NIP (1:1:20 AA), (b) MIP (1:1:20 AA) (c) NIP (1:4:20 AA), (d) MIP (1:4:20 AA), (e) NIP (1:8:20 AA), (f) MIP (1:8:20 AA), (g) NIP (1:8:10 AA), (h) MIP (1:8:10 AA)

In the second part of the study, MAA-TRIM-DMSO polymer formulation with the 1:8:20 were used for the creation of recognition sites of template molecule, 5-FU. Fig.3.14 shows the synthesis mechanism of 5-FU-MIP.

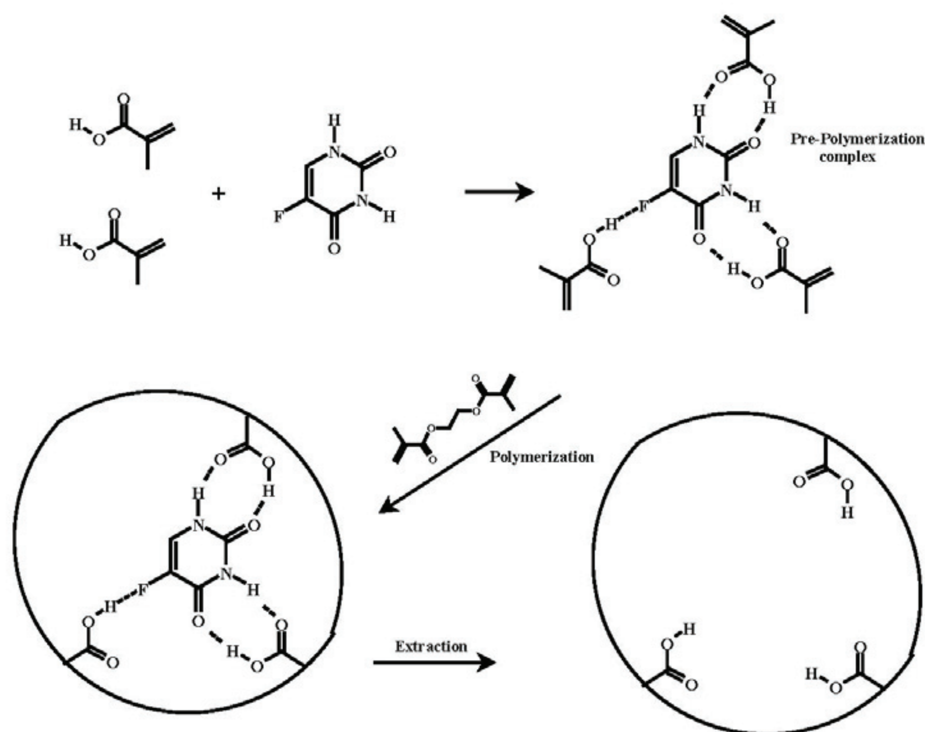


Figure 3.14. Possible synthesis mechanism of 5-FU imprinted polymer. (Puoci et al., 2007)

Total monomer/porogen ratio (w/v) helps in controlling the morphology of polymer. If this ratio is smaller than 5.0 %, the precipitation polymerization takes place and the polymer will be spherical shaped. In the second part of the study, firstly monolithic MIP/NIP polymers were synthesized by using bulk polymerization method (32.5 %.) and spherical polymers were synthesized by using precipitation polymerization method (2.5%). As seen in Figure 3.15 (a) and (b) there is no significant differences between morphology of NIP and MIP monolithic polymers. MIP microspheres pre-polymer solution was prepared at ratio 1:8:10 in 40.0 mL acetonitrile: methanol mixture (20:20 v/v) (Figure 3.15 (c)). By increasing the acetonitrile ratio in porogen mixture (180:20 v/v)), MIP nanospheres were obtained (Figure 3.15 (d)). In this study, non-covalent imprinting approach was used which is based on the formation of non-covalent interactions (hydrogen bonding, hydrophobic and ionic interactions) of functional

monomer and template molecule. This approach provided easy preparation of the template monomer complex and easy removal of the templates from the polymers.

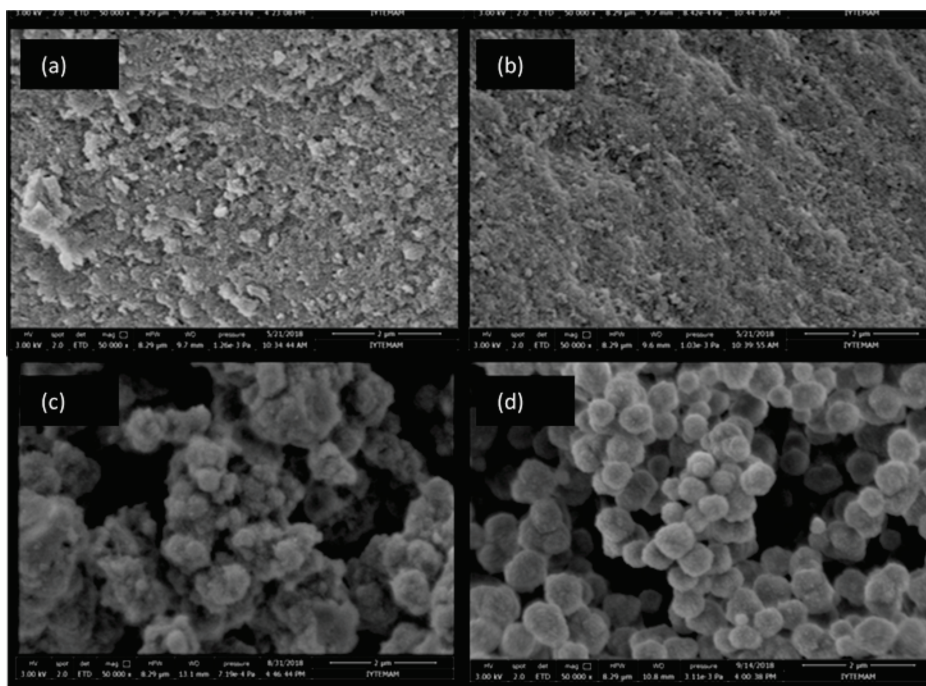


Figure 3.15. SEM images of NIP/MIP monoliths (a) NIP (1:8:20 MAA), (b) MIP (1:8:20 MAA), (c) MIP (1:8:10 AA, (20:20 v/v)), (d) MIP (1:8:10 AA (20:180 v/v)).

3.4. Rebinding Experiments

All synthesized MIP/NIP monoliths with AA-TRIM-DMSO formulations at different ratios: (1:1:20, 1:4:20, 1:8:20 and 1:8:10) were investigated according to their sorption capacities against 5-FU. As seen in Figures 3.16, 3.17 and 3.18, with the increase in 5-FU concentration, it is observed that MIP has better sorption capacity than NIP monolith due to the presence of special binding sites of MIP monolith.

The maximum difference of sorption capacities between MIP and NIP monoliths with 1:1:20 ratio is seen at 250.0 mg/L (Figure 3.16) is an unusual result which needs further experiments to explain. In the rest of the experiments 1:1:20 AA ratio was chosen to prepare the SPE sorbents.

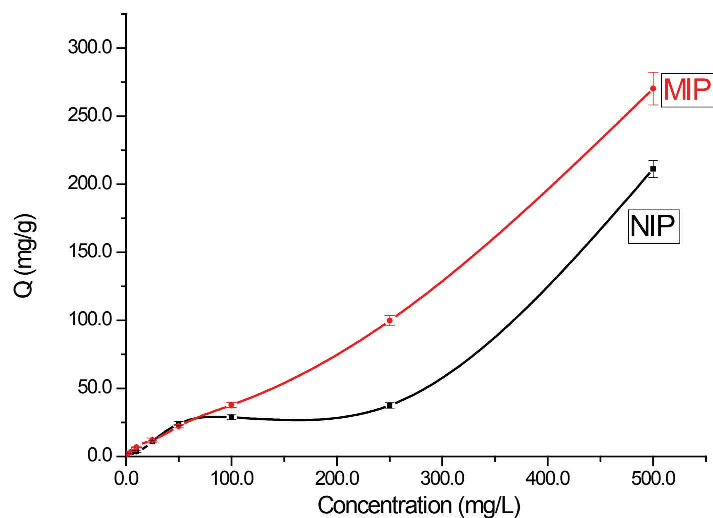


Figure 3.16. Rebinding characteristics assay of MIP/NIP monoliths (1:1:20 AA), (Agilent 1200 Series HPLC-DAD 250 mm C30 column (YMC C30 (250 mm x 4.6 mm)), 90:10 water:acetonitrile mobile phase 0.8 mL/min flow rate, 220 nm), (n=3).

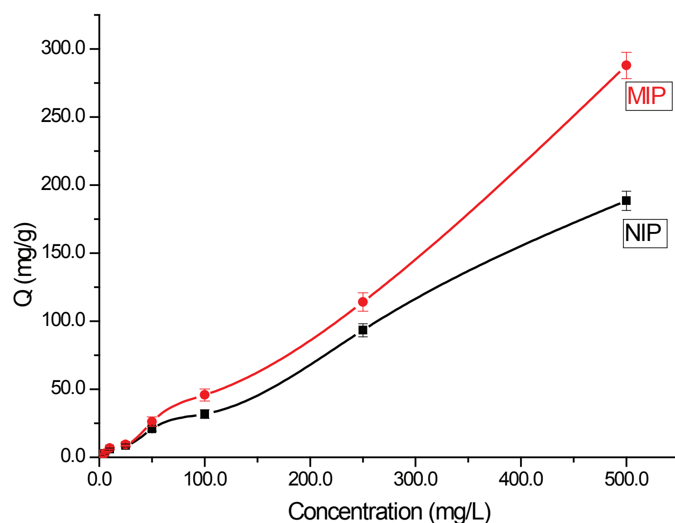


Figure 3.17. Rebinding characteristics assay of MIP/NIP monoliths (1:4:20 AA), (Agilent 1200 Series HPLC-DAD 250 mm C30 column (YMC C30 (250 mm x 4.6 mm)), 90:10 water:acetonitrile mobile phase 0.8 mL/min flow rate, 220 nm), (n=3).

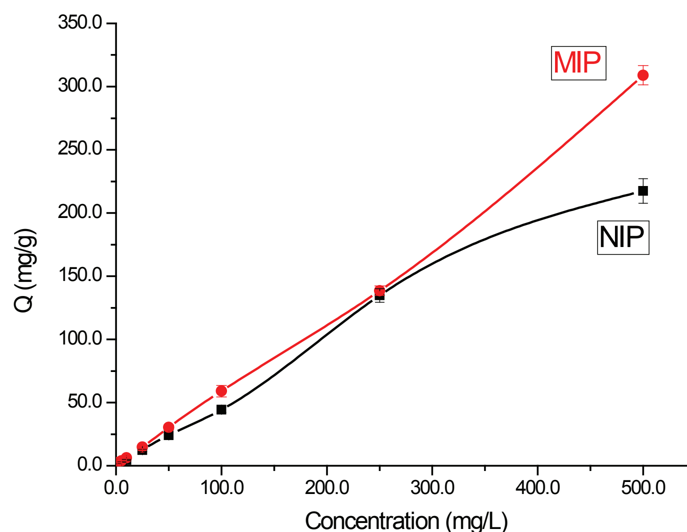


Figure 3.18. Rebinding characteristics assay of MIP/NIP monoliths (1:8:20 AA), (Agilent 1200 Series HPLC-DAD 250 mm C30 column (YMC C30 (250 mm x 4.6 mm)), 90:10 water:acetonitrile mobile phase 0.8 mL/min flow rate, 220 nm), (n=3).

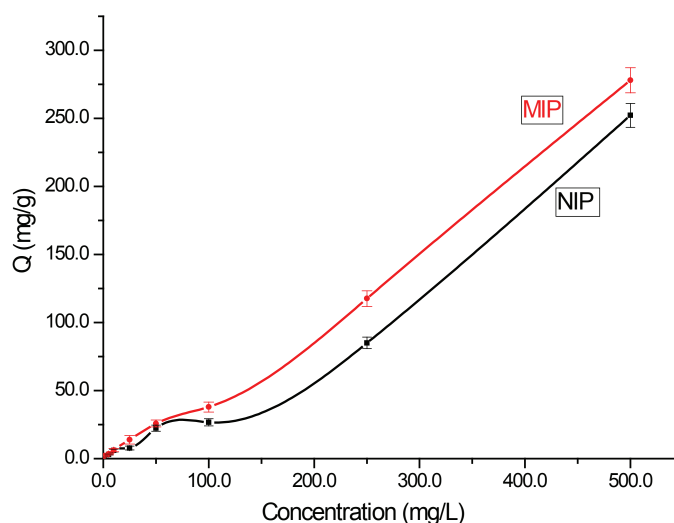


Figure 3.19. Rebinding characteristics assay of MIP/NIP monoliths (1:8:10 AA), (Agilent 1200 Series HPLC-DAD 250 mm C30 column (YMC C30 (250 mm x 4.6 mm)), 90:10 water:acetonitrile mobile phase 0.8 mL/min flow rate, 220 nm), (n=3).

Monolithic synthesis of MIP/NIP by using MAA as functional monomer reveals highest sorption capacity at 250.0 mg/L of initial concentration of 5-FU (Figure 3.20). Moreover, NIP shows increasing trend in sorption of 5-FU with increasing initial concentration of 5-FU. The results show the necessity of further experiments to

enlighten the observed phenomenon. On the other hand, obtained MIP/NIP micro/nanospheres were used in rebinding experiments but any differences in sorption of 5-FU between NIP and MIP polymers have not be observed.

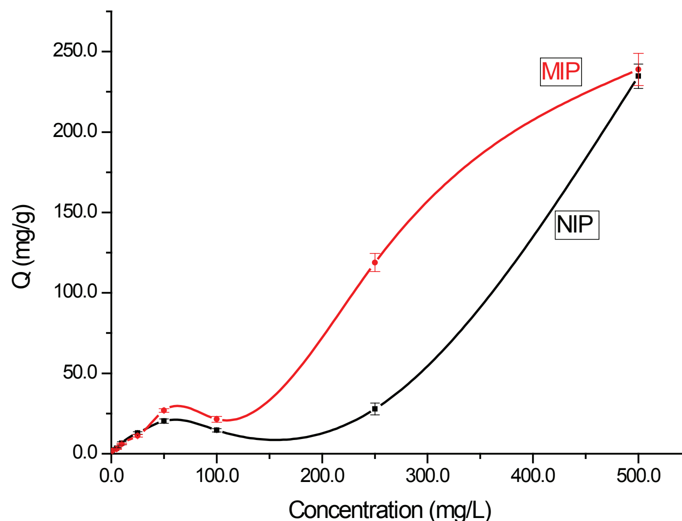


Figure 3.20. Rebinding characteristics assay of MIP/NIP (1:8:20 MAA), (Agilent 1200 Series HPLC-DAD 250 mm C30 column (YMC C30 (250 mm x 4.6 mm)), 90:10 water:acetonitrile mobile phase 0.8 mL/min flow rate, 220 nm), (n=3).

3.5. Cross Selectivity

Anticancer drug 5-fluorouracil (5-FU) and a structuraly related molecule, 5-azacytidine (5AC) were compared according to their sorption capacities to the MIP/NIP monoliths. As shown in Figure 3.21, 5-FU imprinted polymer showed better sorption properties than its corresponding NIP. In addition, it showed very low sorption to 5AC. Structurally related anticancer drug agent 5AC has a bigger molecular size than 5-FU so it cannot enter the cavities which are specific for 5-FU. Sorption capacities of NIP monolith to 5-FU and 5AC showed no significant difference. These two molecules can be captured by the surface of the NIP monolith with non specific interactions. Results show that, MIP monolith has recognition ability to 5-FU provided by memory cavities.

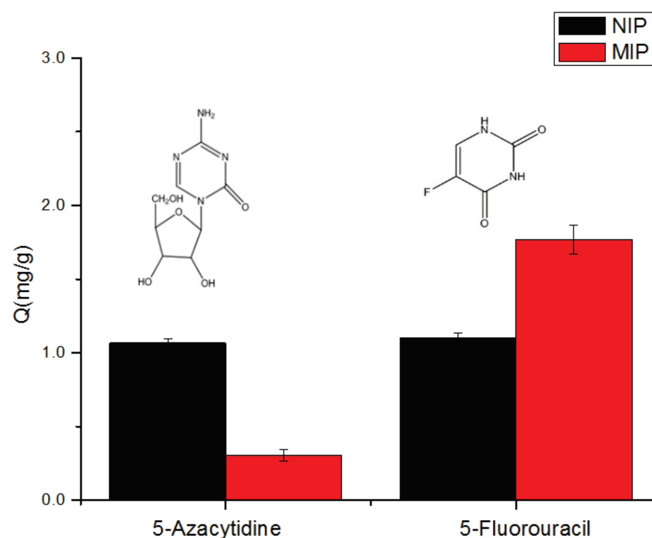


Figure 3.21. Cross Selectivity of MIP/NIP monoliths (1:1:20 AA), (Agilent 1200 Series HPLC-DAD (YMC C30 (250 mm x 4.6 mm)), 90:10 water:acetonitrile mobile phase 0.8 mL/min flow rate, 220 nm), (n=3).

3.2. SPE Optimization Parameters

The pH of the solutions have a significant role in the sorption efficiency. The net charge of amine groups of the polymer matrix and active sites of the 5-FU molecule can be affected from pH changes. It determines the hydrogen bonding ability between the template molecule and the amine groups of the sorbent. Solution pH was changed in a wide range (1.0, 3.0, 5.0, 7.0, 10.0) and efficiency of the MIP/NIP monoliths on the sorption at each pH was investigated. All of the sorbents showed lower sorption efficiency at pH 1.0 and pH 10.0. Hydroxyl groups of 5-FU can be protonated in acidic medium. It increases the solubility of 5-FU in water and weakens the hydrogen bonding between template and monomer. At pH 10.0, reduction of sorption efficiency can be explained by deprotonation of 5-FU. As seen in Figures 3.22 to 3.26, the best sorption efficiencies were obtained at pH 5.0 for MIP/NIP (1:1:20 AA) and MIP/NIP (1:4:20 AA), at pH 3.0 for MIP/NIP (1:8:20 AA), at pH 7.0 for MIP/NIP (1:8:10 AA) and MIP/NIP (1:8:20 MAA).

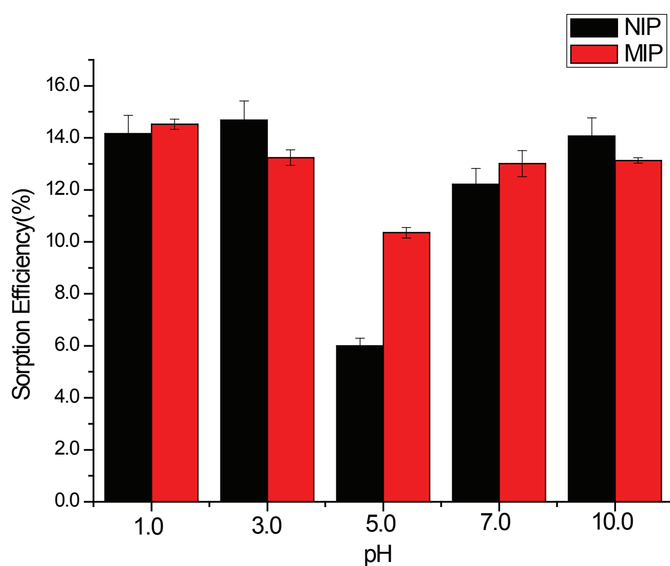


Figure 3.22. Effect of solution pH on the sorption of 5-FU (MIP/NIP, 1:1:20 AA), (Agilent 1200 Series HPLC-DAD (YMC C30 (250 mm x 4.6 mm)), 90:10 water:acetonitrile mobile phase 0.8 mL/min flow rate, 220 nm), (n=3).

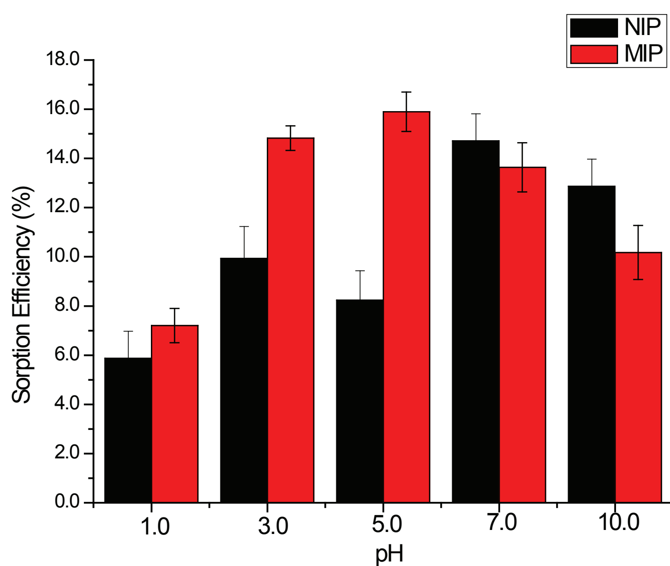


Figure 3.23. Effect of solution pH on the sorption of 5-FU (MIP/NIP, 1:4:20 AA), (Agilent 1200 Series HPLC-DAD (YMC C30 (250 mm x 4.6 mm)), 90:10 water:acetonitrile mobile phase 0.8 mL/min flow rate, 220 nm), (n=3).

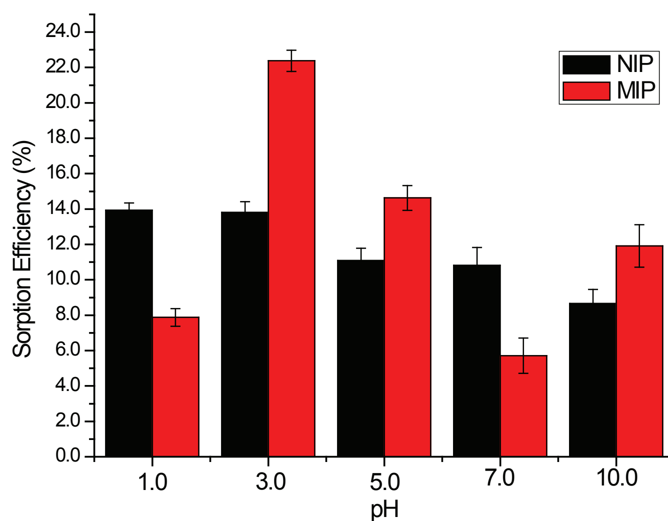


Figure 3.24. Effect of solution pH on the sorption of 5-FU (MIP/NIP, 1:8:20 AA), (Agilent 1200 Series HPLC-DAD (YMC C30 (250 mm x 4.6 mm)), 90:10 water:acetonitrile mobile phase 0.8 mL/min flow rate, 220 nm), (n=3).

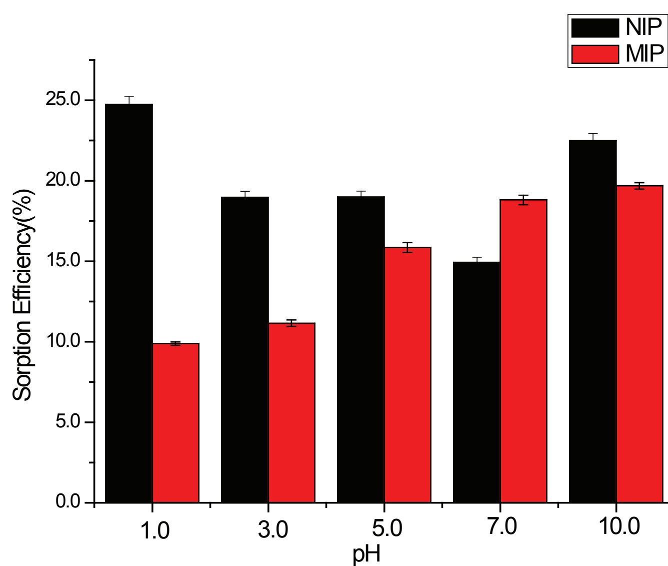


Figure 3.25. Effect of solution pH on the sorption of 5-FU (MIP/NIP, 1:8:10 AA), (Agilent 1200 Series HPLC-DAD (YMC C30 (250 mm x 4.6 mm)), 90:10 water:acetonitrile mobile phase 0.8 mL/min flow rate, 220 nm), (n=3).

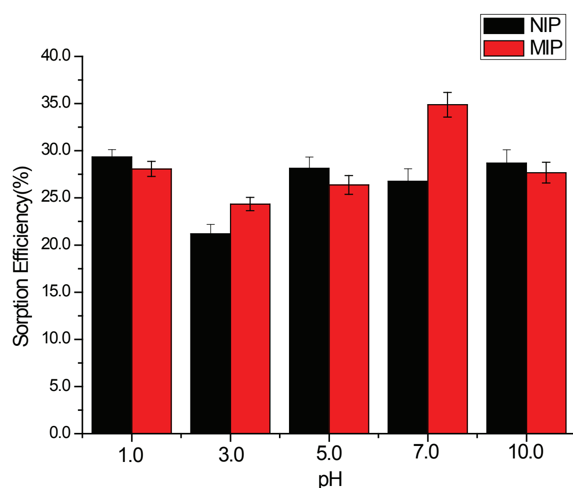


Figure 3.26. Effect of solution pH on the sorption of 5-FU (MIP/NIP, 1:8:20 MAA), (Agilent 1200 Series HPLC-DAD (YMC C30 (250 mm x 4.6 mm)), 90:10 water:acetonitrile mobile phase 0.8 mL/min flow rate, 220 nm), (n=3).

Effect of amount of MIP monoliths were optimized by varying from 5.0 mg to 100.0 mg. The sorption capacities of the sorbents increased by the increasing amount of sorbent. The maximum sorption capacity obtained by using 100.0 mg sorbent. This amount was used for the following experiments.

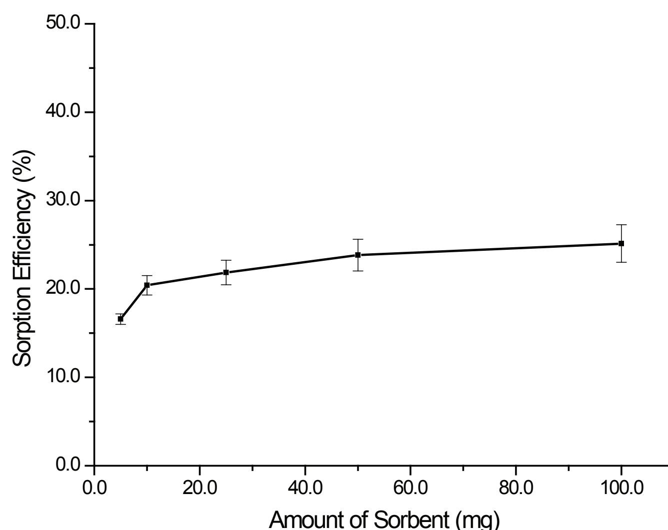


Figure 3.27. Effect of amount of sorbent on the sorption of 5-FU (MIP, 1:1:20 AA), (Agilent 1200 Series HPLC-DAD (YMC C30 (250 mm x 4.6 mm)), 90:10 water:acetonitrile (pH 5.0) mobile phase 0.8 mL/min flow rate, 220 nm), (n=3).

Sorption capacity of MIP monolith at a constant amount (100.0 mg) was investigated by changing the shaking time. Results were obtained at 1.0, 5.0, 15.0, 30.0,

60.0 and 120.0 min. Even at 1.0 min, a very efficient sorption was obtained. Shaking time was closed to be 60.0 min to guarantee the sorption.

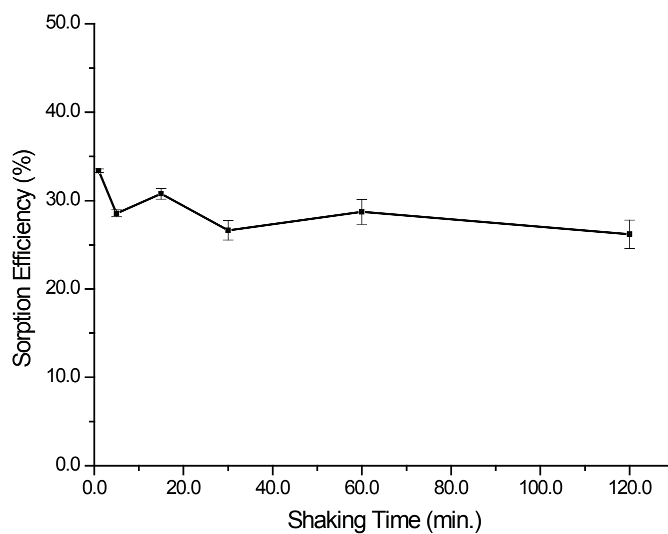


Figure 3.28. Effect of shaking time on the sorption of 5-FU (MIP, 1:1:20 AA) (Agilent 1200 Series HPLC-DAD (YMC C30 (250 mm x 4.6 mm)), 90:10 water:acetonitrile (pH 5.0) mobile phase 0.8 mL/min flow rate, 220 nm), (n=3).

CHAPTER 4

CONCLUSION

The thesis progressed on solid-phase extraction method. Molecularly imprinted polymers were synthesized at five different (template: functional monomer: crosslinker) ratios of (1:1:20 AA, 1:4:20 AA, 1:8:20 AA, 1:8:20 MA, 1:8:10 AA) for selective determination of 5-FU prior to HPLC-DAD determination. Imprinted polymers were prepared using 5-FU as template molecule, AA or MAA as functional monomers and TRIM as crosslinker. Imprinted polymer synthesized at 1:1:20 ratio with the functional monomer acrylamide has shown the best sorption properties among the other imprinted polymers. Imprinted and non-imprinted polymers were compared in terms of sorption capacities and imprinted polymer has shown superior sorption performance. Reason of this performance is the presence of specific binding sites (molecular-sized cavities) in the imprinted polymer for 5-FU. The pH of the solution, amount of sorbent and shaking time were optimized for improving the sorption ability of imprinted polymer. Selectivity and rebinding characteristics of imprinted polymer were also investigated. The extraction ability of imprinted and non-imprinted polymers was investigated to 5-FU and 5AC. The results indicate that MIP monolith has recognition ability to the template molecule. The optimized parameters for sorption were as pH 5.0, amount of sorbent 100.0 mg and shaking time 60.0 min.

Finally, this study can be considered in the intersection of many disciplines such as materials sciences, analytical chemistry, organic chemistry, physical chemistry, nanoscience with the aim of the preparation and the application of SPE sorbents in analytical studies.

REFERENCES

Argemí A., Saurina J., Study of the degradation of 5-azacytidine as a model of unstable drugs using a stopped-flow method and further data analysis with multivariate curve resolution. 2007. *Talanta* 74: 176–182.

Argemí A., Vega A., Subra-Paternault P., Saurina J., Characterization of azacytidine/poly(L-lactic) acid particles prepared by supercritical antisolvent precipitation. 2009. *J. Pharmac. Biomed. Anal*. 50: 847-852.

Badea I., Moja D., Tudose A., Stoicescu D., Determination of the 5-fluorouracil and N1(2'-furanidyl)uracil in the presence of tegafur by zero-crossing first derivative spectrometry. 2002. *J. Pharm. Biomed. Anal.* 30: 1371–1378.

Baker D.R., Capillary Electrophoresis. 1995. *John Wiley, New York*.

Blatný P., Kvasnička F., Application of capillary isotachophoresis and capillary zone electrophoresis to the determination of inorganic ions in food and feed samples. 1999. *J. Chromatogr. A*, 834: 419-431.

Castro E.R., Manz A., Present state of microchip electrophoresis: state of the art and routine applications. 2015. *J. Chromatogr. A,* 1382: 66-85.

Cirillo G., Iemma F., Puoci F., Parisi O.I., Curcio M., Spizzirri U.G., Picci N., Imprintedhydrophilic nanospheres as drug delivery systems for 5-fluorouracil sustained release. 2009. *Journal of Drug Targetting,* 17(1): 72-77.

Cong H.L., Xu X.D., Yu B., Yuan H., Peng Q.H., Tian C., Recent progress in preparation and application of microfluidic chip electrophoresis. 2015. *J. Micromech. Microeng.*, 25.

Dos Santos P. M., Hall A. J., Manesiotis, P., Stoichiometric molecularly imprinted polymers for the recognition of anti-cancer pro-drug tegafur. 2016. *Journal of Chromatography B*, 1021: 197-203.

Fahmy O., Korany M., Maher H., High performance liquid chromatographic determination of some co-administered anticancer drugs in pharmaceutical preparations and in spiked human plasma. 2004. *J. Pharm. Biomed. Anal*. 34: 1099–1107.

Fontanals N., Marce R., Borrull F., Overview of the novel sorbents available in solid-phase extraction to improve the capacity and selectivity of analytical determinations. 2010. *Contributions to science*, 199-213.

Geiger M., Hogerton A.L., Bowser M.T., Capillary Electrophoresis. 2012. *Anal. Chem*., 84: 577-596.

Kenndler E., A critical overview of non-aqueous capillary electrophoresis. Part I: mobility and separation selectivity. 2014. *J. Chromatogr. A*, 1335: 16-30.

Kissinger L., Stemm N., Determination of the antileukemia agents cytarabine and azacitidine and their respective degradation products by high-performance liquid chromatography. 1986. *J. Chromatogr*. 353: 309–318.

Komiyama M., Takeuchi T., Mukawa T., Asanuma H., WILEY-VCH. 2003. Molecular Imprinted From Fundamentals to Applications.

Kovalova L., McArdell C., Hollender J., Challenge of high polarity and low concentrations in analysis of cytostatics and metabolites in wastewater by hydrophilic interaction chromatography/tandem mass spectrometry. 2009. *J. Chromatogr. A*, 1216(7): 1100-1108.

Lu G., Crihfield C.L., Gattu S., Veltri L.M., Holland L.A., Capillary Electrophoresis Separations of Glycans. 2018. *Chem. Rev*. 118: 7867−7885.

Mahnik S., Rizovski B., Fuerhacker M., Mader R., Determination of 5-fluorouracil in hospital effluents. 2004. *Anal. Bioanal. Chem*. 380: 31–35.1419–1425.

Malá Z., Gebauer P., Boček P., Recent progress in analytical capillary isotachophoresis. 2013. *Electrophoresis*, 34: 19-28.

Martino M., Clinical studies of three oral prodrugs of 5-fluorouracil (capecitabine, UFT, S-1): a review. 2002. *The oncologist*, 7(4): 288-323.

Mikšík I., Sedláková P., Capillary electrochromatography of proteins and peptides. 2007. *J. Sep. Sci.*, 30: 1686-1703.

Puoci F., Iemma F., Cirillo G., Picci N., Matricardi P., Alhaique F., Molecularly imprinted polymers for 5-fluorouracil release in biological fluids. 2007. *Molecules*, 12(4), 805-814.

Pyell U., Advances in column technology and instrumentation in capillary electrochromatography. 2000. *J. Chromatogr. A*, 892: 257-278.

Rubino F.M., Floridia L., Pietropaolo A.M., Tavazzani M., Colombi A., Measurement of surface contamination by certain antineoplastic drugs using high-performance liquid chromatography: applications in occupational hygiene investigations in hospital environments. 1999. *Med. Lav.* 90: 572–583.

Rustum A., Hoffman N., High-performance liquid chromatographic determination of 5-azacytidine in plasma. 1987. *J. Chromatogr*. 421: 387–391.

Schmaus G., Schierl R., Funck S., Monitoring surface contamination by antineoplastic drugs using gas chromatography-mass spectrometry and voltammetry. 2002. *Am. J. Health Syst. Pharm*. 59: 956–961.

Shewach D.S., Kuchta R.D., Introduction to Cancer Chemotherapeutics. 2009. *Chem. Rev.* 109: 2859–2861.

Silva M., Micellar electrokinetic chromatography: A review of methodological and instrumental innovations focusing on practical aspects. 2013. *Electrophoresis*, 34: 141-158.

Silvertand L.H.H., Toraño J.S., van Bennekom W.P., de Jong G.J., Recent developments in capillary isoelectric focusing. 2008. *J. Chromatogr. A*, 1204: 157-170.

Sinha V., Kumar R., Bhinge J., A Stability-Indicating RT-HPLC Assay Method for 5-Fluorouracil. 2009. *Indian. J. Pharm. Sci.* 71: 630–637.

Sirén H., Shimmo R., Sipola P., Abenet S., Riekkola ML. Capillary electrophoresis of diuretics and probenecid in methanol. 2008. *J Chromatogr A.* 1198-1199:215-219.

Sun H., Li L., Chen X., Sensitized chemiluminescence determination of fluorouracil in pharmaceutical and biological fluids based on potassium permanganate oxidation in the presence of formaldehyde. 2007. *J. Clin. Lab. Anal*. 21: 213–219.

Thursten D.E. Chemistry and pharmacology of anticancer drugs. 2007. *British Journal of Cancer.* 97: 1713.

Tichy T.H., Current challenges in European oncology pharmacy practice. 2010. *J. Oncol. Pharm. Pract.* 16: 9–18.

Yang Y., Liu Q., Tao W., Nie L., Yao S., Improved determination of 5-fluorouracil and its prodrug tegafur in pharmaceuticals by large-volume sample stacking in CE. 2007. *Journal of Seperation Science.* 30: 3296–3301.

Zhao M., Rudek M. A., He P., Hartke C., Gore S., Carducci M. A., Baker S. D. Quantification of 5-azacytidine in plasma by electrospray tandem mass spectrometry coupled with high-performance liquid chromatography. 2004. *Journal of Chromatography B*, 813(1-2): 81-88.

Zhao M., Rudek M., He P., Hartke C., Gore S., Carducci M., Baker S., Quantification of 5-azacytidine in plasma by electrospray tandem mass spectrometry coupled with high-performance liquid chromatography. 2004. *J. Chromatogr. B*. 813: 81–88.

Zheng X. F., Lian Q., Yang H., Wang X. Surface molecularly imprinted polymer of chitosan grafted poly (methyl methacrylate) for 5-fluorouracil and controlled release. 2016. *Scientific reports*, 6: 21409.

Zhu Z., Lu J.J., Liu S., Protein separation by capillary gel electrophoresis: a review. 2012. *Anal. Chim. Acta*, 709: 21-31.