DEVELOPMENT OF NOVEL SOLID PHASE EXTRACTION (SPE) SORBENTS AND SOLID PHASE MICROEXTRACTION (SPME) FIBER COATINGS FOR DETERMINATION OF ENDOCRINE DISRUPTING COMPOUNDS (EDCs)

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ABSTRACT

DEVELOPMENT OF NOVEL SOLID PHASE EXTRACTION (SPE) SORBENTS AND SOLID PHASE MICROEXTRACTION (SPME) FIBER COATINGS FOR DETERMINATION OF ENDOCRINE DISRUPTING COMPOUNDS (EDCs)

This thesis is composed of four chapters. In the first part of the study, molecularly imprinted polymer (MIP) was prepared as SPE sorbent for selective determination of BPA prior to HPLC DAD analysis. The adsorption capacity and selectivity of imprinted polymers were investigated. To improve the MISPE method, the parameters including pH of sample solution, adsorption time, amount of sorbent, desorption solvent were examined. The extraction efficiency of BPA imprinted polymer was investigated by using the spiked samples of ultrapure, drinking and tap water.

The second part of the thesis was about determination of estrogen hormones. For this purpose, amino modified silica and molecular imprinted silica were prepared and their SPE performances were compared. The proposed methodology was validated through the analysis of real water samples.

The preparation of MIP nanoparticles encapsulated in electrospun polystyrene fibers as the SPME fiber coating was the subject of the third part of the thesis. Developed fibers were used for selective extraction and analysis of parabens in water samples. The optimization parameters affecting the extraction and desorption of parabens were investigated. The validity of the proposed method was verified via spike recovery tests.

Finally, fibers having amino functionality prepared by the sol-gel based electrospinning process were used for determination of BPA. The effect of solution pH, extraction time, agitation speed and ionic strength on the extraction performance were investigated. Validity was checked via the application of the proposed methodology on real samples.

ÖZET

ENDOKRİN BOZUCU KİMYASALLARIN TAYİNİ İÇİN YENİ KATI FAZ EKSTRAKSİYON (SPE) SORBENTLERİNİN VE KATI FAZ MİKRO EKSTRAKSİYON (SPME) FİBER KAPLAMALARININ GELİŞTİRİLMESİ

Bu tez dört bölümden oluşmaktadır. Çalışmanın birinci kısmında, bisfenol A nın HPLC-DAD analizi öncesi tayini için moleküler baskılanmış polimerler (MIP) SPE sorbenti olarak sentezlenmiştir. Baskılanmış polimerlerin adsorpsiyon kapasitesi ve selektivitesi araştırılmıştır. MISPE metotunu geliştirmek için örnek çözelti pH'sı, çalkalama zamanı, sorbent miktarı, desorpsiyon çözeltisi gibi parametreler test edilmiştir. BPA ile baskılanmış polimerin ekstraksiyon verimi ultra saf su içme suyu ve çeşme suyu analizleri ile anlaşılmıştır.

Çalışmanın ikinci kısmı estrojen hormonlarının tayini hakkındadır. Bu amaç için yüzeyi amino- grubu ile modifiye edilmiş silika ile yüzeyi moleküler olarak baskılanmış silika hazırlanmış ve bunların SPE performanları kıyaslanmıştır. Önerilen yöntem gerçek örneklerin analizi ile valide edilmiştir.

MIP nano parçacıkları gömülmüş elektrodukma polistren fiberleri SPME fiber kaplaması olarak hazırlanması bu tezin üçüncü kısım konusudur. Geliştirilen fiberler sularda bulunan parabenin selektif ekstraksiyonunda ve analizinde kullanılmıştır. Paraben ekstraksiyonunu ve desorpsiyonunu etklileyen parametreler araştırılmıştır. Önerilen yöntemin geçerliliği, gerçek örnekler ile doğrulanmıştır

Son olarak, sol-jel esaslı elekrospun işlemi ile hazıranmış amino işlevselliğine sahip fiberler BPA'nın tayini için kullanılmıştır. Çözelti pH, ekstraksiyon zamanı, çalkalama hızı ve iyonik kuvvetin ekstraksiyon perfonması üzerinde ki etkisi araştırıldı. Önerilen metodolojinin geçerliliği, gerçek örnekler üzerinde uygulama yoluyla kontrol edilmiştir.

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CHAPTER 1

INTRODUCTION

1.1. Endocrine System

Endocrine systems, also referred to as hormone systems, are found in all mammals, birds, fish, and many other types of living organisms. Hormones are released by glands and travel throughout the body, acting as chemical messengers. Hormones interface with cells that contain matching receptors in or on their surfaces. The hormone binds with the receptor, much like a key would fit into a lock the hormones, or keys, need to find compatible receptors, or locks, to work properly. Although hormones reach all parts of the body, only target cells with compatible receptors are equipped to respond. Once a receptor and a hormone bind, the receptor carries out the hormone's instructions by either altering the cell's existing proteins or turning on genes that will build a new protein. Both actions create reactions throughout the body. Researchers have identified more than 50 hormones in humans and other vertebrates.

The endocrine system regulates all biological processes in the body from conception through adulthood and into old age, including the development of the brain and nervous system, the growth and function of the reproductive system, as well as the metabolism and blood sugar levels. The female ovaries, male testes, and pituitary, thyroid, and adrenal glands are major constituents of the endocrine system. (Figure 1.1).

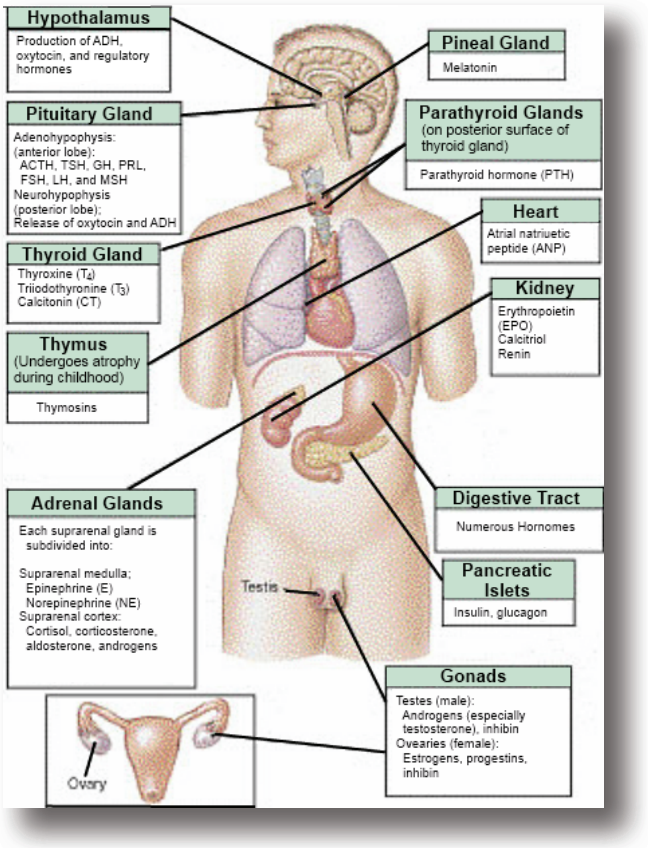

Figure 1.1. Principles and Explorations, Teaching Transparencies.
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1.2. Endocrine Disrupting Compounds (EDCs)

An endocrine-disrupting compound was defined by the U.S. Environmental Protection Agency (EPA) as "an exogenous agent that interferes with synthesis, secretion, transport, metabolism, binding action, or elimination of natural blood-borne hormones that are present in the body and are responsible for homeostasis, reproduction, and developmental process." Endocrine system disrupting compounds (EDCs) can affect hormones in three different ways (Fig 1.2). In the first mechanism, EDCs may mimic the natural hormones and binds the active side of cells, so they can easily alter the response (agonistic effect). In the second case, these EDCs compounds can behave as blocking agent and inhibits the interaction of natural hormones with receptors (antagonistic effect). In the last mechanism, both hormones- blocking and hormone-mimicking chemicals cause a change in the conformation of the receptor by binding to the same receptor (Rahman et al., 2009).

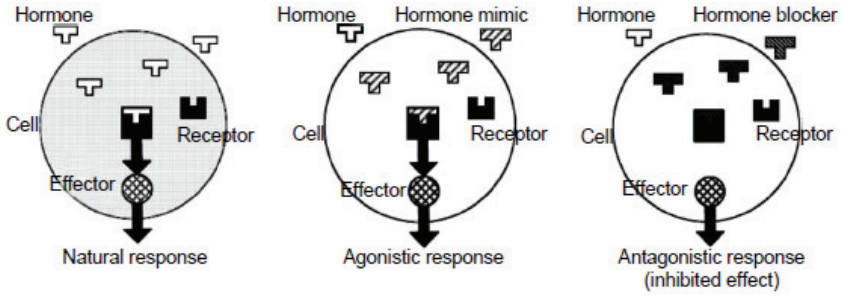


Figure 1.2. The influence mechanism of Endocrine Disrupting Compounds (EDCs) (Birklett 2003).

Endocrine disrupting compounds (EDC) are one of the current issues taking great attraction of worldwide organizations who deals with protecting human health and environment, such as WHO and EPA. Endocrine disrupting compounds are both man-made and natural exogenous chemicals which cause some disorders in human body by interfering normal hormone working principle. Hormones in endocrine system control many of the processes in body, such as early processes (cell differentiation and organ formation), normal functioning of tissues during adulthood. These exogenous substances when included in endocrine system during early, mid, or late prenatal or postnatal period can actualize its own disruption by two ways: It can directly affect hormone-receptor protein complex or specific protein which is responsible for hormone delivery to a specific place at an exact time (WHO/UNEP 2012).

As all the chemicals, endocrine disrupters have a dose response relationship. However, unlike other chemicals, EDCs have non-monotonic dose response curves (Fig.1.3) that completely different from the view of 'the dose make poison' (in monotonic curves). Even if at the quite low doses, EDCs may be toxic for both humankind and wildlife. The response may be huge at lower doses when compared to higher doses (EPA 2015).

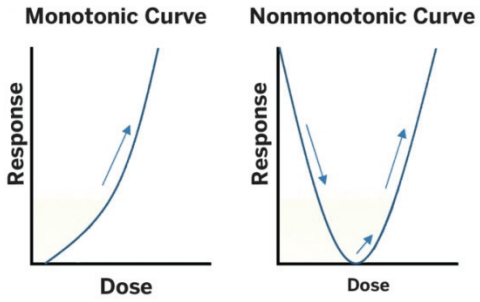


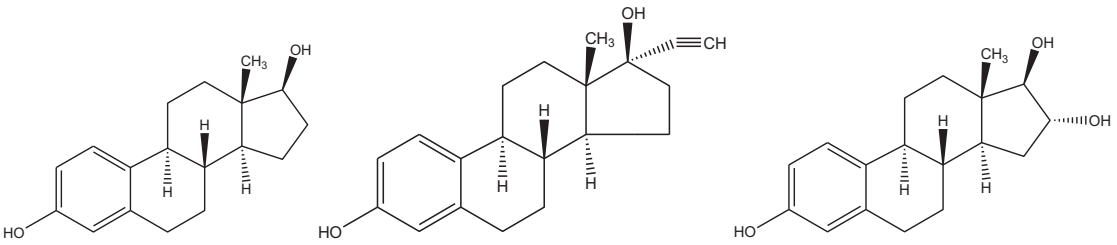
Figure 1.3. Examples of monotonic and non-monotonic curves.
(Source: EPA 2015)

Endocrine disrupters are classified into some groups: household product ingredients, personal care products (cosmetic ingredients), food additives, flame retardants, plastics, pesticides ingredients, antimicrobials, biogenic compounds, industrial additives, solvents, metals, byproducts, medicals, metabolites.

1.2.1. Hormones

Steroid hormones (estrogens, progestogens, androgens, and corticosteroids) are considered the most potent active EDCs present in the environment; they are formed naturally by humans and wildlife and are produced synthetically (Lai et al 2000). The hormones interfere with endocrine function, and their presence in the environment has been observed to produce estrogenic effects, such as fish feminisation, changes in reproduction and behaviour, a decrease in the number of spermatozoids, increased incidences of breast cancer in women, and increases in certain anomalies in the human reproductive system, even at low concentrations (Purdom et al., 1994).

In this study, new methods were developed for determination of β-estradiol, 17α-Etthylestradiol and estrone. The molecular structure molecular mass and pKa values were given Figure 1.4.



β-estradiol
Mw= 272.28 g/mol
pKa=10.71

17α-ethylestradiol
Mw= 296.40 g/mol
pKa=10.71

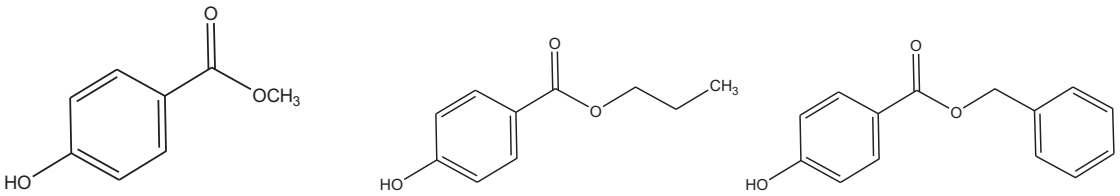
estrone
Mw= 272.38 g/mol
pKa=10.71

Figure 1.4. The molecular structure, mass and pKa values of β-estradiol, 17α-ethylestradiol and estrone.

1.2.2. Personal Care Products(PCPs)

Personal care products (PCPs) are a diverse group of compounds used in soaps, lotions, toothpaste, fragrances, and sunscreens. The primary classes of PCPs include disinfectants (triclosan), fragrances (musks), insect repellants (DEET), preservatives (parabens) and UV filters (methylbenzylidene camphor). Unlike pharmaceuticals which are intended for internal use, PCPs are products intended for external use on the human body and thus are not subjected to metabolic alterations; therefore, large quantities of PCPs enter the environment unaltered through regular usage (Ternes et al., 2004). Many of these compounds are used in large quantities, and recent studies have indicated many are environmentally persistent, bioactive, and have the potential for bioaccumulation (Peck, 2006; Mackay and Barnthouse, 2010). Acute toxicity of TCS and biphenylol has been examined in invertebrates, fish, amphibians, algae, and plants. TCS is more toxic to similarly studied trophic groups in comparison to other disinfectants (Palenske et al., 2010, Lyndall 2010, Orvos et al., 2010). Parabens (alkyl-p-hydroxybenzoates) are antimicrobial preservatives used in cosmetics, toiletries, pharmaceuticals, and food (Daughton and Ternes, 1999). There are currently seven different types of parabens in use (benzyl, butyl, ethyl, isobutyl, isopropyl, methyl, and propyl). Of the seven different types of parabens currently in use, benzylparaben appears to be most acutely toxic (Madsen, 2009; Terasaki et al., 2009; Bazin et al., 2010) Methyl- and ethylparaben appear to be least acutely toxic

In this study, new methods were developed for determination of some personal care products. The molecular structure molecular mass and pKa values of studied PCPs were given in figure 1.5.



Methyl Paraben
Mw= 152.15 g/mol
pKa= 8.5

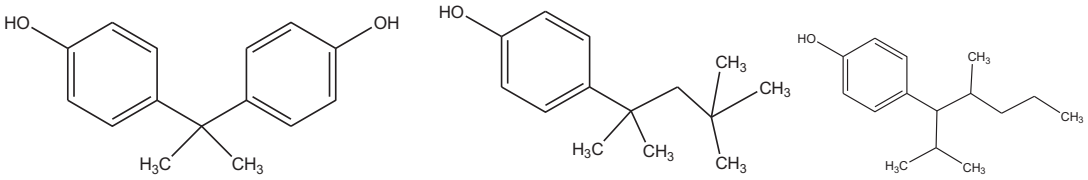
Propyl Paraben
Mw= 180.2 g/mol
pKa= 8.5

Benzyl Paraben
Mw= 228.247 g/mol
pKa= 8.5

Figure 1.5. The molecular structure, mass and pKa values of studied PCPs

1.2.3. Alkylphenols and Bisphenol A.

Alkylphenols are a class of nonionic surfactants that are used extensively as detergents, emulsifiers, wetting agents, and dispersing agents in industrial, agricultural, and household applications. Because alkylsubstituted phenols are relatively polar substances with an –OH group, they are highly soluble in water, which increases their potential to pollute water (Ferrera ve diğ, 2013). Several groups of authors have reported that this class of compound exhibits bioaccumulation in aquatic organisms (Ferguson and Brownawell, 2003) and chronic toxicity (Staples et al., 2004) and can mimic natural hormones and disrupt endocrine functions by interacting with estrogen receptors. Bisphenol A (BPA) is used extensively in the industrial and is present in a diverse range of manufactured products. It is a monomer used in the manufacture of epoxy, polycarbonate, and polyester styrene resins. Such resins are widely used in canned-food and beverage packaging and in dental resins, which leads to potential human exposure to BPA (Ferrera et al, 2013). In this study, new methods were developed for determination of some personal care products. The molecular structure molecular weight and pKa values of studied alkyphenols and bisphenol A were given in Figure 1.6.



Bisphenol A
Mw= 228.291 g/mol
pKa= 9.6

4-Octylphenol
Mw= 206.329 g/mol
pKa= 10.33

Nonylphenol
Mw= 220.35 g/mol
pKa= 10.7

Figure 1.6. The molecular structure, weight and pKa values of Bisphenol A, 4-oktylphenol and nonyphenol

1.2.4. Other EDCs

Brominated flame retardants include polybrominated diphenyl ethers (PBDEs), polybrominated biphenyls (PBBs), brominated cyclohydrocarbons, decabromodiphenyl ethers(DeBDEs), hexabromocyclododecanes (HBCDs), and tetrabromobisphenol A (TBBPA) Because of their widespread presence in the environment and their potential toxicity to humans and animals, increasing concern has prompted many countries to ban some of these compounds.

A huge number of chemicals have been identified as endocrine disruptors, among them several pesticides (Acephate, atrazine, Chlorfenviphos, Cypermethrin, Deltamethrin, Dieldrin, Toxaphene..etc). Pesticides are used to kill unwanted organisms in crops, public areas, homes and gardens, and parasites in medicine. Human are exposed to pesticides due to their occupations or through dietary and environmental exposure (water, soil, air) (Mniff et al., 2011)

1.3. The effects of EDCs

Reproductive function of humankind can be affected crucially and adversely by endocrine disruptors during preconception or pregnancy, or during childhood and puberty of offspring. Considering reproduction system, chronic exposure from pregnancy to adulthood is more critical than acute exposure to endocrine disruptors for humankind (Diamanti-Kandarakis 2011).

First disruption has been seen from a synthetic estrogen called diethylstilbestrol which used for prevention of miscarriage of pregnant women. This chronic exposure

during pregnancy has caused to daughters of these women, who take these pills, had unexpectedly gynecologic neoplasm and vaginal adenocarcinoma (Herbst A.L., Ulfelder H., and C. 1971). The later studies have shown that these two are not only the disorders that could be observed due to disruption; the others are characteristic T-shaped uterus, infertility, preterm birth, menstrual irregularity, ectopic pregnancy and poor pregnancy outcome concerned to spontaneous abortion (Kaufman et al. 2000, R.H. 1982, Palmer et al. 2001). In addition to these, puberty timing in girls has changed and caused thelarche and menarche in an earlier time due to estrogen mimics and antiandrogens which are considerably related to environmental factors (Herman-Giddens et al. 2008, Selevan, et al. 2008, Jacobson-Dickman and Lee 2009).

Male reproductive system is also affected from endocrine disrupters. There are three major diseases; poor semen quality and infertility, urogenital tract abnormalities and testicular germ cancer. Some endocrine disrupting chemicals existences are related to come down with these illnesses. For example, polychlorinated biphenyls, phthalates, and non-persistent pesticides have an inverse effect on sperm parameters (Dallinga et al. 2002, Hauser et al. 2003, Hauser et al. 2006, Juhler R.K. et al. 1999).

Endocrine disrupting chemicals also act as thyroid hormones. These hormones have many functions in both vertebrates and invertebrates as regulation of development, tissue growth, and metabolism (Heyland and Moroz 2005). Especially during pregnancy, fetus' growth and its brain development are greatly affected by disruption of thyroid hormones with a result of neurological and cognitive deficiencies (Boas et al. 2006).

The most important public health problem in these days is obesity epidemic. In addition to change in food intake, physical activity, and genetic predisposition; endocrine disrupters can also have an effect on obesity as an exogenous factor by impairing body's natural weight control system (Baillie-Hamilton 2002). 'Obesogens', that have reversely effect on regulation of lipid metabolism, are responsible for overweight and obesity in human life if any exposure has occurred during development (Grun et al. 2006).

EDCs are also the reason of some metabolic disorders. Even at low doses, Bisphenol A can decrease the glycose level which is responsible for a rise in insulin in blood (Alonso-Magdalena et al. 2005). The other metabolic disorders that can be met because of endocrine disruption are some hearth diseases, diabetes, insulin resistance, disturbed glycogen secretion.

1.4. The way of exposure to EDCs

Chemicals that can be generally classified as endocrine disrupters are substances that are greatly used in daily life. Exposure can be occurring from contaminated foods, contaminated groundwater or drinking water, combustion sources or contaminants in consumer products (Town 2015).

Many researchers have shown that wastewater treatment plants (WWTPs) are major contributors to the presence of EDCs in the environment, where they enter via domestic and industrial discharges. These plants remove EDCs only partially. As a consequence, these compounds have been found in the effluents from WWTPs, and they can therefore reach the surface and the groundwater. Because of the nonpolar and hydrophobic nature of many EDC, that they can be absorbed onto particulate materials. This behaviour suggests that the general effect of wastewater treatment processes should be to concentrate organic pollutants in the sewage sludge, whereas mechanical separation techniques, such as sedimentation, should result in significant removal of organic pollutants from the aqueous phase to primary and secondary sludges. As a result, the treated wastewater is discharged relatively free of EDCs; however, the EDCs are absorbed into sewage sludge, which could constitute a new source of pollution. The sludge from WWTPs can be applied to agricultural fields as a fertiliser.

The analysis of EDCs are diffucult because they usually exist at low concentrations (ngL^{-1}) or in complex martixes. Especially, biological samples contain large amount of interfering compounds so it is necessary to use extensive extraction procedure to remove EDCs from these samples. As a consequence, it is important to develop a fast and efficient analytical method for trace analysis of target compounds in environmental and biological samples

1.5. Determination of EDCs

In recent years, numerous analytical methods have been developed for the identification and determination of endocrine disrupting chemicals. In the work of Jıang et al. (2007), a novel and simple imprinted amino-functionalized silica gel material was synthesized by combining a surface molecular imprinting technique with a sol–gel process on the supporter of activated silica gel for solid-phase extraction-high performance liquid chromatography (SPE-HPLC) determination of bisphenol A (BPA).

Zhao and coworkers (2010) have applied bamboo-activated charcoal SPE-rapid resolution-LC-ESI-MS/MS for trace determination of tetrabromobisphenol A and bisphenol A in real-world environmental water samples. In another study, an extraction-preconcentrating procedure based on the use of a molecularly imprinted polymer (MIP) as selective sorbent has been developed for the determination of several phenolic compounds (bisphenol- A, bisphenol-F and 4-nitrophenol) and phenoxyacid herbicides (2,4-D, 2,4,5-T and 2,4,5-TP) in honey samples. Liquid chromatography with diode array detection (LC–DAD) and electrospray ionization-ion trap mass spectrometry (LC–IT-MS) were used for the separation, identification and quantification of these analytes (Hernandez et al. 2009). In the work of Sheng and coworkers in 2012, a new method was developed to embed the synthesized dummy molecularly imprinted polymers (DMIPs) into the coating of stir bar directly by sol-gel technique. The selective absorption and anti-interference ability of DMIPs-coated bar were investigated by extracting BPA from the mixture of BPA and its analogues (4-tert-butylphenol, 4,4-dihydroxybiphenyl, and 3,3,5,5-tetrabromo-bisphenol A) as well as comparing with the bars coated with non-imprinted polymers (NIPs) or PDMS. In another study, the analysis of bisphenol A nonylphenol, estrone, estradiol and other endocrine disrupting compounds were performed by SPE cartridges combined with GC-MS (Liu et al, 2003). Yang et al. (2005) used SPME on-fiber derivatization technique for determination of some steroid hormones (estrone, estradiol, testosterone) and APEOs in water and biological samples prior to GC-MS analysis. Matejicek and coworkers (2013) synthesized molecularly imprinted polymer for selective determination of estrogen and alkylphenols in water and sediments by using LC-MS/MS. In a study in which various estrogens were identified, Supel-Q capillary column SPME unit was combined with LC-MS/MS and waste water analysis was performed (Mitani et al. 2005). Hu and coworkers (2010) synthesized SPME fibers coating by molecular imprinting method for identification of various estrogens in fish and shrimp specimens by HPLC-DAD. Palacios et al. (2012) identified bisphenol-like endocrine disruptors on recycled paper using Q-TOF-MS coupled with focused ultrasonic solid-liquid extraction (FUSLE) and reverse phase ultra-performance liquid chromatography (UPLC). In a study conducted in Turkey, Muz and colleagues (2012) used SPE cartridges prior to LC-ESI-MS/MS analysis prior to estrogen and progesterone hormones. There is also a study about distributions of various EDCs in Van Lake using commercial SPE cartridges (Oğuz and Kankaya 2013).

In study of Pedrouzo et al. (2009), commercial SPE cartridges and HPLC-MS / MS were used for identification of 11 personal care products in surface and waste waters. In another SPE study, Chen et al. (2011) developed an analytical method for determination of triclosan in surface and wastewater by using commercial SPE cartridges and GC-MS. Kasprzyk-Hordern and coworkers (2007) used commercial OasisMCX (polymeric mixed-mode strong cation exchanger sorbent) SPE cartridges combined with UPLC-MS/MS for detection of drugs and personal care products in the water. Similarly, Zhang (2011) used commercial SPE cartridges and HPLC-MS / MS systems for the identification of 31 endocrine disrupting chemicals in water. In a study conducted by Diamadopoulos and coworkers (2009), it has been reported that various personal care products, drug compounds and hormones (triclosan, 2,4-dichlorophenol, 2,3,4-trichlorophenol, estrone, 17- -ethinyl estradiol, clofibric acid, and carbamazepine) were determined by commercial SPME fibers and GC-MS. In another SPME study, an analytical method was developed for determination of paraben, triclosan and chlorophenol in water by using GC-MS / MS system and commercially available fibers (Regueiro et al., 2009). Strittmatter et al. (2012) used thin film microextraction (TFME) consisting of C18 / SCX matrix and electrospray ionizing mass spectroscopy (DESI-MS) to determine carbamazepine, carbamazepine-d10, triclosan and triclosan-d3 in waste water. Zhao and colleagues (2011) performed a method by using ionic liquid / ionic liquid dispenser liquid-liquid microextraction (IL-DLPME) before HPLC-ESI-MS / MS for determination of triclosan and triclocarban in environmental water samples. In the study of Lee, Peart and Svoboda (2005) acidic medicines, personal care products and alkyl phenols in sewage waters identified by using Oasis MAX SPE.

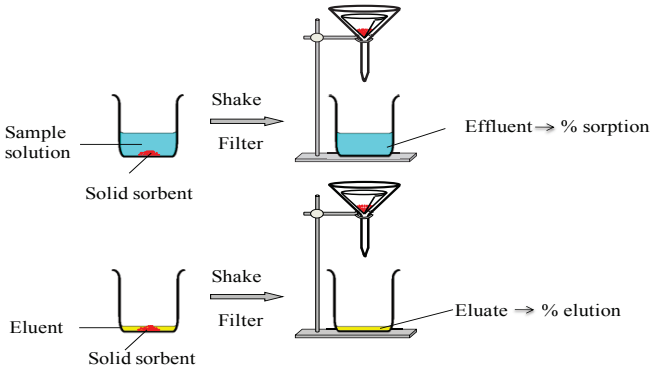
1.6. Solid Phase Extraction (SPE)

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

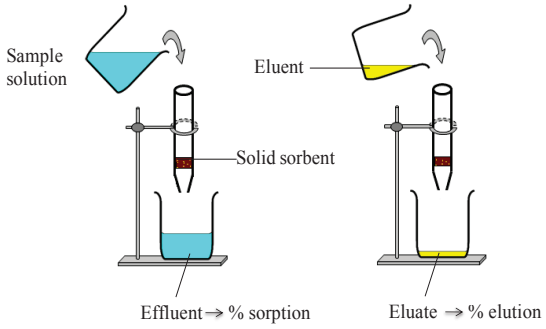
Solid-phase extraction (SPE) is one of the most common techniques used for extraction and pre-concentration of analytes in liquid samples because of many advantages such as high enrichment factor, high recovery, rapid phase separation, low cost, low consumption of solvents. Solid phase extraction (SPE) 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 solvent. There are basicly two type of SPE; namely, the batch and the column modes. When SPE is performed on a batch type, solid sorbent is weighed and put into the liquid sample solution. To obtain efficient mass transfer of the solutes, large surface area is provided by the particles in solid phase. After shaking process, two phases easily can be separated by filtration (Figure 1.2 (a)). In column type SPE, the column is loaded with the sample solution for the sorption of the analyte by the solid phase (Figure 1.2 (b)). A higher percentage of extraction is expected in SPE column compares to 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 eluated by using, generally, a smaller volume of a proper eluent. The analyte concentration in the eluate 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 1.7. Schematic illustration of the SPE modes (a) batch type, (b).column type

SPE has both advantages and disadvantages over the other methods mentioned above. 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 concentration, type, and 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.

1.7. Solid Phase Microextraction (SPME)

The solid-phase microextraction (SPME) was introduced in early 1990s. SPME, incorporates several sampling, extraction, concentration and sample introduction processes into a single step (Kataoka 2010; Zhang et al. 1994). In addition to time saving features of single (multipurpose) step, the other advantage of SPME is that it is a solvent free method for desorption in gas chromatography (GC). Generally, only two essential steps exist: partitioning of the analyte between coating and sample matrix and desorption of the extracted analyte into an analytical instrument (Zhang et al. 1994). The first studies about SPME were started by Janusz Pawliszyn to develop a solvent free solid-phase extraction method for volatile organic compounds (Zhang et al. 1994). The major problem associated with plugging in the classical solid-phase extraction method focused on the studies on head space extractive sorbents. This approach did not suffer from plugging limitations. Coatings of commercial GC capillary columns were the examples of the first developed SPME active phases for extraction of the volatile organic compounds (VOCs) (Lord and Pawliszyn 2000). Figure 1.2 illustrates the general view of a commercial solid-phase microextraction fiber and fiber holder. The fiber consists of a fused silica core coated with an active outside layer. The fiber with the extracting phase is protected within stainless steel piercing needle for repetitive use. The stainless-steel needle is contained in special designed syringe like fiber holder.

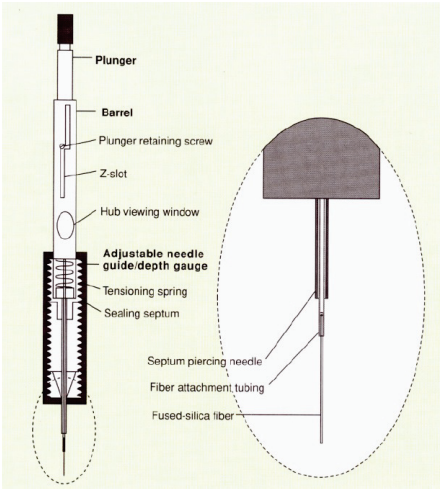


Figure 1.8. Typical SPME device. (Source: Zhang et al. 1994)

1.7.1. Types of SPME

SPME can be used in two general approaches, namely, direct mode and headspace mode (Koning et al. 2009). Direct mode (Figure 1.3a) depends on the direct immersion of the fiber active phase in the solution containing the nonvolatile analyte(s). When equilibrium is established, the fiber is withdrawn, and the concentrated analytes are introduced into the sample introduction port of an analytical instrument such as high performance liquid chromatograph (HPLC), inductively coupled plasma mass spectrometer (ICP-MS) or capillary electrophoresis (CE). Primarily, the headspace mode is used for volatile analytes. Extraction of the analyte(s) from the sample is achieved just by inserting the active phase of the fiber on the top of the sample without direct contact with the solution (Figure 1.3b). Extracted analyte(s) are thermally desorbed on injection port of a GC which provides both qualitative and quantitative information (Mester et al. 2001). The headspace mode includes extraction and sequential desorption (to GC) steps and eliminates elution step with solvents. There are various derivatization agents which make possible the headspace extraction of some non volatile and semi-volatile analytes. The acting mechanism depends on the changing polarity (which also affects the volatility) of the analyte. In addition, derivatization has a function of enabling better chromatographic separations.

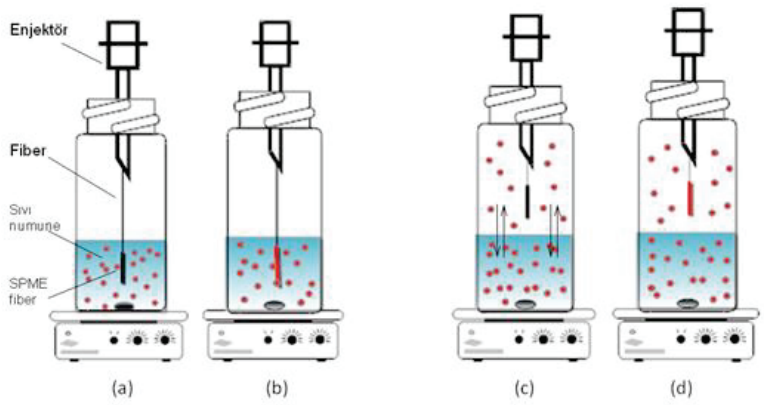


Figure 1.9. SPME extraction modes a) direct extraction, b) equilibrium in direct mode, c) headspace extraction d) equilibrium in headspace mode

1.7.2. Advantages and Disadvantages of SPME

SPME is a solvent free technique which is one of the most important benefits of the method. In addition, short application time, analyte concentration ability, improved detection limits, repetitive use of the same fiber (up to 100 extractions) and selectivity are other important features. Moreover, it is practical to use with a wide range of samples such as air samples, solid samples, food samples and aqueous samples (Risticevic 2009). In contrast to the great deal of advantages limitations arise especially with lack of analyte specific fiber coatings. Since commercially available fibers are limited to some polar, non-polar and semi-polar characters the extraction of a fiber is similar for analogous compounds (Nerin et al. 2009; Zhang et al. 1994). Other drawbacks are poor reproducibility in analysis and production of fibers (Dietz et al. 2006).

1.7.3. Commercially Available Fibers

Commercially available fibers are divided into three major categories, namely, polar, semi-polar and nonpolar fibers. The coatings are inspired from commercial gas chromatographic capillary column fillings. Polydimethylsiloxane (PDMS) is the most popular non-polar coating for SPME. Polydimethylsiloxane/Divinylbenzene (PDMS/DVB) is semi-polar coating. Polar coatings are polyacrylate (PA) and Carbowax/Divinylbenzene (CW/DVB) (Mester et al. 2001; 2005). Production of fibers with distinctive coatings reduces the possibility of extracting interferences and increases

the extraction selectivity. Another classification of the coatings depends on homogeneity of the active phase. One of them is homogeneous pure polymer coatings (Pawliszyn 1999). PDMS and PA are examples of homogeneous coatings. The stability of these coatings via organic solvents is enhanced by cross linking (bonding) of polymers. The second type of coatings is the porous particles embedded in a partially cross-linked polymeric phase. These coatings are not robust as homogeneous types, but they are more selective. Examples of blended coatings are PDMS/DVB, CW/DVB and PDMS/Carboxen. Blending of the polymer enhances the total capacity of the fiber by increasing the porosity of the coatings. In addition, increasing the porosity of the polymer particles in fiber amplifies analyte retention on fiber. Moreover, pore size of the polymer particle in coating affects selectivity of the fiber (Pawliszyn 1999).

1.7.4. Fiber Coatings (Literature survey)

Development of a new type of fiber coating is a growing area in SPME technologies, especially to extend the application areas, matrixes and analyte types. Generally, commercial fibers are convenient for GC applications, but the main drawback is the thermal instability of the phase during desorption. Some fibers can also be used with HPLC (PDMS). However, desorption into HPLC requires solvents which destroy surface coating. The studies related to production of the new fiber coatings are extended to develop solvent-resistive and thermally stable active coatings as well as to enhance mechanical strength of the phase. In addition, developing the new functionality enhances the selectivity for specific analytes. Especially, sol-gel route offers the opportunity for attachment of various functionalities to the fiber which expands the working area of SPME to biological applications. It can be mentioned that; the major working areas are sol-gel based modifications of fiber surface by attachment of sol-gel active functional groups onto fused silica. Molecular imprinting is another surface modification method frequently used specially to prepare analyte selective coatings. Furthermore, immobilization of nanoparticles, particularly carbon nanotubes, is promising in SPME coatings (Augusto et al. 2010).

1.8. Molecular Imprinted Polymers (MIPs)

Complexes of molecules in solutions or gases have relatively small lifetimes that they have nearly zero concentration in solutions. However, some of the molecules, namely receptors, can recognize only one type of substances among other species. In the crowd of solutions, receptors can distinguish their own partner molecule and make stable complexes with quite high concentrations.

There are lots of naturally occurring receptors in body. These receptors are responsible for many processes which are essential for existence. Without these molecules living being cannot survive. The superiority of these substances makes scientists to create new molecules by imitating natural receptors. These artificial receptors have advances among the natural ones. These man-made molecules do not only deal with proteins. For a variety of compounds, a specific molecule can be created. Also, stability, flexibility and activity in different conditions can be determined by humankind. In addition to all these, by designing, one can create a substance that has the sites completely suits to an analyte. In general, this can be named as 'molecular recognition' which is the key idea of 'molecular imprinted polymers'.

For the treatment of waste water, the usage of the receptor is a common method. However, its cost is quite high. For industries dealing with the production of NSAIDs, separation and purification of the product and clarification of their waste water cost more than the production process. Here MIP can be put into use as an inexpensive method with quite high yield. Being cheap is not only advance of MIP. They are very easy to prepare in a short time. Also they have high stability and activity during a wide range of conditions in addition to robustness. Selective and strong binding sites against analyte in water conditions with the other qualities of MIP make it a favorable method for water treatments.

Figure 1.7 shows three basic steps of molecular imprinting: pre-polymerization, polymerization, and removal of template. In pre-polymerization step, monomer and template molecule let to connect to each other via covalent or non-covalent interaction (Makoto Komiyama et al. 2003).

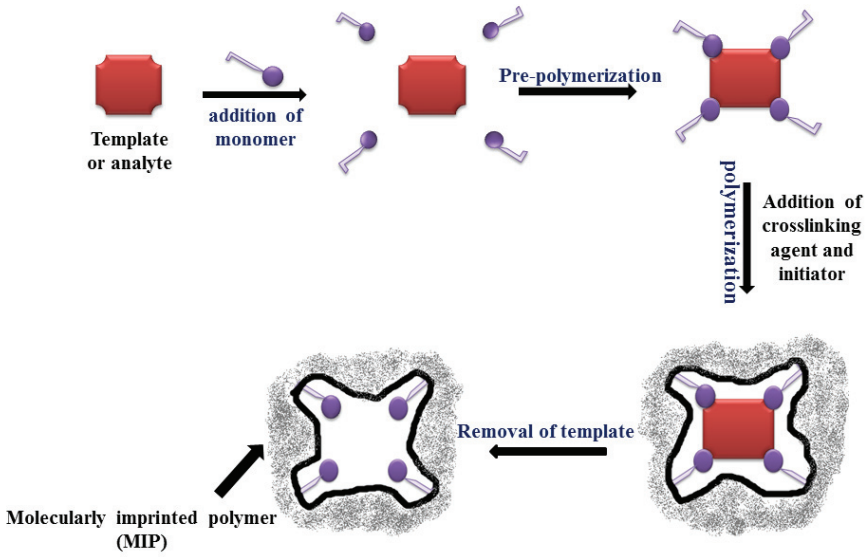


Figure 1.10. Schematic illustration of molecular imprinting.

These interactions give specific name of the total process, semi-covalent imprinting or non-covalent imprinting. Non-covalent approach is based on polar interactions such as H-bonding and electrostatic interactions, whereas semi-covalent imprinting occur from covalent bonding. In semi-covalent approach covalent bonds are formed during the pre-polymerization step, but rebinding is achieved by non-covalent attraction as in non-covalent approach. (Nuria Fontanals, Rosa Maria Marce, and Borrull 2010) Even if the clearer structure of cavities and free polymerization conditions are supplied by covalent imprinting, generally non-covalent imprinting is preferred. The reason originates from the fast removal, rebinding, and release of template in non-covalent imprinting. However, restricted conditions during polymerization make non-covalent imprinting not an easy process at the beginning (Makoto Komiyama et al. 2003).

Also, the type of the solvent that is chosen as reaction medium determines the type of imprinting process. There are two types of solvents used during polymerization: non-to moderately polar/aprotic solvents/porogens (DCM, toluene, chloroform, acetonitrile) and polar protic solvents (methanol, ethanol, water). Polar protic solvents decrease the polarity of the interactions between the template and monomer, so they are used for covalent imprinting. In contrast to protic solvents, aprotic solvents (porogens) support and stabilize the H-bonding during non-covalent interaction (Nuria Fontanals, Rosa Maria Marce, and Borrull 2010).

In the polymerization step crosslinking agent and initiator are added into the reaction mixture with a fixed ratio and let them to polymerize. Crosslinking agent controls the morphology of MIP and supply robustness to the polymer while stabilizing the specific binding sites (imprinted region). The addition of initiator under proper condition changes the destiny of polymerization step if radical copolymerization chosen as the method. Free radical copolymerization is initiated by the thermal decomposition of radical initiator and molecular oxygen is taken away from the reaction mixture in order to prevent from trapping of radical. Removal of oxygen is supplied by degassing with argon or nitrogen gas or freeze-and-thaw cycle. In some cases application of high temperatures for initiation can be harmful to non-covalent interaction between monomer and template. This time photo-initiation by UV-light can be applied under low temperatures. In addition to these, the usage of UV-absorbable monomer supplies the initiation even if the absence of initiator. If these processes are not applied, the polymerization cannot be started (Makoto Komiyama et al. 2003).

After the achievement of polymerization, the solid particles, now called as MIP's, are filtered, then washed until any template observed at chromatographic applications. The most remarkable problem during template removing is template bleeding in trace analysis. When analyte molecule used as template and not removed completely before the usage of MIP as sorbent, higher amount of analyte than expected can be observed at real samples. To overcome this problem, a dummy molecule can be used as template. Dummy molecules resemble the target anaylte in terms of size, shape and functionality, but gives different chromatographic separation than template. Thereby, the bleeding molecule and the anayte molecule can be discriminated during chromatographic processes (Nuria Fontanals, Rosa Maria Marce, and Borrull 2010).

Before starting the synthesis of this special polymer, physical nature of the product must be determined according to needs for the experimental procedures and conditions. Some important parameters should be considered. The crosslink ratio with respect to the total number of monomer and the volume of the solvent determines and changes all the physical morphology of MIP (Figure 1.8) (Peter A.G Cormack and Elorza 2004).

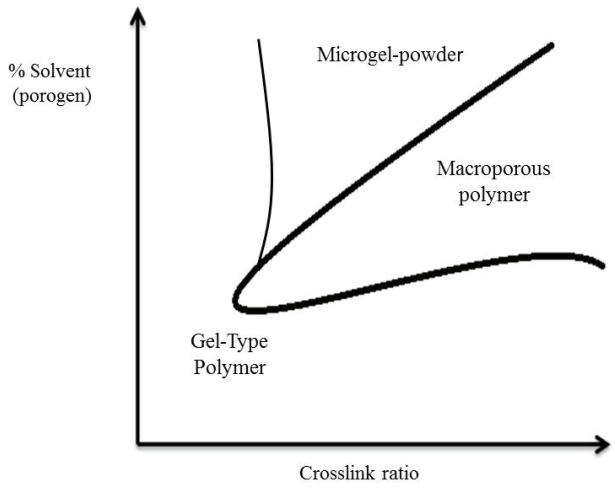


Figure 1.11. Polymer pseudo diagram.

High crosslink ratio at low percentages of solvent or vice versa produce gel-type polymer. This type of MIP can swell in solvents that have thermodynamically good properties. However, it supplies low specific surface area which is not useful for the experiments that deal with the great amount of analyte in working solution. In addition to that, when crosslink ratio is low, its poor mechanical strength may restrict the experimental conditions. Macroporous polymer synthesis is most widely used method for the preparation of MIP. When compare to gel-type polymers, it has more specific surface area and has more robustness due to the high crosslink ratio. When solvent ratio is increased, microgel powders can be synthesized via precipitation polymerization. This method supplies spheres that have radius around micrometers. In generally, the ratio of all reagents used for MIP synthesis should have a ratio to have usable polymer according to need for the process. Finding exact ratio is the most important part and the most time-consuming process that may continue several weeks. This can be achieved only by trial-and-error experiments (Cormack and Elorza 2004).

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 we were used in our works, 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 only 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. Compare to monoliths the spheres supply higher surface area. However, for SPE process, these micro and nano-spheres are not large enough to be used (Nuria Fontanals, Rosa Maria Marce, and Borrull 2010).

Adding the template to the synthesis supplies us 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 called as Non Imprinted Polymer (NIP). By comparing the results of two polymers, selectivity can be clarified.

1.9. Sol-Gel Chemistry for Thermally Stable Coatings

As aforementioned, commercial SPME coatings are inspired from GC capillary column packings. The active filling inside the wall of the GC column is not chemically bonded. Mostly, static coatings are applied. For SPME fibers, the same approach has limitations for thermal stability of the phase (Pawliszyn 1999). Thus, SPME fibers commonly suffer from limitation due to the repetitive use. Another drawback is due to film coating thinner than what is needed. To overcome these weaknesses of the method, the active phase should be chemically bonded onto the surface of the fiber. The best approach for the preparation of the stable coatings is the sol-gel based coating methods (Bagheri et al. 2010). This approach provides strong adhesion of the coating onto a silica surface which improves the stability against solvent and heating. The porosity of the silicate matrix presents a large surface area for extraction sites. Moreover, selectivity of the fibers can easily be varied with the nature of the coatings (Yu et al. 2002; Alhooshani et al. 2005). Sol-gel active reagents make it possible to extend variation in coating as well as in controlling the volume of the coating.

Generally, the sol-gel based coatings are utilized as follows: the bare silica fiber is dipped into an alkoxide-based sol-gel precursor under acid catalyst. This results in hydrolysis of the precursor and formation of the polymer network attached onto surface of the silica via reaction of surface silanol groups with siloxane groups (Gbatu et al. 1999; Kumar et al. 2008). The addition of a hydroxy terminated sol-gel active polymer and surface derivatizing agent to the sol-gel solution produces a porous organic-inorganic hybrid chemically bonded phase on the surface of the fiber. The functional groups can vary according to the analytes. The thickness of the coating is simply controlled by the number of times of successive dippings of the fiber into the sol-gel solution.

1.9.1. Sol-Gel Coating Step

1.9.1.1. Hydrolysis

The first step in the sol-gel process is the hydrolysis reaction which occurs while the alkoxide precursor is mixed with water in the presence of alcohol to achieve sufficient homogenization. Hydrolysis leads to the formation of silanol groups. Intermediates produced in the alcohol–water medium include silanols, ethoxy silanols and oligomers of low molecular weight which were formed during the first stages of the process. Hydrolysis reactions can be catalyzed by either acid or base. Two hydrolysis processes give different structures and morphology; acid catalysis forms linear weakly cross-linked polymer whereas base catalysis forms more highly branched clusters as a result of rapid hydrolysis step (Brinker et al. 1990).

Figure 1.12 illustrates the hydrolysis reaction that occurs under acid catalysis of the alkoxide based precursor.

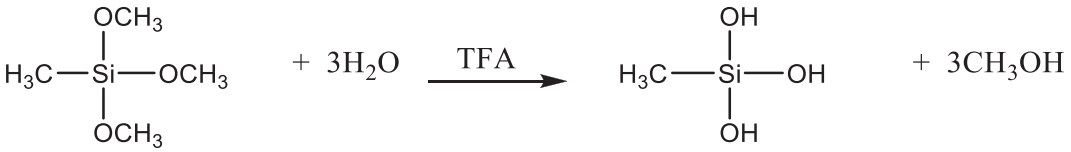


Figure 1.12. Hydrolysis reaction

1.9.1.2.Polycondensation

The second step of sol-gel synthesis is the polycondensation between the two silanol groups and condensation reaction between silanol and alkoxyde groups by releasing a water or alcohol unit, respectively (Figure 1.13).

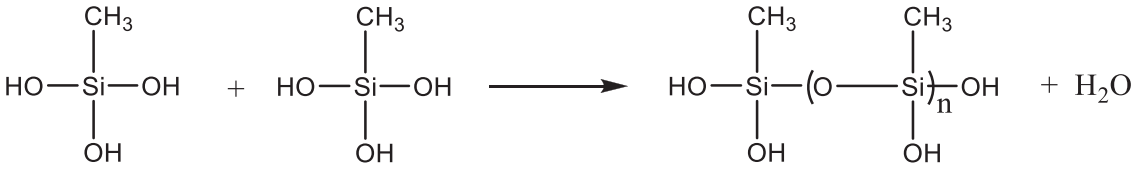


Figure 1.13. Polycondensation reaction

1.9.1.3.Condensation

Further condensation of the silica network with sol-gel active hydroxy terminated organic polymer results in organic- inorganic network (Figure 1.14).

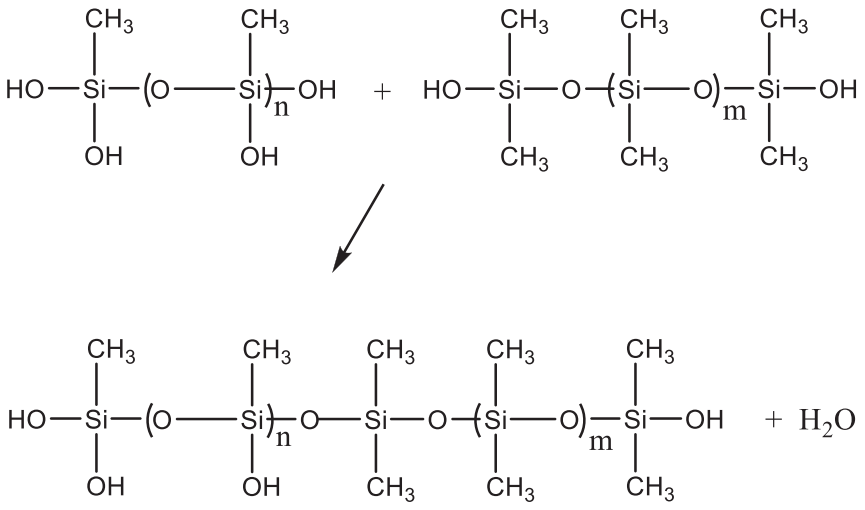


Figure 1.14. Condensation reaction

1.9.1.4.Surface Bonding

Surface bonding on silica fiber is achieved simply by dipping the bare fiber into produced viscous sol-gel. Chemical bonding of polymer network to silica surface occurs through condensation of the surface silanol groups with silanol or siloxane groups in the organic-inorganic polymer network (Figure 1.15).

The final step of the process is deactivation (or derivatization) of the silanol groups using Poly(methylhydrosiloxane) (Figure 1.16). Deactivation process is completed after thermal curing of the resulting fiber. In addition to the deactivation process, the PMHS also controls the polarity of the resulting coating.

Numerous sol-gel based coatings are summarized in the literature (Bagheri et al. 2008; Bianchi et al. 2008; Yu et al. 2004; Zeng et al. 2001; Wang et al. 2000). The most widely used are organic/inorganic based sol-gel approaches. In addition, only inorganic sol-gel with various functional groups is also given.

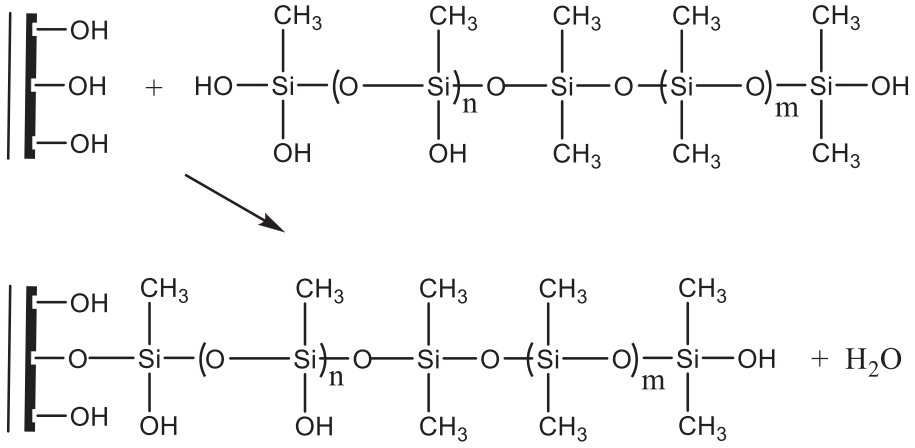


Figure 1.15. Surface bonding of the network

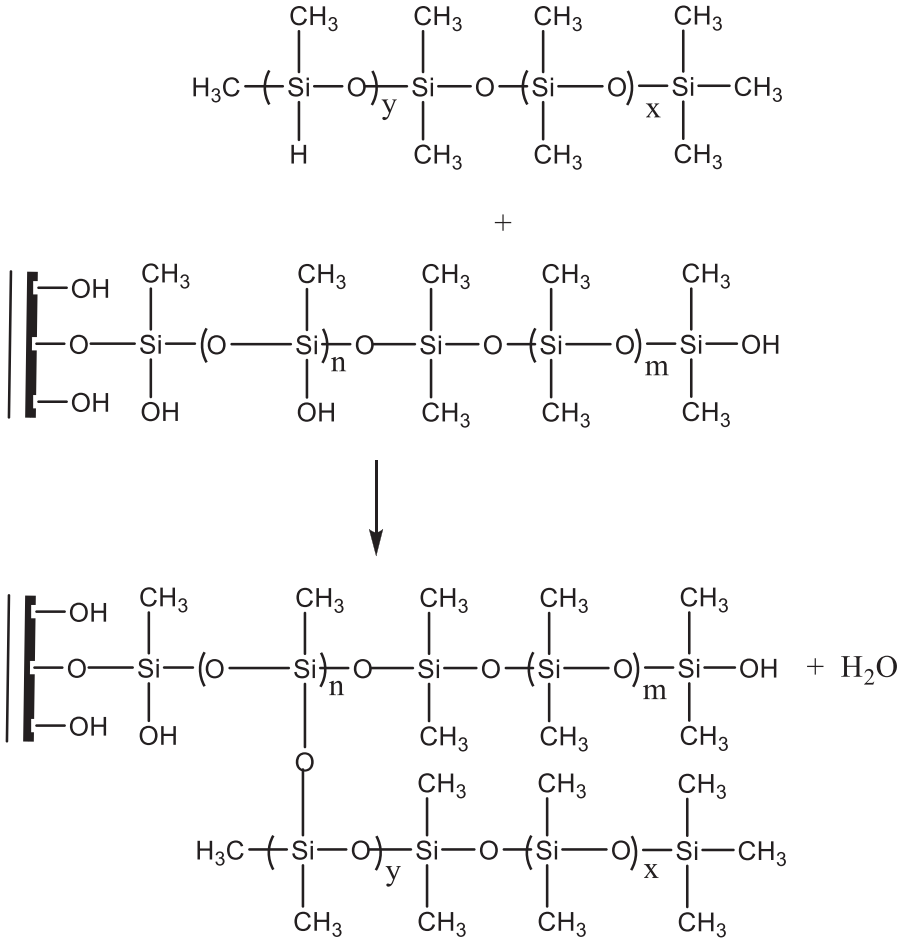


Figure 1.16. Deactivation process

1.10.AIM OF STUDY

The general purpose of this thesis is to develop novel SPE sorbents and SPME fiber coatings for determination of endocrine disrupting compounds. The first part of the thesis includes the preparation of molecularly imprinted polymer, amino group containing and molecularly imprinted amino-functionalized silica gel sorbent as a solid phase extraction material. This part deals with the synthesis, characterization and application of the sorbent for BPA and steroid hormones. The second part of the thesis is related to the development of new SPME fiber coatings. As explained in related parts of the introduction section, the main drawback encountered with the commercial SPME fibers is the production of analyte-specific fiber coatings. Other critical point in SPME process is the stability problem that arises during thermal and solvent desorption. The second part of the thesis includes easily prepared and selective SPME coatings. In this part, sol-gel based amino functionalized electro spun SPME fibers and MIP nanoparticles encapsulated electrospun polystyrene fibers as SPME fibers were aimed to be prepared. Prepared fibers were used to develop novel SPME-HPLC-DAD methodologies for the simultaneous microextraction and determination of the paraben species and bisfenol A in natural waters such as drinking and tap and sea water.

CHAPTER 2

DEVELOPMENT OF A NOVEL MISPE METHOD FOR SELECTIVE DETERMINATION OF BISPHENOL A AS AN EMERGING POLLUTANT

Bisphenol A, 2,2-(4,4-dihydroxydiphenyl) propane, which was firstly synthesized as an artificial estrogen in 1890s and has been one of the most important monomer used worldwide in many products including production of polycarbonate plastics, epoxy and phenol resins, polyesters and flame retardants in nowadays. Human exposes to BPA by the way of dermal, inhalation or their diet. Many studies reveal that BPA is endocrine disrupting compound which has estrogenic effects by binding to the estrogen receptor. Exposure to BPA even at low doses have many adverse health effects in perinatal, childhood and adult periods such as reproduction system problems (fertility, male sexual function, sperm quality, breast cancer, miscarriage etc.), development effects (birth weight, male genital abnormality and metabolic diseases (Type-2 diabetes, Thyroid function). BPA has been eliminated partially in waste water treatment plants (WWTP) and easily pass through the aquatic environment. Moreover , it can exist in sediments and bioaccumalate in biota and finally reach to human as a last step with all negative effects. Therefore, it is very significant to advance a simple analytical methods to determine BPA in environmental samples. To isolate BPA from its matrix or preconcentrate low level concentration prior to its analysis, sample pretreatment is crucial step. Solid Phase Extraction (SPE) is most frequently used an enrichment and purifying method for determination of BPA beacuse it provides many benefits such as low cost, fast, simple manipulation, high preconcentrating factor and does not require large amount of organic solvent. In many studies, commercial SPE cartridges such as C18 and Oasis HLB were used for determination of BPA in real samples. Despite showing high sorption capacity, these traditional sorbents are lack of the selectivity to BPA. In recent years, to overcome this problem, molecularly imprinted polymers were used as SPE sorbent for analysis of BPA in various sample which provides excellent molecular recognition ability, sensitivity and high selectivity. The preparation of molecularly imprinted polymers (MIPs) is based on the interaction of a template molecule with functional monomer to form specific

interactions (non-covalent or covalent approaches). The crosslinker and functional monomer around the template molecule establish a three dimensional network. Later, removal of the template molecule from the synthesized polymer leaves behind specific recognition sites for template molecule or related compound. BPA imprinted polymers have been obtained by using different approaches such as surface imprinting, magnetic particles, precipitation polymerization, bulk polymerization or supercritical polymerization.

2.1. Experimental

2.1.1. Chemicals and Reagents

All chemicals and reagents were analytical grade. Endocrine disrupting compounds, bisphenol-A (BPA, ≥99%), 4-ocytlphenol (4-OP,99 %) and 4-nonylphenol (4-NP) were obtained Sigma Aldrich, while β-estradiol (β-EST) 99% (dry wt.), ca 3% water) and triclosan (TCS, 99%) were purchased from Alfa Aesar. .1000.0 mg/ml of stock solutions of endocrine disrupting compounds were prepared in methanol as monthly in amber bottles and stored at –20 °C in refrigerator. All studied solutions were prepared daily by appropriate diluting the stock solution. Doubly distilled ultra-pure water was used in all experiments. All solutions that were used through the study firstly were filtered from 0.25 μm cellulose acetate filter paper or 0.25 μm polyamide filter paper (depend on the solvent system) and degassed for 15.0 min in ultrasonic bath prior to HPLC analysis. pH of the solutions were adjusted by using NH_3 and HNO_3 at different concentrations In the synthesis of BPA imprinted polymer, acrylamide (AA, ≥99%, Sigma Aldrich) as funtional monomer, Trimethylolpropane trimethacrylate (TRIM, Aldrich) as crosslinker, HPLC grade acetonitrile (Sigma Aldirch) as a porogen and inititatior 2,2’ Azobis(2,4-dimethyl valeronitrile (AIVN, Alfa Aesar) were used. The other solvents were HPLC grade that used through the study. Tap water, bottled water and sea water samples were obtained and filtered to remove particles.

2.1.2. Instrumentation and Apparatus

The analysis of BPA and other endocrine disrupting compounds were performed by using Agilent 1200 Series High Pressure Liquid Chromatography system with diode-array UV detection (HPLC–DAD) equipped with. The analytical column was C30 (YMC, 250 mm x 4.6 mm). Isocratic elution was applied which consisted of 20% ultrapure water (adjusted to pH 3.0 with acetic acid):80% methanol at a flow rate of 0.8 mL/min. The injection volume was 20µL, and the DAD wavelength was set at 220.0 nm. The optimized operation conditions for HPLC was given Table 2.1.

The pH adjustments were performed with Ino Lab Level 1 pH meter (Weilheim, Germany). To obtain effective mixing, IKA yellow line OS 5 basic orbital shaker (Staufen, Germany) was used. The surface morphology and particle size of synthesized materials was realized with Quanta 250FEG Scanning Electron Microscope (SEM)

Table 2.1. Operation conditions for HPLC analysis

HPLC	Agilent 1200
Analytical column	C30 (250 mm x 4.6 mm, 5 µm)
Mobile phase	80% methanol, 20% UPW (adjusted to pH: 3.0 with acetic acid)
Flow rate	0.8 mL/min
Column temperature	30 ºC
Sample volume	20 µL
Selected wavelength	220.0 nm

2.1.3. Synthesis of Bisphenol A imprinted polymers

Synthesis of uniform sized MIP microspheres as well as NIP polymers were performed by precipitation polymerization as reported our previous work (Olcer et al. 2017). In the synthesis of MIP/NIP particles, non-covalent approach was performed by dissolving 1.0 mmol bisphenol A (template molecule) and 4.0 mmol acrylamide

(functional monomer) in 100.0 ml acetonitrile solution for 1.0 hour at room temperature. Under argon gas, 20.0 mmol TRIM (crosslinker) and 2.0 % mole AIVN(inititatior) were added to reaction mixture. All reaction system was sealed and the polymerization was started at 60 ˚C with stirring for 24 h. Subsequently, the obtained particles were washed with acetonitrile to remove unreacted species and dried at 100 ˚C for 12 h in oven. Finally, to remove template molecule BPA, the polymers wash with methanol-acetic acid (9:1,v/v) mixture and methanol respectively. Same experimental protocol was applied for NIP particles except for the addition of BPA. Rebinding Experiments

The binding capacities and the dissociation constants for BPA imprinted and corresponding non-imprinted polymers were realized by rebinding experiments. In batch type procedure, ten milliliters of each BPA solutions with different concentrations (1.0-250.0 mg/L) were mixed with 10.0 mg MIP/NIP particles in 20.0 ml vials and then were shaken at 480.0 rpm for 24 h. After binding, the mixtures were filtered and free BPA concentrations in supernatant were determined by HPLC-DAD (Agilent1200).

The adsorption capacity Q and dissociation constant K_d were calculated using equations 1 and 2

$$Q = \frac{(C_0 - C_f)\mathbb{V}}{m} \qquad \text{Equation 1}$$

$$\frac{Q}{c_f} = -\frac{1}{\mathcal{K}_d} Q + \frac{Qmax}{\mathcal{K}_d} \qquad \text{Equation 2}$$

Here, C_0 and C_f (µmol/L) are the initial and the final concentration of BPA, V (L) is the total volume of sample , m (g) is the mass of MIP/NIP particles Q and Qmax (µmol/g) are amount of BPA adsorbed at equilibrium and saturation, respectively. The dissociation constant (Kd) and maximum number of binding sites (Qmax) were determined by the Scatchard plot.

2.1.4. Cross Selectivity

Selectivty studies were performed with BPA and structurally related compound 4-Octyphenol and 4-nonylphenol at concentration of 50.0 mg/L. 10.0 ml of this mixture was added into 10.0 mg of MIP/NIP particles. Sorption was achieved on an orbital shaker at 480rpm and 60.0 min. All mixtures were filtered through cellulose acetate membranes prior to analysis. Effluents were analyzed with HPLC-DAD at 220 nm.

2.1.5. Optimization of MISPE conditions

Various experimental parameters such as the effect of pH of sample solution, amount of sorbent, adsorption time, desorption solvent, reusability and interferences were optimized to obtain best extraction efficiency for determination of BPA. All studied parameters are given in Table 2.2.

Table 2.2.Experimental parameters

Analyte concentration	1.0 mg/L
pH	3.0, 7.0, 10.0
amount of sorbent	5.0, 10.0, 25.0, 50.0, 100.0 mg
Desorption solvent	Acetonitrile, ACN Methanol, MET M1: MeOH: HOAc, 9:1, v/v , M2: MeOH : H_2O (pH 3.0 HOAc)
adsorption time	1.0, 5.0, 15.0, 30.0, 60.0, 120.0 min.
Reusability	10 cycle
Interferences	Estradiol and Triclosan

2.1.6. Method Validation and Real Samples

After the determination of optimum parameters, the calibration curve was obtained by analysing BPA standards after performing the MISPE procedure using eigth different concentrations (10.0-1000 ng/mL). Limit of detection (LOD) was calculated by analyzing the least concentrated standard 10 times with HPLC-DAD. The sorption efficiency of sorbents was investigated by using the spiked samples of drinking and tap water. The tap water and drinking water samples were filtered to remove particles and then stored at 4 ˚C. Finally, developed MISPE procedure was applied for water samples which were spiked with BPA at three different concentration namely 0.1, 1.0 μg/mL.

2.2. Results and Discussion

2.2.1. Preparation and Characterization of BPA printed polymer

In this study, AA-TRIM-ACN polymer formulation with the typical 1:4:20 template:monomer:cross-linker molar ratio was used for selective uptake of template molecule, BPA. Fig. 2.1 shows the possible reaction mechanism of BPA-MIP synthesis.

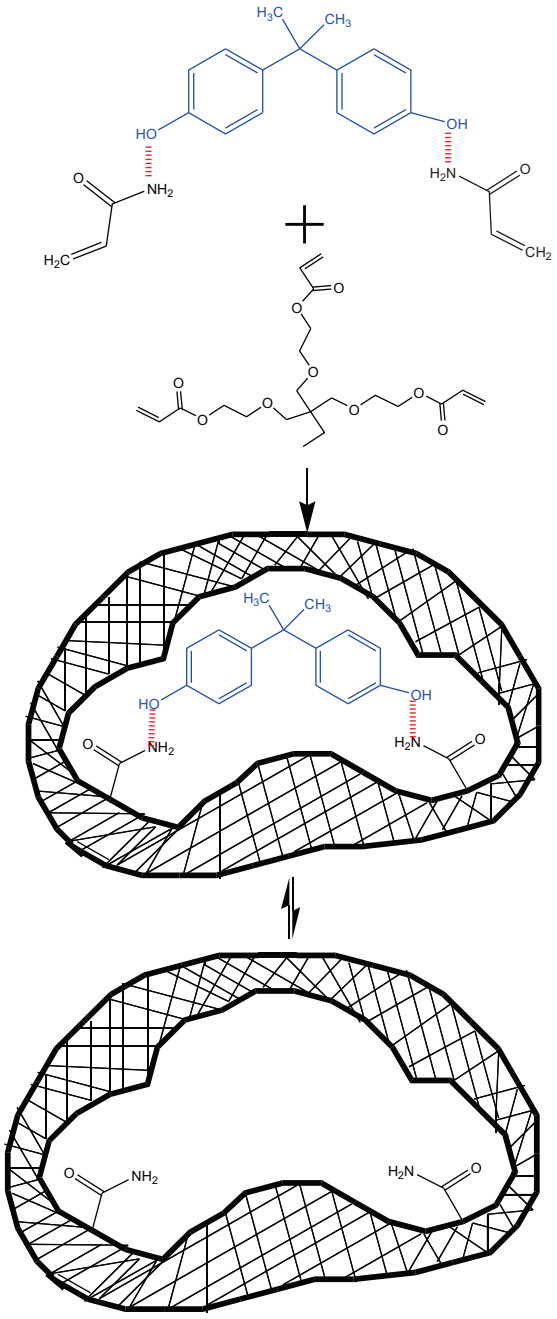


Figure 2.1. Possible sythesis mechanism of BPA imprinted polymer beads

Functional monomer, acrylamide , is responsible for the binding interactions in the imprinted binding sites. The reason of chosing acrylamide as functional monomer in this study is the phenolic aromatic ring in BPA that has weak acidic property and interact with the basic amine moiety in acrylamide (pK_a=7.9) The SEM image of synthesized BPA-imprinted polymer is given in Figure 2.2. Spherical polymer particles were obtained by precipitation polymerization. The first step in synthesis of MIP is to decide he polymerization strategy. Precipitation polymerization strategy is simple and easy way to obtain MIP beads. Especially, it eliminates the crushing and sieving steps and provides homogenous binding sites in comparison to bulk polymerization.

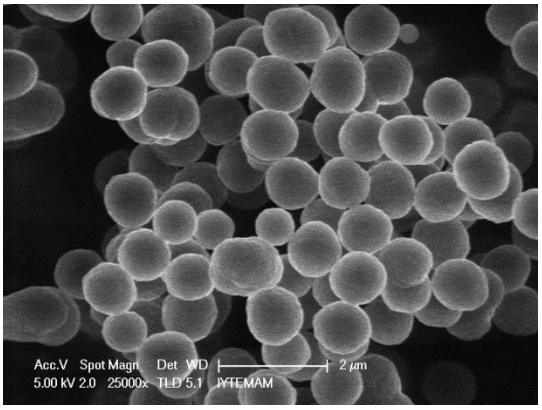


Figure 2.2. SEM image of BPA imprinted polymer beads

Besides that, total monomer/porogen ratio (w/v) helps in controlling the morphology of polymer. If this ratio is smaller than 5 %, the precipitation polymerization takes place and the morphology of polymer matrix could be spherical shaped particles. In our synthesis route applied, this ratio was calculated as 2.5 %.

In this study, non-covalent imprinting approach was used which is based on formation of non-covalent interactions (hydrogen bonding, hydrophobic and ionic interactions) of functional monomer and template molecule. This approach provides both easy preparation of the template monomer complex, easy removal of the templates from the polymers.

2.2.2. Rebinding Experiments

The adsorption capacities of MIP and NIP sorbents for BPA were determined by means of rebinding experiments. As clearly seen Figure 2.3. The BPA -imprinted polymer shows higher sorption capacity for BPA than corresponding NIP polymer. The reason of weaker adsorption capacity of BPA on NIP can be explained with the absence of BPA-specific interactions. On the other hand, the amount of bound BPA to MIP was increased with an increase in the initial concentration of BPA. This result proved the presence of special cavities in MIP matrix.

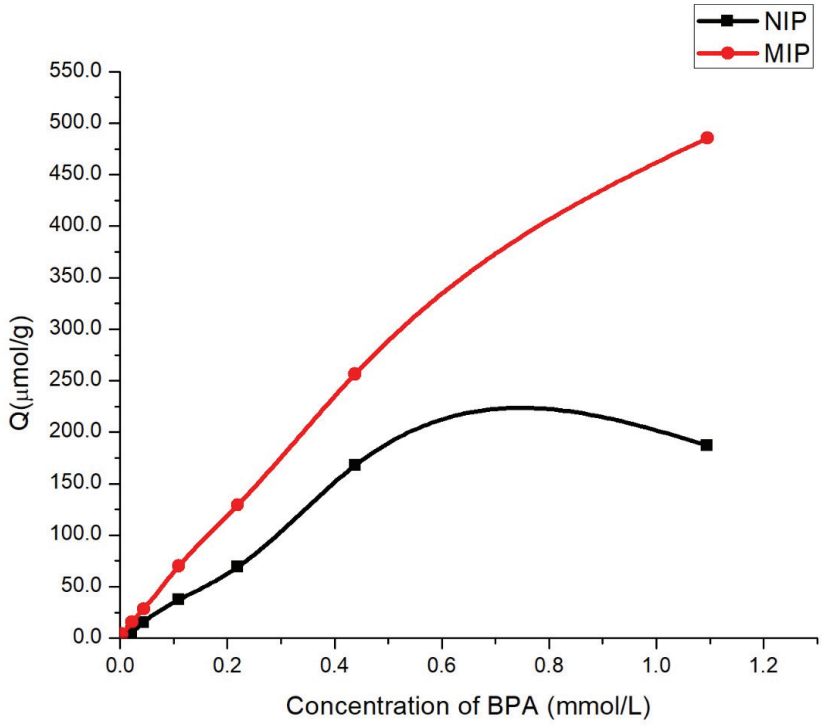


Figure 2.3. Binding Isoterms of BPA to NIP and MIP

The binding affinity and theoretical number of binding sites for BPA was evaluated by Scatchard analysis. The dissociation constants K_d and specific site capacities Q_{max} can be obtained from the slope and intercept of Scatchard plots. As shown in Figure 2.4, the Scatchard plot of MIPs has two- linear part compared to NIP polymer. The results revealed that the affinity of binding sites of MIP is heterogenous and two classes of

binding sites, high (specific) and low affinity(non-specific) binding are formed in BPA imprinted polymer matrix.

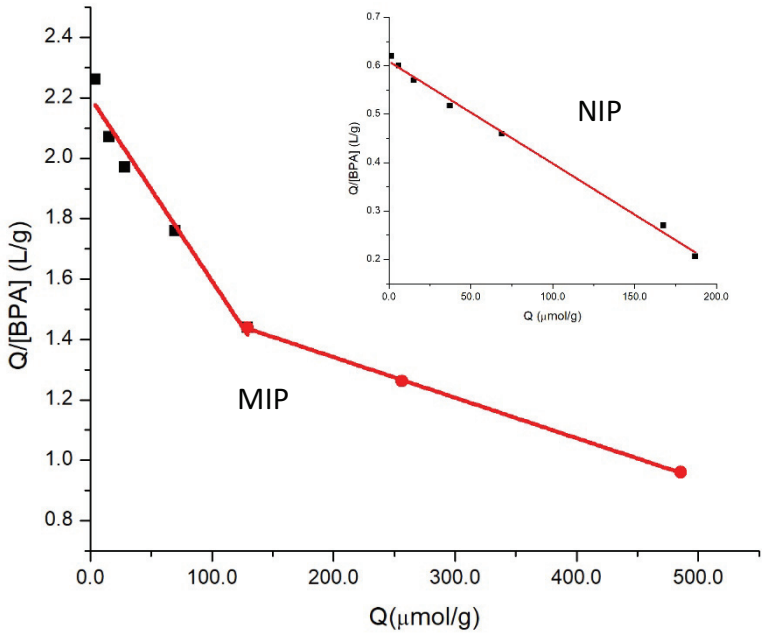


Figure 2.4. Bindindg Isoterms of BPA to NIP and MIP

From Scatchard analyses, dissociation constants (K_d) and saturation capacity values Q_{max} were calculated as 166.9 and 367.0 µmol/g, respectively for the high-affinity binding sites, and 769.0 µmol/L and 1237.4 µmol/g, respectively for the low-affinity binding sites for MIP. Furthermore, the Kd and Qmax values were 59.0 µmol/L and 94.4 µmol/g respectively for NIP. These results indicated that BPA imprinted polymer has high selectivity to BPA.

2.2.3. Cross Selectivity

In the presence of structurally related compounds 4-Octylphenol and 4-Nonylpenol, the specific binding characterictics of MIP and NIP to BPA were verified. As shown in Figure 2.5. BPA imprinted polymer has shown an obviously higher sorption capacity towords BPA compare to NIP. There were no differences in the sorption capacity of MIP and NIP for 4-OP and 4-NP. The phenol group of BPA can interact with

two amine moieties of MIP by hydrogen bonding interaction while there is only one phenolic sites for 4-OP and 4-NP. These molecules can be captured on the surface of polymer matrix with non specific interaction. These results have indicated that MIP matrix have memory cavities with specific binding sites which provides recognition ability to BPA molecule.

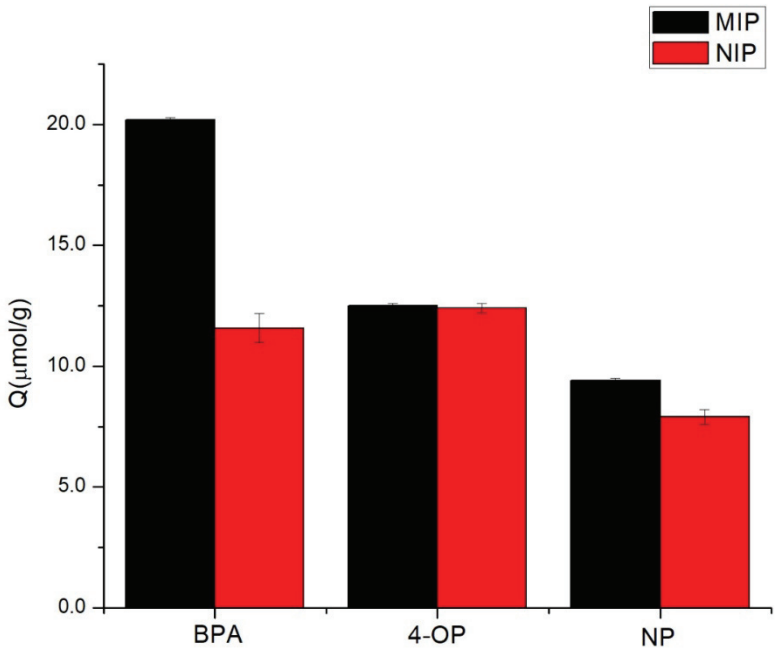


Figure 2.5. Sorption capacity of MIP and NIP in the presence of 4-OP and 4-NP. (Agilent 1200 Series HPLC-DAD system, YMC C30 column (25cm×4.6mm) column, 20: 80 water (pH 3.0): methanol mobile phase, 0.8 ml/min flow rate, 220 nm)

2.2.4. Optimization of MISPE conditions

The pH of the sample solution plays a critical role for SPE process because it can not only change the net charge of amine groups in polymer matrix but also determines existing forms of BPA molecule and then affects the hydrogen bonding between amine and phenolic moieties. To investigate higher sorption efficiency results, different pH of the sample solutions (3.0 to 10.0) were examined. The results are given in Figure 2.6. As clearly seen the maximum the sorption efficiency value (> %95.0) wasobtained at pH 7.0. The lowest sorption efficieny was observed at pH 3.0. This can be explained with the protonation of hydroxyl groups of BPA can increase its solubility in water so the

hydrogen bonding between the amine and phenolic hydroxyl groups was interfered and weakened at acidic medium. On the other hand, the reduction in sorption efficiency of BPA at pH 10.0 can be explained due to the deprotonation of BPA at high pH value. With these results, the pH of solution was adjusted to 7.0 for further experiments.

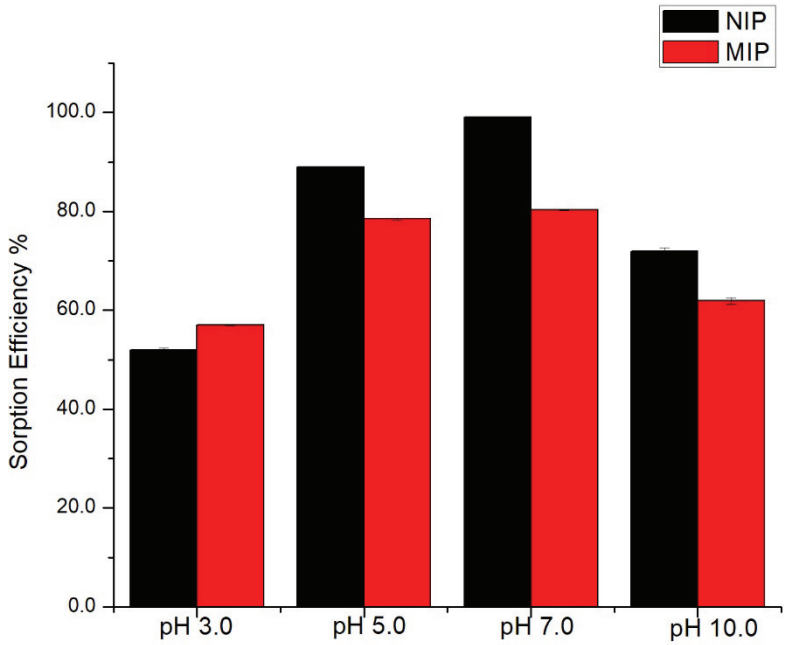


Figure 2.6. The effect of solution pH on the sorption of 1.0 µg/mL of BPA Agilent 1200 Series HPLC-DAD system, YMC C30 column (25cm×4.6mm) column, 20: 80 water(pH 3.0): methanol mobile phase, 0.8 ml/min flow rate, 220 nm)

The effect of amount of MISPE sorbent was investigated by varying from 5.0 mg to 100.0 mg. As illustrated in Fig 2.7., the percentage sorption increased with increase of sorbent amount and reached maximum at 10.0 mg of MISPE sorbent. In following experiments, 10.0 mg MISPE sorbent were used to quantitative sorption of BPA

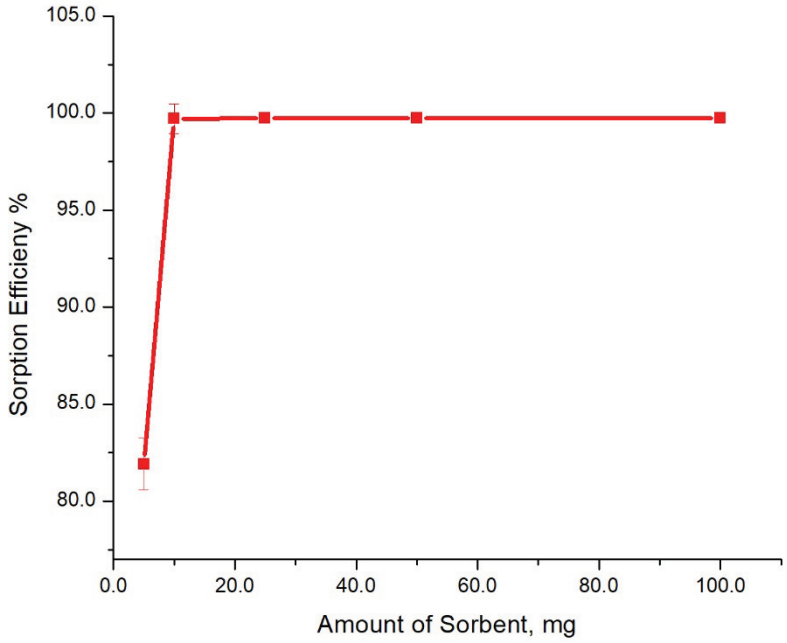


Figure 2.7. The effect of amount of sorbent on the sorption of 1.0 µg/mL of BPA Agilent 1200 Series HPLC-DAD system, YMC C30 column (25cm×4.6mm) column, 20: 80 water (pH 3.0): methanol mobile phase, 0.8 ml/min flow rate, 220 nm)

Adsorption time is one of the key parameters affecting the sorption capacity. The effect of shaking time on the sorption of BPA was investigated at 1.0, 5.0, 15.0, 30.0, 60.0 and 120.0 min. As shown in Fig. 2.8, BPA -imprinted sorbent had a fast kinetics and very high sorption capacity (> %90.0) was obtained even in 1.0 min. To quarantee the sorption, 30.0 min was chosen as shaking time.

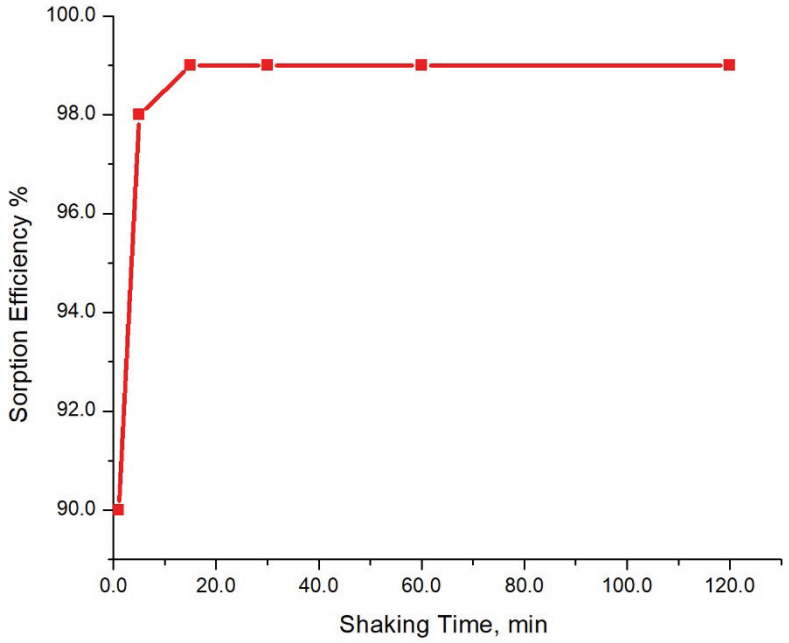


Figure 2.8. The effect of shaking time on the sorption of 1.0 µg/mL of BPA Agilent 1200 Series HPLC-DAD system, YMC C30 column (25cm×4.6mm) column, 20: 80 water(pH 3.0): methanol mobile phase, 0.8 ml/min flow rate, 220 nm)

The choice of elution solvent is a crucial step in desorption process. Eluents may destroy the specific interaction of template molecule and polymer matrix. In this study, two common organic solvents, acetonitrile and methanol and two mixture, M1 (methanol:acetic acid mixture (9/1 v/v)) and M2 (methanol:water at pH 3.0 with acetic acid 8/2 (v/v)) were used as desorption solvents. Figure 2.9. shows the recoveries of BPA with different eluents. As seen, acetonitrile had the least elution ability. On the other hand, the mixtures M1 and M2 gave the best recoveries because these solvents include acetic acid which disturbs the hydrogen bonding between BPA and MIP. The M2 mixture also mobile phase used in HPLC analysis throughout the study Therefore, M2 was used as desorption solvent for simplicity.

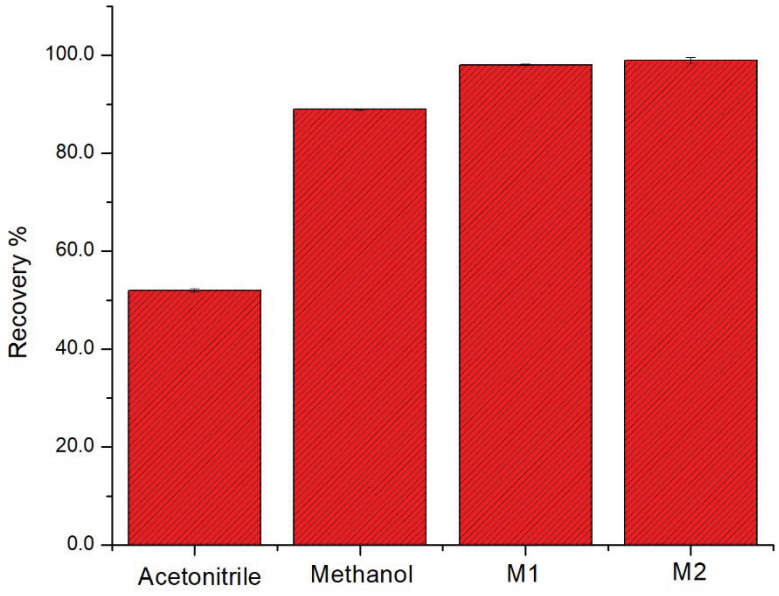


Figure 2.9. The effect of desorption solvent Agilent 1200 Series HPLC-DAD system, YMC C30 column (25cm×4.6mm) column, 20: 80 water(pH 3.0): methanol mobile phase, 0.8 ml/min flow rate, 220 nm)

The reusability of the BPA imprinted sorbent was also investigated because regeneration is another significant factor for evaluating the performance of MISPE sorbent. As seen in Figure 2.10 the binding efficiency still remained as 85% after six MISPE cycles, and BPA-MIP could be effectively regenerated.

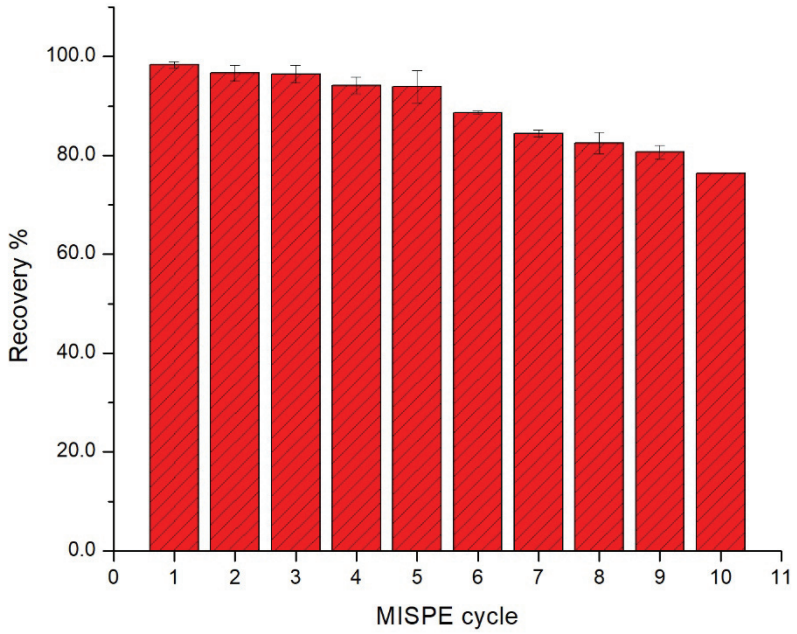


Figure 2.10. The reusability of MISPE sorbent Agilent 1200 Series HPLC-DAD system, YMC C30 cloumn (25cm×4.6mm) column, 20: 80 water(pH 3.0): methanol mobile phase, 0.8 ml/min flow rate, 220 nm)

Two known endocrines disrupting compounds TCS and β-EST were used for competitive recognition studies. The added concentration for TCS and β-estradiol was 1.0 µg/mL. The experiments were conducted at the optimum conditions. The chromatogram obtained after MISPE application is given in Figure 2.11. The recovery results of BPA in presence of these compounds are given Table 2.3. The results demonstrated that there is no interference at the studied compounds in analysis of BPA

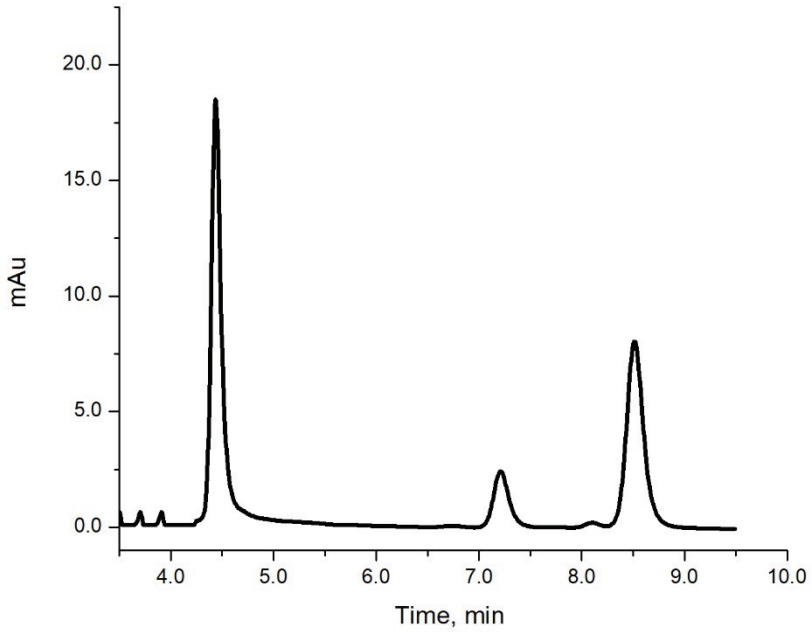


Figure 2.11. Chromatograms obtained after extraction with MISPE procedure. Agilent 1200 Series HPLC-DAD system, YMC C30 column (25cm×4.6mm) column, 20: 80 water(pH 3.0): methanol mobile phase, 0.8 ml/min flow rate, 220 nm)

Table 2.3. Recovery Results obtained after MISPE procedure. (Solution pH: 7.0, sorption time: 30 min, sorbent amount: 10.0 mg, sample volume: 10.0 mL, , eluent: Methanol:Water at pH 3.0 with acetic acid 8/2 (v/v), desorption time: 30 min, n=3)

Compound	**Recovery %**	**RSD %**
BPA	98.7	2.1
B-EST	74.3	3.4
TCS	89.8	2.8

2.2.5. Method Validation and The analysis of spiked samples

The percent sorption and recovery values at two spike concentrations are given in Table 2.4. The results indicate that the proposed methodology is practical to use in ultrapure drinking water and tap water with the percentage recovery and sorption values above 98%. The MISPE sorbent was not affected from matrix.

Table 2.4. Spike sorption and recovery results obtained using MIP for water samples (solution pH: 7.0, sorption time: 30 min, sorbent amount: 10.0 mg, sample volume: 10.0 mL, eluent: Methanol:Water at pH 3.0 with acetic acid 8/2 (v/v), desorption time: 30 min, n=3).

	Sorption % BPA	
	0.1 mg/L	1.0 mg/L
UPW	99,6 (±0,3)	99,8 (±3,1)
Bottled water	99,0 (±0,2)	98,2 (±1,1)
Tap Water	99,5 (±0,9)	98,8 (±1,4)
	Recovery % (mg/L) BPA	
	0.1 mg/L	1.0 mg/L
UPW	99.1 (±0,1)	99.8 (±0.7)
Bottled water	98.2 (±0,2)	98.2 (±0.9)
Tap Water	99.0 (±0,1)	98.8 (±0.1)

CHAPTER 3

TWO DIFFERENT SYNTHESIS APPROACHES FOR DETERMINATION OF ESTROGENS

3.1. Experimental

3.1.1. Chemicals and Reagents

All chemicals and reagents were analytical grade. Endocrine disrupting compounds, estrone (EST, ≥99%), 17α-ethynylestradiol (99 %) were obtained Sigma Aldrich, while β-estradiol (β-EST) 99% (dry wt.), ca 3% water) was purchased from Alfa Aesar. .1000.0 mg/ml of stock solutions of endocrine disrupting compounds were prepared in methanol in monthly in amber bottles and stored at –20 °C in refrigerator. All working solutions were prepared daily by appropriate diluting of the stock solution. Doubly distilled ultra-pure water was used in all experiments. All solutions first were filtered through 0.25 μm cellulose acetate or 0.25 μm polyamide membrane (depending on the solvent system) and degassed for 15.0 min in ultrasonic bath prior to HPLC analysis. Solution pH was adjusted by using NH_3 and HNO_3 at different concentrations. During the synthesis 3-aminopropyltriethoxysilane (APTES, ≥99%, Sigma Aldrich) as functional monomer and surface modification reagent, tetraethyl orthosilicate (TEOS, Aldrich) as crosslinker, HPLC grade THF, toluene (Sigma Aldirch) as porogen. The other solvents were also HPLC grade. Tap water, bottled water samples were filtered immediately after collection to remove particles.

3.1.2. Instrumentation and Apparatus

The analysis of estrogens was performed by using Agilent 1200 Series High Pressure Liquid Chromatography system with diode-array UV detection (HPLC–DAD) equipped with an the analytical column was C30 (YMC, 250 mm x 4.6 mm). Isocratic elution was applied which 20% ultrapure water (adjusted to pH 3.0 with acetic acid)-80% methanol at a flow rate of 0.8 mL/min. The injection volume was 20 μL, and the DAD

wavelength was set to 220.0 nm.The optimized operation conditions for HPLC are given in Table 3.1.

The pH adjustments were performed with Ino Lab Level 1 pH meter (Weilheim, Germany). To obtain effective mixing, IKA yellow line OS 5 basic orbital shaker (Staufen, Germany) was used. The surface morphology and particle size of synthesized materials was examined with Quanta 250FEG Scanning Electron Microscope (SEM)

Table 3.1. Operating conditions for HPLC analysis

HPLC	Agilent 1200
Analytical column	C30 (250 mm x 4.6 mm, 5 μm)
Mobile phase	20% UPW (adjusted to pH: 3.0 with acetic acid)-80% methanol,
Flow rate	0.8 mL/min
Column temperature	30 ºC
Sample volume	20 μL
Selected wavelength	220.0 nm

3.1.3. Synthesis of surface modified silica and β-estradiol imprinted silica

The procedure utilized in the functionalization of the silica surface was compiled from literature (Liu et al. 2002) with some modifications. The outline of the functionalization of silica gel surface is illustrated in Figure 3.1. The first step is the activation of the silica surface to convert the siloxane groups to silanol. For this purpose, 5.00 g of silica was treated with 50.0 mL of 0.010 M acetic acid for 1 h and then washed with ultrapure water until a neutral filtrate was obtained. This step was completed after drying the activated silica at 120 °C for 24 h in an oven. The amine modification step was carried out by mixing 5.0 g of activated silica, 3.0 mL of APTES and 9.0 mL toluene in a two necked 25 mL flask. A condenser having anhydrous $CaCl_2$ drying tube at the top was connected to the reaction flask. The reaction was proceeded under an inert

atmosphere provided with N_2 bubbled through the side arm of the flask. The mixture was stirred for 24 h at 100 rpm under constant reflux in an oil bath at a temperature of 110 °C. After the reaction had been completed, amine-treated silica was washed sequentially with 10.0 mL portions of acetone and toluene and then dried in an oven at 50 °C overnight.

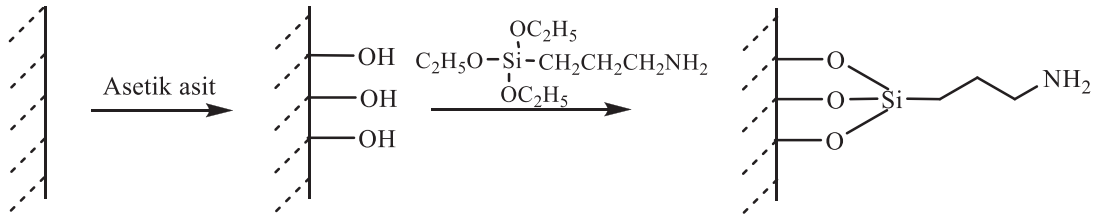


Figure 3.1. The modification of silica surface .

In the second part of the study, to obtain β-est imprinted silica, β-est (template molecule) was dissolved in 5.0 mL THF/water mixture. After 30 min., 4.0 mmol APTES (functional monomer) was added to reaction medium to obtain hydrogen bonding between template molecule and monomer. Under constant mixing rate 16 mmol TEOS (cross linker) added to reaction mixture. After 30 min mixing 1.0 g activated silica gel and 1.0 mL acetic acid were added and allowed to mix for 18 hour. After filtration, the obtained sorbent was dried in oven at 100 ºC overnight. To remove the template molecule from the silica matrix, the sorbent was washed with methanol-acetic acid (MeOH: HOAc, 9:1, v/v) mixture methanol:water (MeOH : H_2O (pH 3.0 HOAc)) and metanol respectively. Finally the sorbents were dried at 100 ºC for 12 hour. Corresponding non imprinted silica gel was sythesized with same protocol except the addition of β-est. The synthesis mechanism was given in Figure 3.2. The sorbents sythesized were given names (MIP)silica and (NIP)silica, respectively.

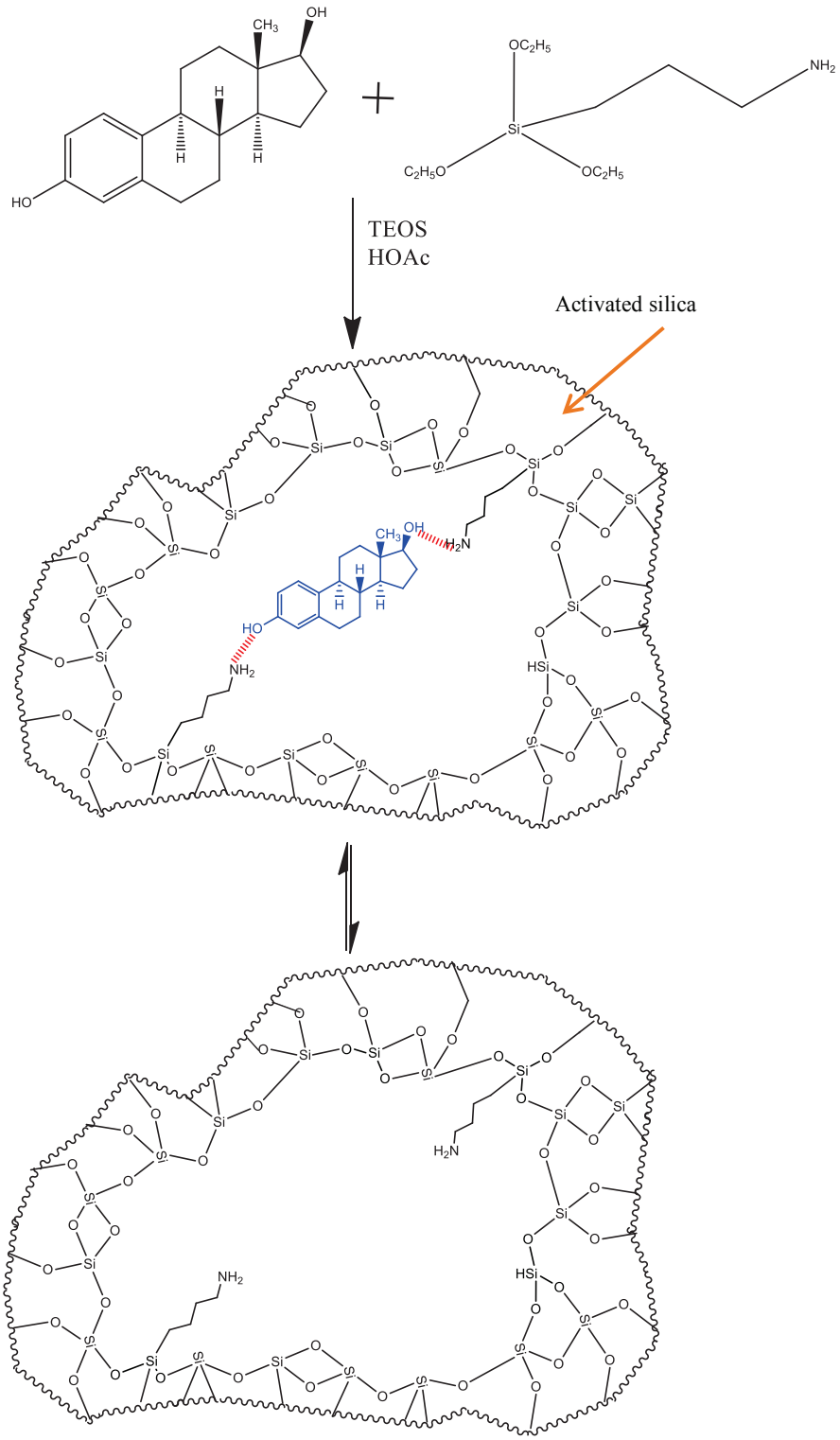


Figure 3.2. Synthesis mechanism of β-estradiol imprinted silica

3.1.4. Comparison of sorption behaviour of modified silica and β-Estradiol imprinted silica

Sorption studies were performed through a batch process for all the sorbents prepared; namely, silica, (NH_2) silica (MIP) silica and (NIP)silica. Batch sorption studies were carried out at 25 °C with each sorbent separately after the initial pH of solutions was adjusted to, 3.0, 7.0 and 10.0 with dilute HNO_3 or NH_3. After pH adjustment step, 10.0 mg sorbent was added into each of the 20 mL amber vials containing 20.0 mL aliquots of 1.0 mgL^{-1} β-estradiol solution. The mixtures were placed in orbital shaker and shaken for 60 minutes, then they were filtered. The filtrates were analyzed for β-estradiol concentration by HPLC-DAD.

3.1.5. Rebinding Experiments of (MIP)/(NIP)silica

To prove (MIP)silica have specific cavities but (NIP)silica not, ten milliliters of β-estradiol solutions with different concentrations (1.0-250.0 mg/L) were mixed with 10.0 mg (MIP)silica/(NIP)silica particles in 20.0 mL vials and then were shaken at 480.0 rpm for 24 h. After binding, the mixtures were filtered, and β-estradiol remained in the solution was determined by HPLC-DAD (Agilent 1200).

3.1.6. Cross Selectivity

Selective recognition studies were performed with β-estradiol and structurally related compounds 17α-ethynylestradiol and estrone at concentration of 50.0 mg/L. 10.0 ml of this mixture was added into 10.0 mg of MIP/NIP silica sorbent. Sorption was achieved on an orbital mixture at 480rpm and 60.0 min. Filtration was made in previous sections. Effluents were analyzed with HPLC-DAD at 220 nm

3.1.7. MISPE Optimization Parameters

Various experimental parameters such as pH of sample solution, amount of sorbent, adsorption time, desorption solvent, reusability and interference compounds were optimized to obtain best sorption efficiency for determination of β-estradiol. Herein,

all the sorption procedures were realized using orbital shaker at 480 rpm. Effluents and eluates were analyzed using HPLC-DAD.

Firstly, pH of the working solution is determined. For this purpose, 3.0, 7.0 and 10.0 pH solutions were prepared at the concentration of 1.0 mg/L (10 mL) and added to the 10.0 mg of MIP/NIP silica sorbents. In a separate experiment, MIP silica amount was investigated by changing it from 5.0 to 100.0 mg for again 10 mL of 1.0 mg/L β-Estradiol solutions. After that, adsorption time is determined for the time interval from 1.0 to 120.0 min. by keeping the other parameters constant. To elute the analyte molecules, retained by MIPs, nearly completely the type of the eluent is very important. For this, acetonitrile (ACN), MeOH, MeOH:HOAc and MeOH:H_2O were used for the as eluents for the desorption of 1.0 mg/L β-Estradiol. Reusability of the synthesized sorbent was checked via sorption/desorption cycle for 1.0 mg/L bisphenol A and 10.0 mg MIP. After each cycle the sorbent was dried in an oven. This protocol applied 10 times for the same sorbent. Lastly, the extraction ability of obtained sorbent was compared with commercially available SPE cartridge including LC-C8 ve LC-PHEN by using pre-determined parameters. For comparative purpose, 50.0 mg of (MIP)silica sorbent was placed into empty SPE cartridges After all cartidges were pretreated with 10 ml methanol and 10 ml pure water, 10.0 ml of 1.0 mg/L mixture of estrogen solution was loaded onto the commercial and (MIP)silica SPE cartridges. Then commercial columns were eluted with methanol and (MIP)silica SPE cartridge was eluted with methanol-acetic acid-water. The elution were analysed by HPLC and UV detection at 220 nm. All studied parameters were given in Table 3.2

Table 3.2. Summary of the parameters and ranges used throughout the study

Studied Parameters	Working Range
pH	3.0, 7.0, 10.0
Sorbent amount (mg)	5.0, 10.0, 25.0, 50.0 and 100.0
Shaking Time (min)	1, 5, 15, 30, 60 ve 120 min
Desorption Solvents	(ACN), MeOH, MeOH : HOAc (9/1) ve MeOH : H_2O (pH3.0)
Reusability of sorbent	10 sorption/desorption
SPE cartidge	LC-C8 and LC-PHEN

3.1.8. Method Validation

After the determination of optimum parameters, the calibration curve was sobtained by analysing β-estradiol standard solutions after performing the MISPE procedure using seven different concentrations (25.0-1000 ng/mL). Limit of detection (LOD) was calculated by analyzing the least concentrated standard 10 times with HPLC-DAD. The sorption efficiency of sorbents was investigated by using the spiked samples of drinking and tap water. The tap water and drinking water samples were filtered to remove particles and then stored at 4 ˚C. Finally, developed MISPE process applied for water samples. The water samples were spiked with β-estradiol at the concentration 0.1 and 1.0 µg/mL.

3.2. Results and Discussion

In this study, two different synthesis approaches were followed. In first study, silica surface was modified with amino groups, whereas in second study imprinted amino-functionalized silica gel material was synthesized by combining a surface molecular imprinting technique. The SEM images of obtained (NH_2)silica and (MIP)silica were given Figure 3.3.

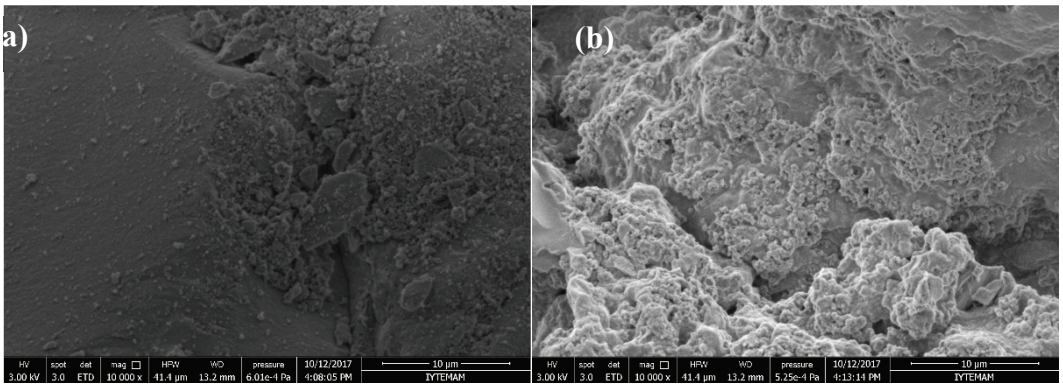


Figure 3.3. SEM images of (a) (NH_2)silica and (b) (MIP)silica

3.2.1. Comparison of sorption behaviour of modified silica and β-Estradiol imprinted silica

Batch type sorption experiments were performed with silica, (NH_2)silica, (MIP)silica and (NIP)silica. The sorption behavior of all sorbents was investigated at different pH values. As seen in figure 3.4, bare silica showed the lowest sorption efficiency against the β-estradiol. On the other hand, the highest sorption efficiency was obtained at pH7.0 with (MIP)silica. (NIP)silica and (NH_2)silica showed similar sorption trend. The reason of showing higher sorption values of (MIP)silica is to have specific binding sites in it which belong to β-est. Moreover, (NIP)silica and (NH_2)silica can only interact nonspecifically with β-est.

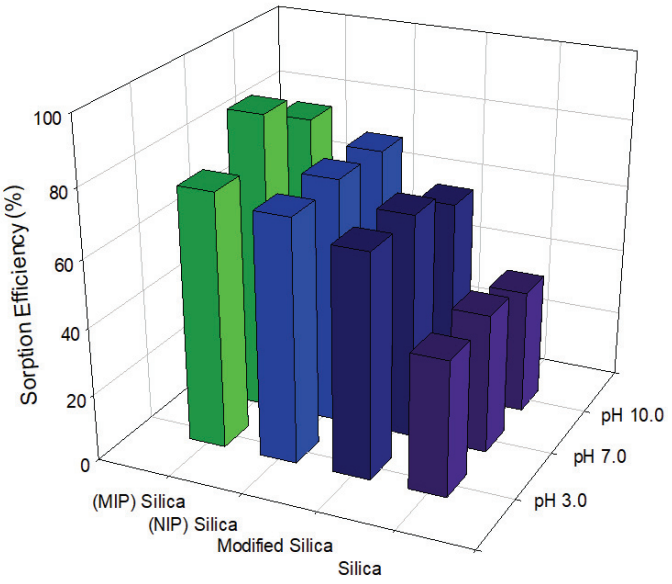


Figure 3.4. The sorption efficeny of Silica, (NH_2)silica, (MIP)silica and (NIP)silica at different pH values (Agilent 1200 Series HPLC-DAD 250 mm C30 column (YMC C30 (250mm x 4.6 mm)) , 20:80 Water acetic acid (pH 3.0):methanol– mobil phase 0.8 mL/min flow rate, 220 nm).

3.2.2. Rebinding Experiments of (NIP)/(MIP)Silica

The adsorption capacities of (MIP)silica and (NIP)silica sorbents for β-est were determined by using rebinding experiments. As clearly seen Figure 2.3. The β-est imprinted silica shows higher sorption capacity for β-est than corresponding (NIP)silica. The reason of weak adsorption capacity of β-est on (NIP)silica is only existence of non-specific interactions. On the other hand, the amount of bound β-est to (MIP)silica increased with increase in the initial concentration of β-est.This result proved the presence of special cavities in MIP silica matrix. (Figure 3.5)

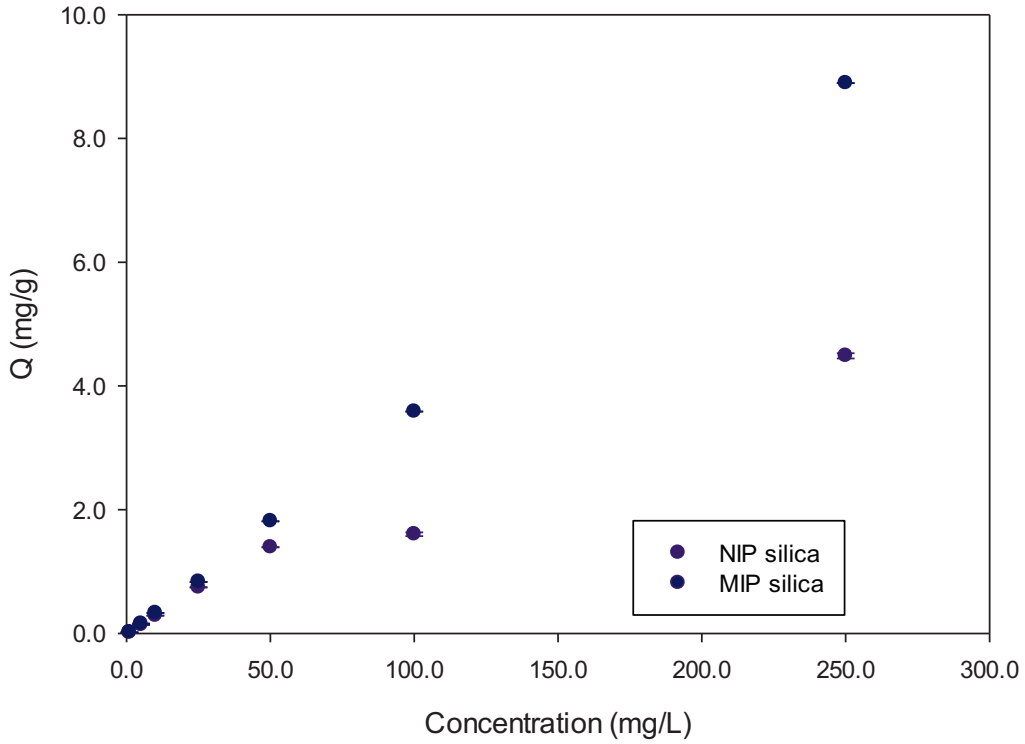


Figure 3.5. Rebinding characteristics assay of (MIP) silica ve (NIP) silica (Agilent 1200 Series HPLC-DAD 250 mm C30 column (YMC C30 (250mm x 4.6 mm)) , 20:80 Water acetic acid (pH 3.0):methanol–mobil phase 0.8 mL/min flow rate, 220 nm).

3.2.3. Cross Selectivity

The extraction ability of MIP and NIP silica was investigated in the presence of 17α-ethynylestradiol and estrone. As seen in figure 3.6 all MIP silica showed high selectivity to all estrogen species. Moreover, these results exhibit that prepared MIP silica have recognition ability to common structure of estrogens.

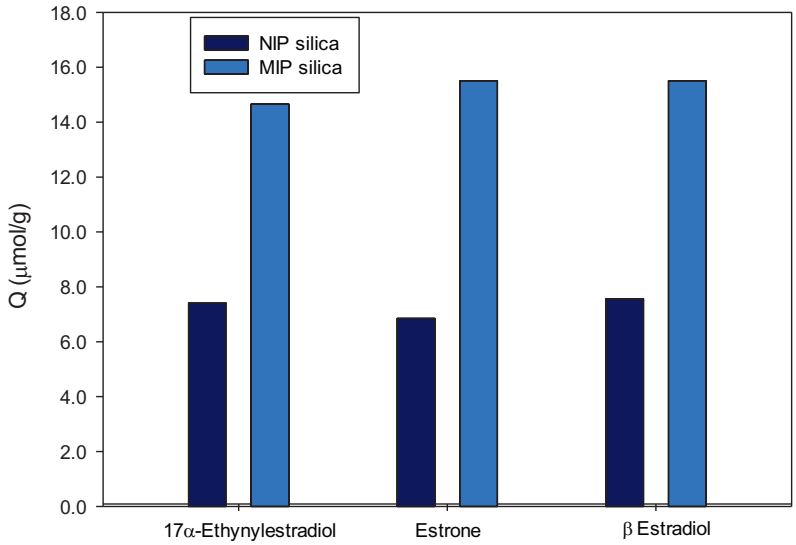


Figure 3.6. Cross Selectivity of MIP(silica) and (NIP)silica (Agilent 1200 Series HPLC-DAD 250 mm C30 column (YMC C30 (250mm x 4.6 mm)) , 20:80 Water acetic acid (pH 3.0):methanol–mobil phase 0.8 mL/min flow rate, 220 nm).

3.2.4. SPE Optimization Parameters

To obtain higher sorption efficiency results, different pH of the sample solutions ranging from 3.0 to 10.0 were examined. The results are given in Figure 3.7. As clearly seen that the sorption efficiency values of β-Est increased with increase of pH of sample solution and maximum sorption efficieny (> %95.0) were obtained at pH 7.0. When the pH of sample solution was 3.0, the lowest sorption efficieny was observed. This can be explained the protonation of hydroxyl groups of β-Est can increase its solubility in water so the hydrogen bonding between the amine and phenolic hydroxyl groups was interfered and weakened at acidic medium. On the other hand, the reduction in sorption efficiency of β-Est at pH 10.0 can be explained due to the deprotonation of BPA at high pH value. If all results were taken into consideration, the pH of solution was adjusted 7.0 for further experiments.

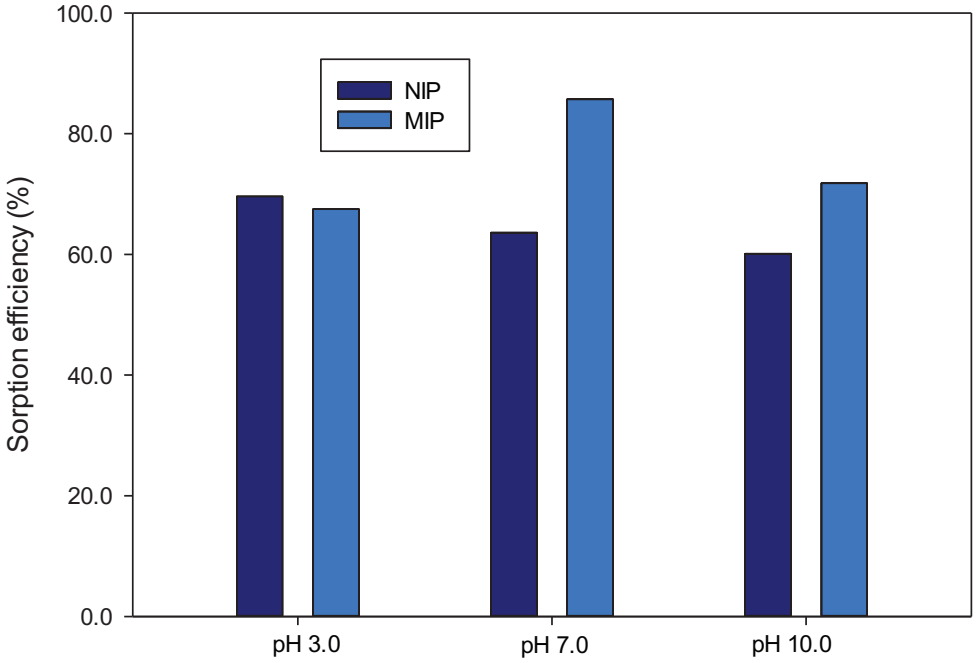


Figure 3.7.The effect of solution pH on the sorption of β-Est (Agilent 1200 Series HPLC-DAD 250 mm C30 column (YMC C30 (250mm x 4.6 mm)) , 20:80 Water acetic acid (pH 3.0):methanol–mobil phase 0.8 mL/min flow rate, 220 nm).

The effect of amount of sorbent on sorption of β-Est was investigated. The result was given Figure 3.8 . As realized from figure, by using 50.0 mg (MIP)silica sorbent, the maximum sorption efficiency was obtained. Further experiments were performed by using this value

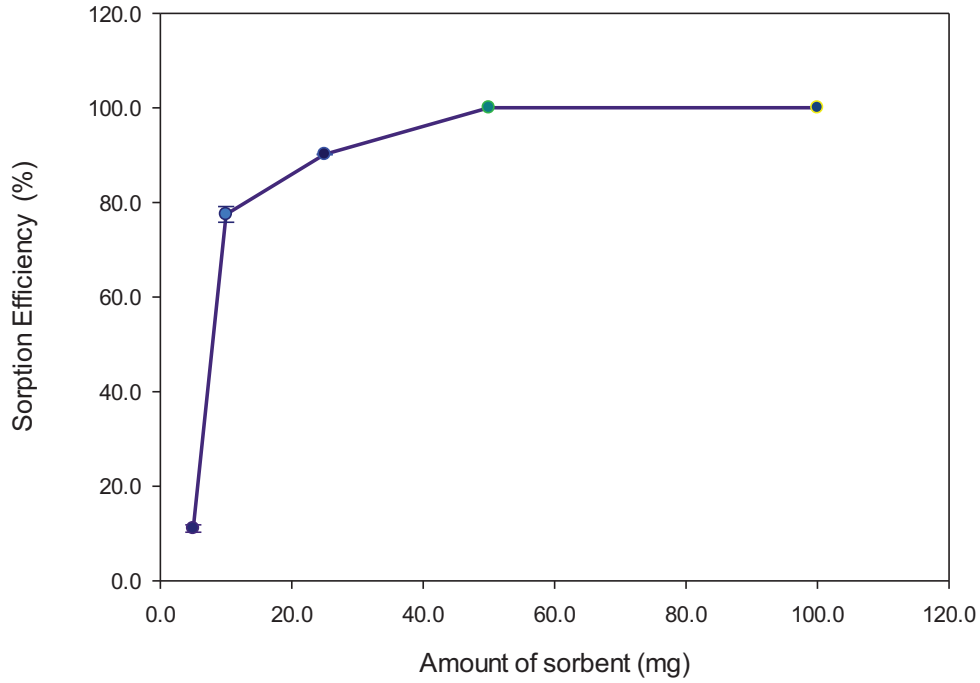


Figure 3.8. The effect of amount of sorbent on the sorption of β-Est (Agilent 1200 Series HPLC-DAD 250 mm C30 column (YMC C30 (250mm x 4.6 mm)) , 20:80 Water acetic acid (pH 3.0):methanol–mobil phase 0.8 mL/min flow rate, 220 nm).

Shaking time is another key parameter which effects the sorption efficiency. To understand the effect of shaking time, different time intervals were investigated As seen in figure 3.9 MIP silica reaches highest sorption values at 30.0 min but to guarantee the sorption 60.0 min chosen as shaking time.

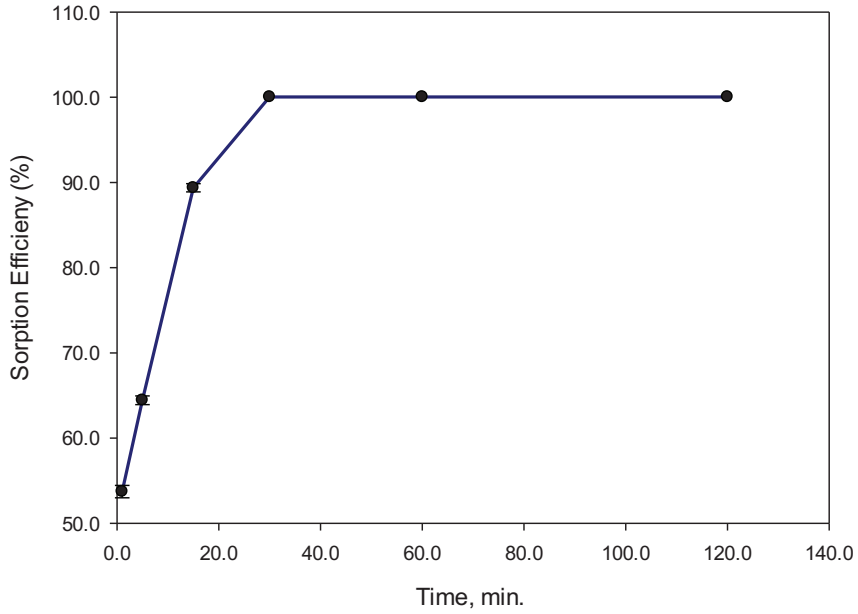


Figure 3.9. The effect of shaking time on the sorption of β-Est ((Agilent 1200 Series HPLC-DAD 250 mm C30 column (YMC C30 (250mm x 4.6 mm)) , 20:80 Water acetic acid (pH 3.0):methanol–mobil phase 0.8 mL/min flow rate, 220 nm).

The chosen of elution solvent is a crucial step in desorption process. Elution solvent which destroys the specific interaction of template molecule and polymer matrix. In this study, two common organic solvents, acetonitrile and methanol and two mixture, M1 (Methanol:Acetic acid mixture (9/1 v/v)) and M2 (Methanol:Water at pH 3.0 with acetic acid 8/2 (v/v)) were used as desorption solvents, respectively. Figure 3.10. shows the recoveries of β-Est with different eluting solvent. As seen in Fig 3.10, Acetonitrile had the least elution ability. On the other hand, the mixtures M1 and M2 gave best recovery results because these solvents include acetic acid which disturb the hydrogen bonding between BPA and MIP polymer. M2 mixture was also used as mobile phase throughout the study Therefore, M2 was used as desorption solvent.

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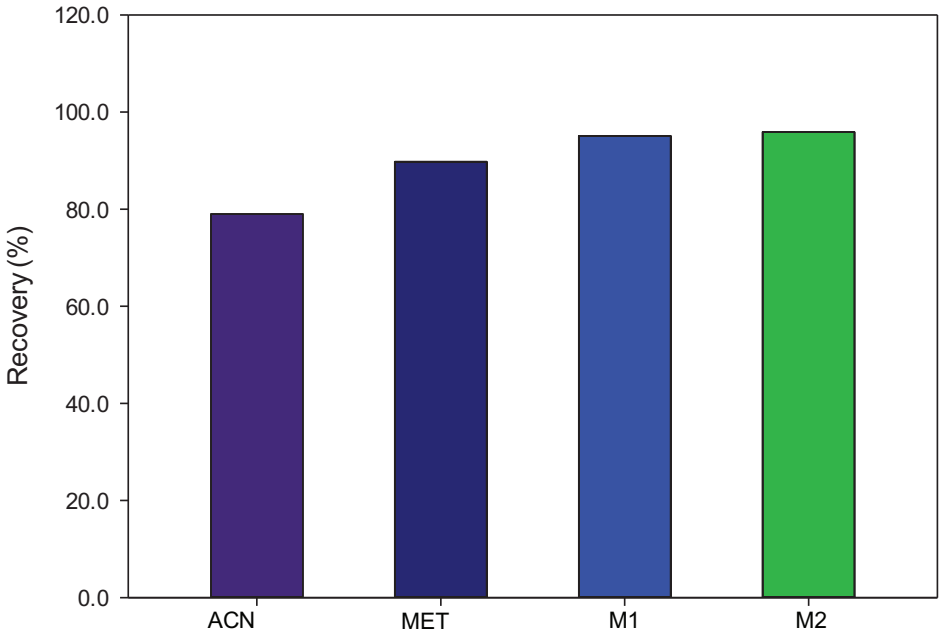

Figure 3.10. Selection of desorption solvent (Agilent 1200 Series HPLC-DAD 250 mm C30 column (YMC C30 (250mm x 4.6 mm)) , 20:80 Water acetic acid (pH 3.0):methanol–mobil phase 0.8 mL/min flow rate, 220 nm).

The reusability of the β-Est imprinted sorbent was also investigated because regeneration is another significant factor for evaluating the performance of (MIP) silica sorbent. As seen in Figure 3.11 the binding efficiency still remained as 85% after seven SPE cycles, and (MIP) silica could be effectively regenerated.

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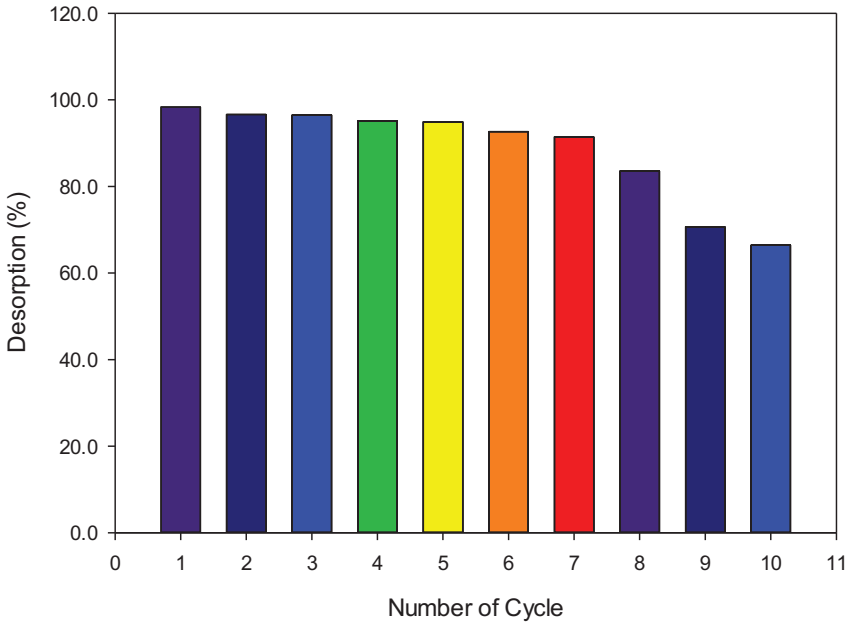


Figure 3.11. Reusability of (MIP)silica sorbent (Agilent 1200 Series HPLC-DAD 250 mm C30 column (YMC C30 (250mm x 4.6 mm)) , 20:80 Water acetic acid (pH 3.0):methanol–mobil phase 0.8 mL/min flow rate, 220 nm).

The sorption performance of prepared the(MIP)silica in another work was compared via commercial LC-C8 and LC-PHE columns. For this purpose, 50.0 mg sorbent SPE was loaded onto empty cartridges and sorption procedures were performed for 3 steroid hormones using SPE manifold. As shown in Figure 3.12, it was understood that in all three sorbents, MIP silica showed high selectivity for steroid hormones

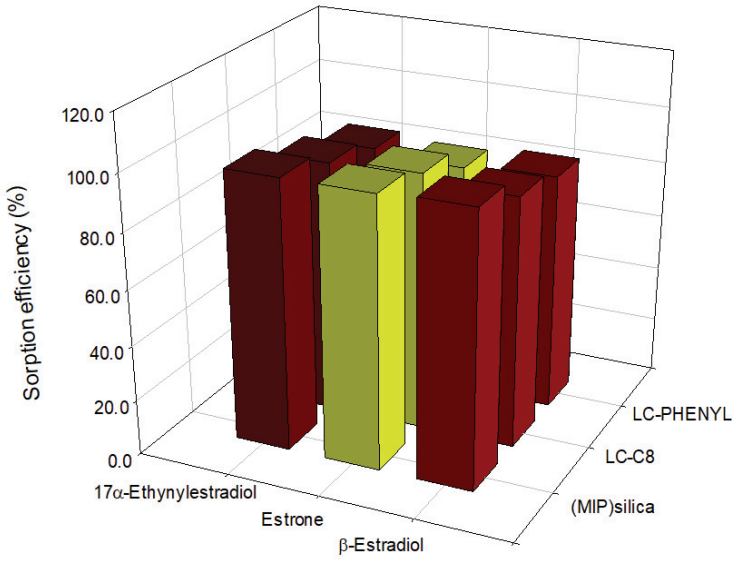


Figure 3.12. The Comparison of the sorption performance of (MIP)silica sorbent with commercial (Agilent 1200 Series HPLC-DAD 250 mm C30 column (YMC C30 (250mm x 4.6 mm)) , 20:80 Water acetic acid (pH 3.0):methanol–mobil phase 0.8 mL/min flow rate, 220 nm).

3.2.5. Method Validation

The applicability of the methodology was also tested by spike tests using drinking water and İYTE tap water. the addition of β-est, estrone and 17α-Ethynylestradiol at concentrations of 0.1 and 1.0 μg / mL to bottled water and fountain water from the IYTE campus was followed by sorption / desorption cycle. The percent sorption and recovery values at two concentration values were given in Table 3.3, 3.4, 3.5, 3.6 indicate that the proposed methodology is practical to use with drinking water and tap water with the percentage recovery and sorption values in the range of 100-95.8% for estrogens.

Table 3.3. Percent sorption of spiked estrogens in bottled and tap water. (Sorption parameters; concentration: 0,1 μg / mL, pH: 7.0, shaking time: 60 min, amount of sorbent: 50.0 mg.

	Sorption %		
	β-est	Estrone	17α-Ethynylestradiol
Bottled water	100,0 (±2,1)	99,0 (±0,8)	97.6 (±0,8)
İYTE Tap water	100,0 (±1,1)	99.8 (±2,7)	98.8 (±2,4)

Table 3.4. Recovery of spiked estrogens in bottled and tap water. (Sorption parameters; concentration: 0,1 μg / mL, pH: 7.0, shaking time: 60 min, amount of sorbent: 50.0 mg.

	Recovery %		
	β-est	Estrone	17α-Ethynylestradiol
Bottled Water	99,8 (±1,1)	98,3 (±0,8)	95.8 (±1,6)
İYTE Tap water	95,0 (±0,8)	97.4 (±1,7)	98.3 (±0,7)

Table 3.5. Percent sorption of spiked estrogens in bottled and tap water . (Sorption parameters; concentration: 1,0 μg / mL, pH: 7.0, shaking time: 60 min, amount of sorbent: 50.0 mg)

	Sorption %		
	β-est	Estrone	17α-Ethynylestradiol
Bottled water	100,0 (±2,1)	99,0 (±0,8)	97.6 (±0,8)
İYTE Tap water	100,0 (±1,1)	99.8 (±2,7)	98.8 (±2,4)

Table 3.6. Recovery of β-est , Estrone ve 17α-Ethynylestradiol.(Sorption parameters; concentration: 1,0 μg / mL, pH: 7.0, shaking time: 60 min, amount of sorbent: 50.0 mg)

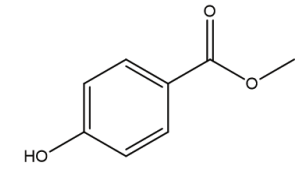
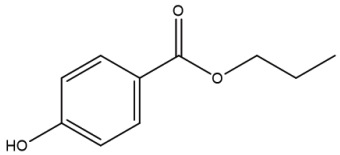
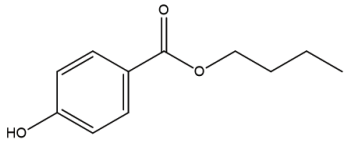
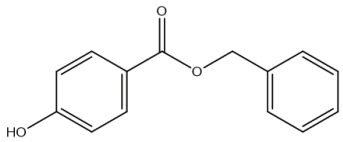
	Recovery %		
	β-est	Estrone	17α-Ethynylestradiol
Bottled water	99,6 (±0,3)	95.4 (±1.2)	97.3 (±1,8)
İYTE Tap water	98,4(±0,5)	96.8 (±2,3)	96.4 (±1.3)

CHAPTER 4

DEVELOPMENT OF MOLECULARLY IMPTRINTED POLYMER BASED SOLID PHASE MICROEXTRACTION (SPME) COATINGS FOR DETERMINATION OF PARABENS

The esters of hydroxybenzoic acid, generally known as parabens are widely used as antimicrobial preservatives in food, cosmetic products and pharmaceuticals because of their broad antimicrobial spectra, chemical stability and low cost. Mostly used paraben species and their K_{ow}, p_{Ka} and aqueous solubility are shown in Table 4.1. Among them, methylparaben and propyl paraben are the most commonly used together in products. (Núñez et al., 2008). Despite widespread application of parabens as a harmless preservative because of low toxicity, several recent studies have revealed that parabens can show endocrine system disrupting effects, a link between parabens and the risk of breast cancer, a relation with male infertility and other side effects. It is known that various companies produce paraben-free products beacuse of these facts. According to European Union, the commercial products should contain maximum concentrations of 0.4% w/w for each paraben and a limit of of 0.8% % (w/w) for total parabens. Therefore, it very significant to develop a method for the determination of parabens which can be emerging pollutants with adverse effects on human, animal and environment.

Table 4.1. Mostly used Parabens, structure, pKa, K_{ow} and water solubilities

Compounds	pKa	Kow	water solubility
 methyl paraben	8.31	1.86	5600
 propyl paraben	8.23	2.88	1200
 butyl paraben	8.22	3.23	600
 benzyl paraben	8.18	3.54	600

4.1. Experimental Procedure

4.1.1. Chemicals

All the chemicals and reagents were prepared from analytical grade. Ultrapure water (18.2 MΩ, Millipore) was used throughout the study. Glassware and plastics were washed firstly with acetone and then detergent and finally ultrapure water.

Methylparaben(MP) (Methyl 4-hydroxybenzoate) (99%), Propyl Paraben (PB) (Propyl 4-hydroxybenzoate) (99%) and Benzyl Paraben (BP) (Benzyl 4-hydroxybenzoate) (99%) were obtained from Sigma-Aldrich 1000.0 mg/ml of stock solutions of Parabens were prepared in methanol as monthly in amber bottles and stored at –20 °C in refrigerator. All studied solutions were prepared daily by appropriate diluting the stock solution. Methanol was HPLC grade (Sigma–Aldrich, St. Louis, MO, USA pH adjustments were done by using 1.0 M, 0.1 M, 0.01 M of HNO_3 and NH_3 solutions. All solutions that were used through the study firstly were filtered from 0.25 μm cellulose

acetate or 0.25 μm polyamide membrane (depending on the solvent system) and degassed for 15.0 min in ultrasonic bath prior to HPLC analysis. For the MIP/NIP nanoparticle synthesis, the functional monomer Methacrylic acid (MAA) and crosslinker Trimethylolpropane trimethacrylate (TRIM) were purchased from Sigma–Aldrich, and the initatior azo(bis)-isobutyronitrile (AIBN) were obtained from AlfaAesar. To prepare electrospinable MIP/NIP-SPME fiber coating, Polystyrene (MW: Sigma Adrich) was used as backbone and support material. Fiber cable were kindly provided by HES cable

4.1.2. Instrumentation and Apparatus

The pH adjustments were performed with Ino Lab Level 1 pH meter (Weilheim, Germany). To obtain effective mixing, IKA yellow line OS 5 basic orbital shaker (Staufen, Germany) was used. The analysis of parabens was performed by using Agilent 1200 Series HPLC system with a C30 (YMC, 250 mm x 4.6 mm) column applying isocratic elution with 80% methanol – 20% ultrapure water (adjusted to pH 3.0 with acetic acid) as mobile phase at a flow rate of 0.8 mL/min. The optimized operation conditions for HPLC was given Table 4.2.

Table 4.2. Operating conditions for HPLC analysis

HPLC	Agilent 1200
Analytical column	C30 (250 mm x 4.6 mm, 5 μm)
Mobile phase	20% UPW (adjusted to pH: 3.0 with acetic acid):80% methanol,
Flow rate	0.8 mL/min
Column temperature	30 ºC
Sample volume	20 μL
Selected λ	280

MIP/NIP encapsulated polystyrene nanofiber based SPME fiber coatings were obtained by using NanoScience electrospinning device. The working conditions were given in Table 4.3.

Table 4.3. Operation conditions for Electrospining

Applied voltage	15.0,25.0, 30 KW
Distance,cm	15.0 cm
Flow rate	1.0 mL/h
Needle diameter	0.8 mm, 1.2 mm, 1.0 mm

4.1.3. Synthesis of MIP/NIP nanoparticles

Firstly, MIP/ NIP nano particles were synthesized by procedure of our previous work prior to preparation of the BP molecularly imprinted polymer encapsulated polystyrene-coated silica fiber.

Firstly, pre-polymer solution was prepared by mixing 1.0 mmol BP (template molecule) and and 4.0 mmol MAA (functional monomer) in 200.0 ml acetonitrile for 1.0 hour at room temperature. And then, 16.0 mmol TRIM (crosslinker) and 2.0 % mole AIVN(inititatior) were added to reaction system under Ar gas. The all reaction system was sealed in order to obtain polymerization at 60 ˚C. After 24 hour polymer solution filtrated and obtained MIP nanoparticles were dried at oven at 60 °C. To remove the template molecule, 80:20) methanol: water (acetic acid pH3.0) methanol and mixture were used respectively until no template molecule. After complete removal of template molecule, nanoparticles were dried at oven at 60 °C. The NIP nanoparticles were prepared following the same procedure except for the addition of benzyl paraben. The synthesis mechanism was given figure 4.1.

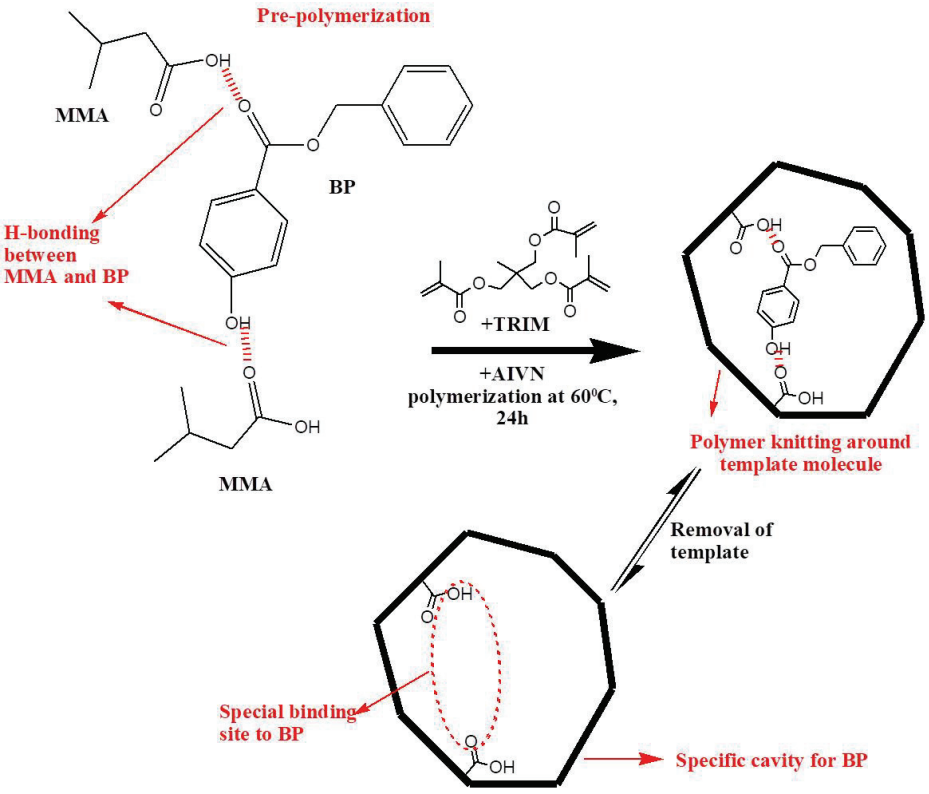


Figure 4.1. The synthesis mechanism for MIP

4.1.4. Synthesis of electrospun SPME fiber coatings

Firstly, the polymer coating of silica fibers was removed by immersing the fibers into acetone for 1.0 hour. After, the surface of bare silica fibers were activated by using 0.1 M NaOH and 0.1 M HCl solution respectively. And then the fiber were silylated for 30.0 min. by exposing them to 10% (v/v) 3-(methacryloxy) propyltrimethoxysilane solution in acetone at room temperature. Finally fibers were washed with methanol and dried prior to coating procedure.

For the electrospinning process, 10 % polystyrene solution in DMF was mixed with MIP and NIP particles by adding 100 % of polystyrene weight. After stirring around 1 hour, a syringe was loaded with prepared solution and electrospinning process was applied at 30 kV potential difference with the flow rate of 1.0 mL/h. 15.0 cm was choosen as the difference between nozel and the metal collector. In order to obtain a homogeneous coating, Silica fiber was inserted into a capillary tube (2.0cm openess for coating) where attached on rotating drum with constant rotating rate of 300.0rpm. The rotating drum was

placed betwen metal collector and nozel The distance between rotating drum and syringe was set as 3 cm. (Figure 4.2) Coated fibers were conditioned at 110 ºC.

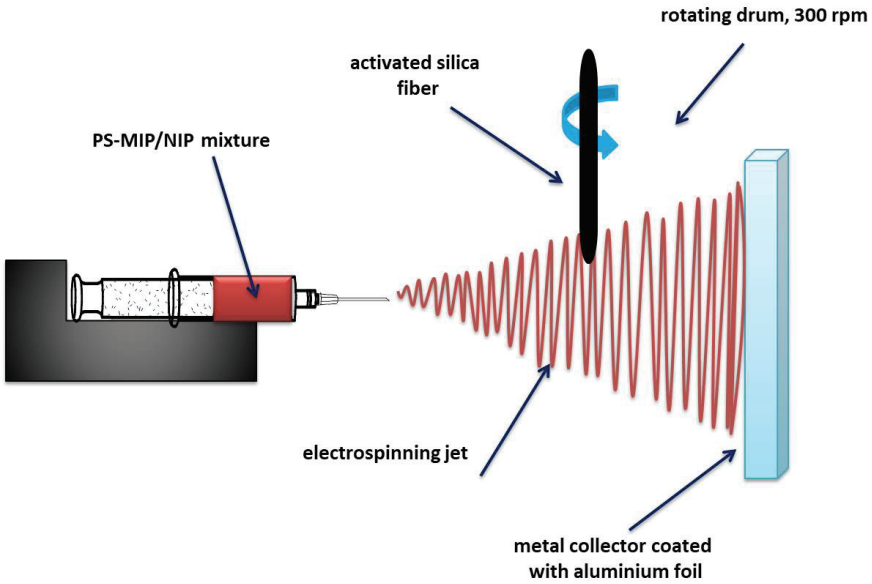


Figure 4.2. Experimental set up for electrospinning process.

4.1.5.. SPME procedure

SPME procedure was performed with MIP and NIP encapsulated polystyrene fibers coated silica fibers for the extraction of MP, PP and BP. After pH adjustment of the solutions, 15.0 mL aqueous mixture containing 10.0 ng/ml parabens were prepared. All extractions were performed at room temperature (25 °C). The extraction time was 60 min and stirring rate was 240 rpm. For desorption, the initial conditions were; desorption time: 60 min, desorption volume: 150.0 µL, desorption temperature: 25 °C, eluent: 80 % methanol – 20 % ultrapure water (adjusted to pH 3.0 with acetic acid). Extraction ability of prepared SPME fibers compared with the polydimethylsiloxane (PDMS) and polydimethylsiloxane–divinylbenzene-carboxen (PDMS-DVB-CAR) fiber which were purchased from Supelco. The extraction ability of the NIP-coated fiber was compared with that of the MIP-coated fiber at the same extraction conditions. Also, selectivity of MIP coated fibers towards the paraben species was examined in the presence of Triclosan (TCS) and Triclocarban (TCC) .The extractions parameters such as extraction pH, extraction time, agitation (stirring) speed, desorption matrix, desorption time, were optimized.

4.1.6. Method Validation

Calibration standard for each paraben was prepared from the range of 2.0 ng/ml to 50.0 ng/ml by applying proper dilution of 1000 mg/ml of stock solution. Besides, the analytical performance of the developed MIP SPME method was tested via determining relative standard deviations of the peak areas for intra-day and inter-day extractions of the paraben species (n=6).

Validity of the proposed method was checked by means of spiked bottled, tap and sea water samples ((10.0 ng/ml in each species).

4.2. Results and Discussion

4.2.1. Characterization of MIP/NIP encapsulated electrospun polystyrene coated SPME fibers

Through the study, precipitation polymerization by using non-covalent strategy was applied which is based on carrying out polymerization under high dilute condition conditions (<5%, w/v monomers/porogen). This technique provides more homogenous binding sites compared to bulk polymerization because it eliminates crushing and sieving steps (Beltran et al. 2010). Also in our previous work (Olcer et al. 2017), it was seen that MIP/NIP particles obtained with different polymerization methods can show difference in morphologies. It was realized in rebinding experiments that the distinction between the sorption capacity of Nano spherical NIP and MIP was more clearly seen compared to monolithic MIP and NIP particles, despite showing higher sorption capacity behavior of both monolithic MIP and NIP particles. In our work, highly diluted conditions (1.25%) was selected to obtain MIP/NIP Nano spherical particles which was easily suspended in polystyrene solution. Also, especially methacrylic acid was chosen as function monomer because of showing the high selectivity to paraben species comparing with 4-VP (Nú˜nez et al.2010). The SEM image of synthesized MIP nanoparticles was given Figure 4.3.

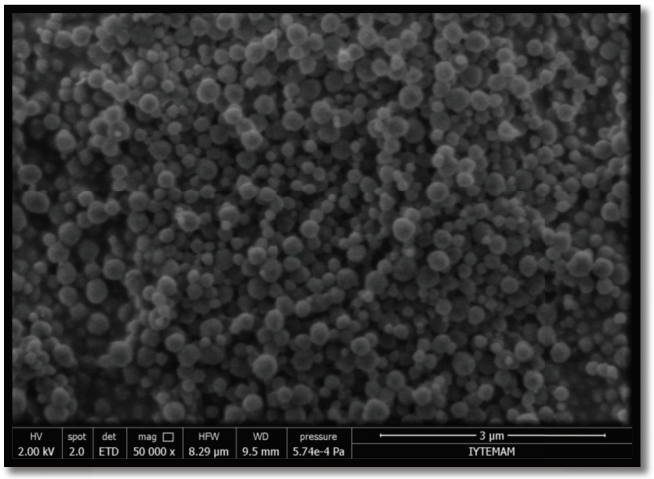


Figure 4.3. SEM image of MIP nanospherical particles.

After electrospinning process, figure 4.4. showed SEM images of MIP nanoparticles encapsulated electrospun polystyrene fibers at various magnifications which were deposited on metallic plate. It is seen that polymer nanoparticles retained on the surface and inside of each polystyrene nanofiber after many mechanical treatments such as crushing, dipping in nitrogen, washing with methanol. Electrospun fiber mats are good candidates for the entrapment of the spherical particles due to having micrometres sized interfibrillar spacing. The presence of porosity of individual fibers may further increase the efficiency of entrapment. Electrospinning of polystyrene from various polar solvents is a well-recognized system for the development of both interior and surface porosity of the individual filament that absolutely increases the surface area to volume ratio of the system. Humidity of the spinning environment was found to be the dominant parameter for the fabrication of porous electrospun PS mats. DMF, solvent in our system, undergoes a liquid-liquid phase separation. PS is a hydrophobic polymer and can be dissolved in DMF; however, water is a nonsolvent. It prefers to be stay in DMF rather than being in water. Since water has higher vapour pressure than DMF, it evaporates earlier. The evaporation of water leaves behind surface and interior porosity. Therefore, MIP and NIP submicron spheres were encapsulated in polystyrene by electrospinning process.

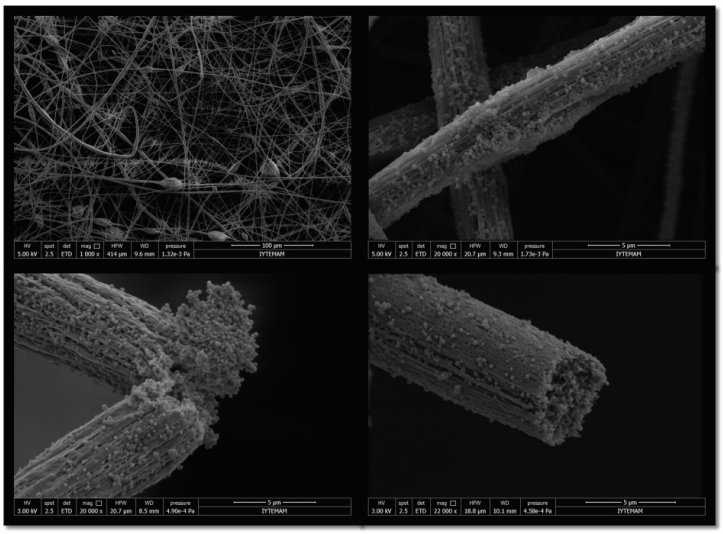


Figure 4.4. SEM images of obtained MIP capsulated polystyrene fibers

The silica fiber should be coated for several times to obtain the wanted thickness. The morphology, homogeneity and thickness of the SPME fiber coating were examined by SEM after coating silica fibers ten times and twenty times, as in given Figure 4.5. Figure d shows the morphology of after 20 times coated SPME fiber under higher magnification value. It can be realized that the polystyrene SPME fiber coating have MIP nanoparticles inside and the surface of each polystyrene fiber. These crazy nanoparticles have high recognition ability to parabens

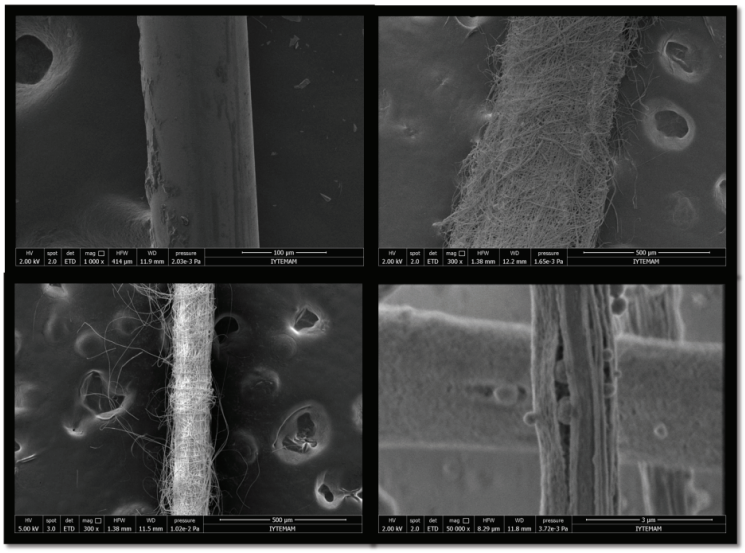


Figure 4.5. SEM images of the (a) bare silica fiber (b) MIP coated fiber ten times (c) MIP coated fiber twenty times with (d)higher magnification

4.2.2. Extraction Ability

The extraction ability of MIP encapsulated polystyrene fiber was examined by comparing with the NIP encapsulated polystyrene fiber which was prepared in same way except for addition on benzyl paraben. Figure reveals the extracted amount of benzyl paraben on both SPME fiber coatings at different initial concentration values. It is clearly seen that the increase in initial concentration of benzyl paraben concluded with increase in extraction amounts for both fiber coating. However, this increase in extracted amount on MIP encapsulated polystyrene coated fiber was higher than corresponding NIP encapsulated polystyrene coated fiber (Figure 4.6). The reason of this should be having specific cavities of the MIP surface. MAA (functional monomer) and TRIM, crosslinking agent in the both MIP-NIP nanoparticles can provide non-specific interaction between template molecule. Moreover, MIP nanoparticles with specific cavities which belongs to template molecule can possess specific interaction (H bonding).

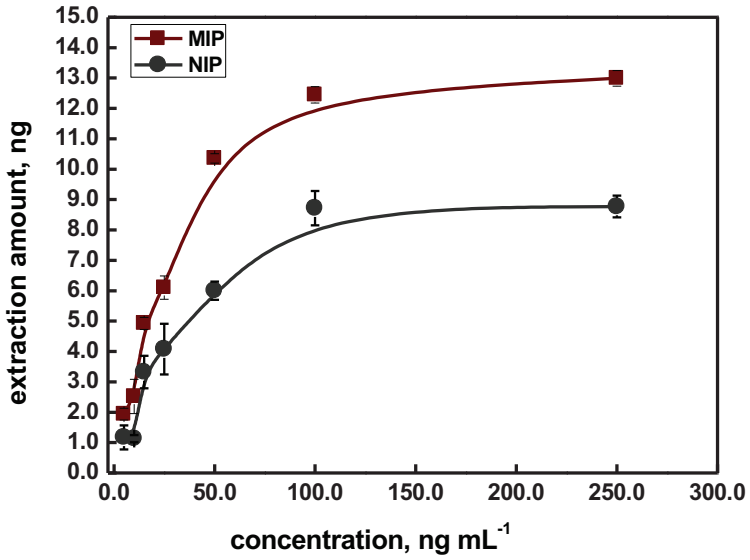


Figure 4.6. Rebinding curves of BP with MIP and NIP coated fiber in spiked UPW. (Extraction conditions: extraction time: 60 min, stirring speed: 400 rpm, solution volume: 15.0 mL. Desorption conditions were; desorption time: 30 min, desorption volume: 150 µL)

The extraction ability of MIP encapsulated polystyrene-coated fiber was compared with commercially available SPME fiber including PDMS, PDMS/DVB/CAR fiber. Figure 4.7 shows the extracted amount of BP by the MIP fiber and two other

commercial SPME fibers at initial concentraton of 10.0 ng/ml. The results show that MIP fiber has higher extraction ability because of specific hydrogen bonding interactions between the carboxylic groups in the MIP coating and hydroxyl moiety in BP.

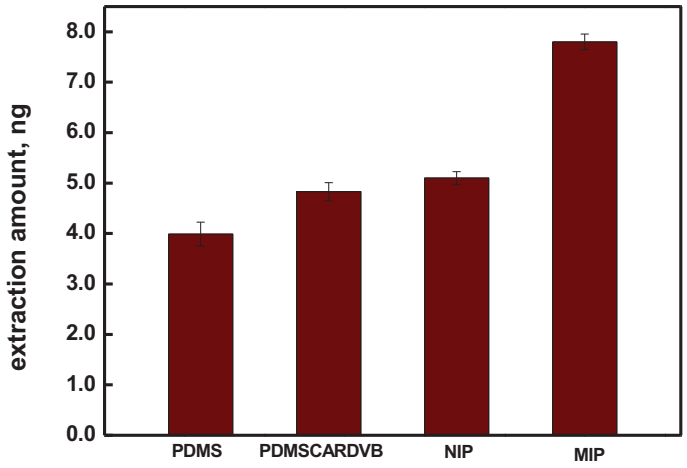


Figure 4.7. The extracted amount of BP on the prepared MIP-coated fiber and on the commercial PDMS, PDMS/CAR/DVB. (Extraction conditions: 10.0 ng/mL BP, at pH 7.0, extraction time: 60 min, stirring speed: 400 rpm, solution volume: 15.0 mL. Desorption conditions were; desorption time: 30 min, desorption volume: 150 μL)

4.2.3. Cross Selectivity

The extraction ability of MIP and NIP encapsulated polystyrene fiber was investigate in the presence of methyl paraben, propyl paraben, triclosan and triclocarban. As seen in figure all MIP fiber showed high selectivity to all paraben species whereas other structurally related compounds TCS and TCC poorly extracted. Moreover, these results exhibit that prepared MIP nanoparticles have recognition ability to common structure of parabens. Comparing with other examined paraben species, BP showed the largest extraction (Figure 4.8)

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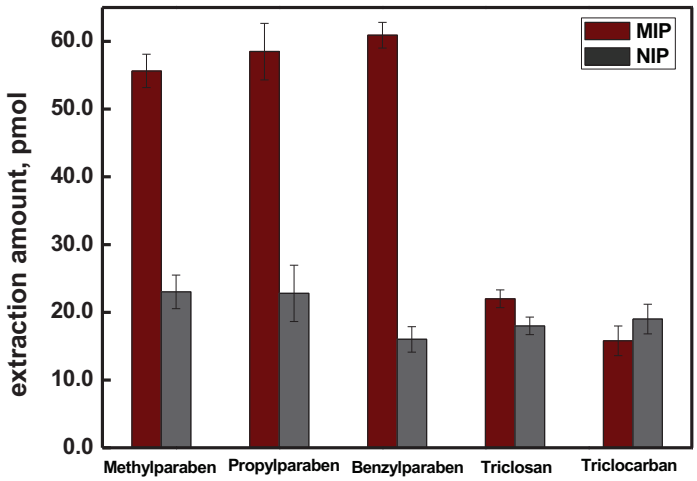

Figure 4.8. Selectivity investigation of the MIP-coated fiber. (Extraction conditions: 10.0 ng/mL BP,MP,PP,TCS and TCC at pH 7.0, extraction time: 60 min, stirring speed: 400 rpm, solution volume: 15.0 mL. Desorption conditions were; desorption time: 30 min, desorption volume: 150 µL)

4.2.4. SPME optimization parameters

One of the most important parameter is pH of the solution. To understand the effect of pH on extraction of BP by the prepared fibers acidic neutral and basic aqueous solutions of BP were prepared. Extraction studies were performed at ambient conditions and the initial pH of solutions was adjusted to 3.0, 7.0 and 10.0. As seen in Figure 4.9 good extraction results obtained at neutral and basic pH values. The dissociation constant of benzyl paraben is 8.37. At acidic pH values, benzyl paraben is its neutral form while TRIM that used for crosslinker has for O atoms so they can establish a H bonding with paraben and therefore there were no difference in extraction of BP on MIP and NIP coatings. On the other hand, under basic conditions parabens dissociate and become ionic and more easily extracted on MIP coating fiber because of the cavities and specific interactions.

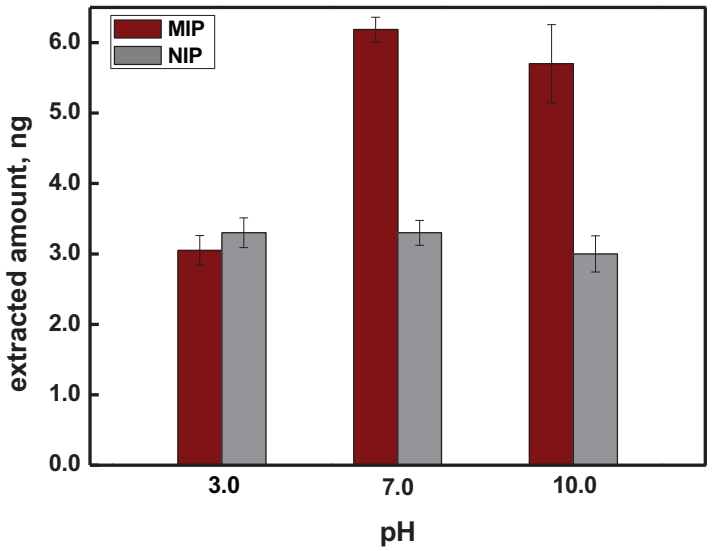

Figure 4.9 .The effect of solution pH on the extraction of BP. (Extraction conditions:, 10.0 ng/mL BP, extraction time: 60 min, stirring speed: 400 rpm, solution volume: 15.0 mL. Desorption conditions were; desorption time: 30 min, desorption volume: 150 µL)

Since the adsorption and desorption kinetics are very significant parameters in SPME (equilibrium method), various extraction times and desorption time were examined for prepared MIP fibers at pH 7.0. Extraction and desorption times of 5, 15, 30, 60, 90, 120 min were tried it is clearly observed that the extraction and desorption reached equilibrium at about 30 min. (Figure 4.10). Not short times were obtained in extraction and desorption of BP. The reason of this could be thickness of the diffusion speed of our analyte into or out of the coating

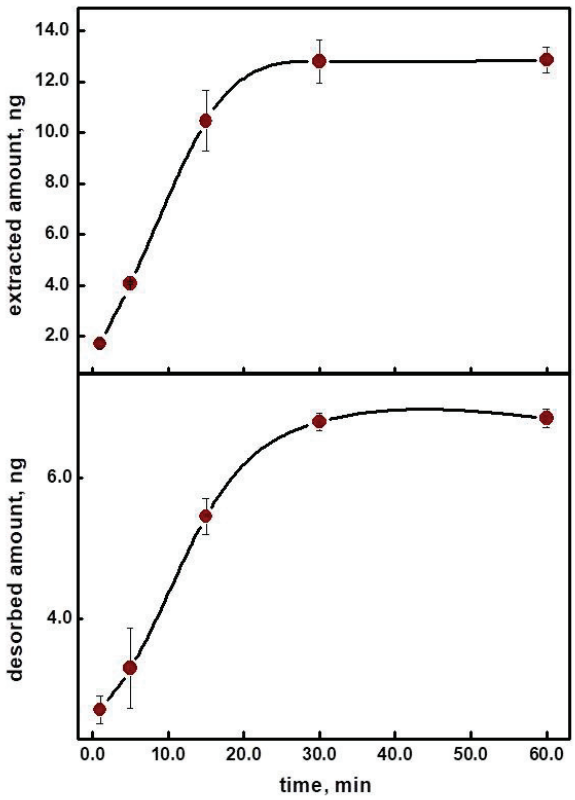

Figure 4.10. The effect of extraction and desorption time on extraction of BP. (Extraction conditions: at pH 7.0, 10.0 ng/mL BP stirring speed: 400 rpm, solution volume: 15.0 mL. Desorption conditions were, desorption volume: 150 µL)

The chosen of desorption solvent is very necessary which should effectively destroy the hydrogen bonding between template molecule and active binding sites of MIP nanoparticles. Therefore, acetonitrile, methanol-acetic acid (9/1) and methanol were selected as desorption solvents. As seen in Figure 4.11, Methanol and acetic acid mixture exibited highest desoption ability which could make hydrogen bonding interaction with the active sites of MIP nanoparticles and finally methanol-acetic acid (9/1) mixture was chosen as desorption solvent that had been used as washing solvent to get rid of the template molecule, benzyl paraben from MIP nanoparticles.

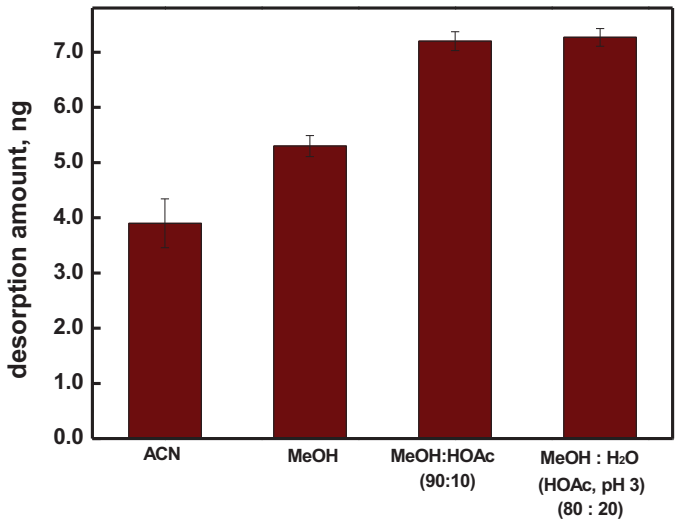

Figure 4.11. The effect of desorption matrix on BP extraction with MIP prepared SPME fiber. (Extraction conditions: at pH 7.0, 10.0 ng/mL BP, extraction time: 60 min, stirring speed: 400 rpm, solution volume: 15.0 mL. Desorption conditions were; desorption time: 30 min, desorption volume: 150 µL)

4.2.5. Method Validation and Sample Analysis

The prepared MIP coated fiber were used to develope a method for determination of parabens by HPLC and UV detection under optimized conditions. The lineraty, detection limits and precion of parabens was examined in order to validate the analytical methodology MIP-SPME-HPLC method linear ranges of 2.0-50.0 ng/mL (Figure 4.12) . The detection limits (LOD), was calculated as three times standard deviations of calibration curves divided by their slopes. The LODs obtained for the parabens were in the range 0.26 -0.29 ng/ml. These results showed that the good performance of coated fiber. Also precison of method was examined by relative standard deviations of the peak areas for intra-day and inter-day extractions of the solution containing 10 ng/ml each compound . The relative standard deviations for the compounds were below 10% for both inter- and intra-day precision study (Table 4.4).

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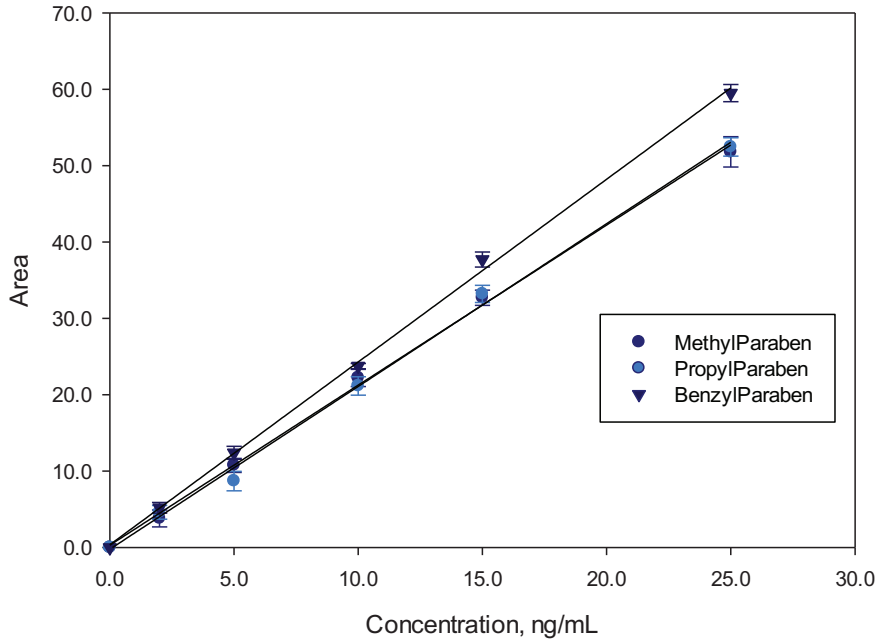

Figure 4.12. Calibration curves for All paraben species

Table 4.4. The linearity, precision and detection limit of developed method

Compounds	Calibration curve				RSD (n=6)	
	Slope	Intercept	Linear range (ng mL^{-1})	LOD (ng mL^{-1})	Intra-day	Inter-day
Methylparaben	2.374	0.580	2.0-50.0	0.29	5.4	3.2
Propylparaben	2.134	0.370	2.0-50.0	0.36	4.8	4.2
Benzylparaben	2.088	0.470	2.0-50.0	0.26	4.2	4.6

The developed MIP-SPME method was applied to analysis of 10.0 μgL^{-1} paraben spiked tap, bottled and sea water samples. The results showed that parabens could not be detected in non-spiked samples but recoveries from spiked samples were above 90% for all paraben species (Table 4.5). The proposed method could be used for selective determination of paraben species.

Table 4.5. Method validation with SPME fibers (Extraction conditions; initial concentration of 10 ng/mL extraction pH: 7.0, extraction time: 60 min, stirring speed: 400 rpm, solution volume: 15 mL, Desorption conditions; desorption time: 30 min, desorption volume: 150 µL, eluent: 90 % MeOH – 10 % H_2O (adjusted to pH 3.0 with acetic acid

Compounds	**Bottled water (ng mL^{-1})**	**Spiked Bottled water (ng mL^{-1})**	**Tap water (ng mL^{-1})**	**Spiked Tap water (ng mL^{-1})**	**Sea water (ng mL^{-1})**	**Spiked Sea water (ng mL^{-1})**
Methylparaben	ND	94.8 ± 1.2	ND	92.2 ± 0.8	ND	99.7±0.4
Propylparaben	ND	96.8 ± 0.9	ND	95.8± 1.9	ND	98.4±0.2
Benzylparaben	ND	95 ±2.3	ND	97.4 ± 1.6	ND	99.8±0.1

CHAPTER 5

AMINE-MODIFIED SPME FIBER COATING FOR DETERMINATION OF BISPHENOL-A PRIOR TO HPLC DAD

5.1. Experimental

5.1.1. Chemicals and Reagents

All the chemicals and reagents were prepared from analytical grade. Ultrapure water (18.2 MΩ, Millipore) was used throughout the study. Glassware and plastics were washed firstly with acetone and then detergent and finally ultrapure water.

All chemicals and reagents were analytical grade. Endocrine disrupting compounds, bisphenol-A (BPA, ≥99%) was obtained Sigma Aldrich, while β-estradiol (β-EST) 99% (dry wt.), ca 3% water) and triclosan (TCS, 99%) were purchased from Alfa Aesar. .1000.0 mg/ml of stock solutions of endocrine disrupting compounds were prepared in methanol as monthly in amber bottles and stored at –20 °C in refrigerator. All studied solutions were prepared daily by appropriate diluting the stock solution. Doubly distilled ultra-pure water was used in all experiments. All solutions that were used through the study firstly were filtered from 0.25 μm cellulose acetate filter paper or 0.25 μm polyamide filter paper (depend on the solvent system) and degassed for 15.0 min in ultrasonic bath prior to HPLC analysis. pH of the solutions were adjusted by using NH_3 and HNO_3 at different concentrations. Methanol was HPLC grade (Sigma–Aldrich, St. Louis, MO, USA pH adjustments were done by using 1.0 M, 0.1 M, 0.01 M of HCl and NaOH solutions. All solutions that were used through the study firstly were filtered from 0.25 μm cellulose acetate filter paper or 0.25 μm polyamide filter paper (depend on the solvent system) and degassed for 150.0 min in ultrasonic bath prior to HPLC analysis. For the. Fiber cable were kindly provided by HES cable

5.1.2. Instrumentation

The pH adjustments were performed with Ino Lab Level 1 pH meter (Weilheim, Germany). To obtain effective mixing, IKA yellow line OS 5 basic orbital shaker (Staufen, Germany) was used. The analysis of parabens was performed by using Agilent 1200 Series HPLC system with a C30 (YMC, 250 mm x 4.6 mm) column applying isocratic elution with 80% methanol – 20% ultrapure water (adjusted to pH 3.0 with acetic acid) as mobile phase at a flow rate of 0.8 mL/min (Table 5.1). The optimized operation conditions for HPLC was given Table 2.2. The surface morphology and particle size of synthesized materials was realized with Quanta 250FEG Scanning Electron Microscope (SEM)

Table 5.1. Operatingconditions for HPLC analysis

HPLC	Agilent 1200
Analytical column	C30 (250 mm x 4.6 mm, 5 μm)
Mobile phase	80% methanol, 20% UPW (adjusted to pH: 3.0 with acetic acid)
Flow rate	0.8 mL/min
Column temperature	30 ºC
Sample volume	20 μL
Selected wavelength	220 nm

5.1.3. Preparation of sol-gel based electrospun amine-modified SPME fiber Coating

In a typical procedure 2.0 mL PDMS (hydroxyl terminated, typical viscosity: 25.000 cSt), 3.0 mL tetrahydrofuran (THF), 1.0 mL APTES and 0.50 mL PMHS was mixed in a 20 mL vial. Into the resulting solution 1.0 mL of TFA (95v TFA+5v water) was added and mixed thoroughly. The resulting sol-gel solution was stirred for 48 h at room temperature. To achieve viscosity adequate for electrospinning process, some of the solvent (THF) was evaporated from the sol-gel solution at 80 ºC for 2 h. Resulting sol-gel solution was loaded into a syringe and electrospinning process was performed at 25 kV potential difference with the feeding rate of 5.0 mL/h. The distance between the syringe and the metal collector was 10 cm. In order to obtain a homogeneous coating on the fiber, the plain silica fiber was attached on rotating drum and constant rotation rate of 300 rpm was applied during coating. The drum was placed between the metal collector and syringe. The distance of rotating drum from syringe was 3 cm. Coated fibers were conditioned at 110 ºC overnight to facilitate the end capping of free silanol groups.

5.1.4. SPME optimization parameters

The first investigated parameter was solution pH. Extraction studies were carried out at 25 °C after the initial pH of solutions was adjusted to 3.0, 7.0 and 10.0 with dilute HNO_3 or NH_3. The conditions for the extractions were; BPA concentration: 25.0 µg/L from, stirring speed: 240 rpm, extraction time: 60 min, solution volume: 15.0 mL. Afterwards, desorption of the extracted analytes was performed. Desorption conditions were; desorption time: 30.0 min, 150 µL (8/2V) Methanol/water (pH 3.0 acetic acid). Desorption solution was analyzed for its arsenic content and species by HPLC-DAD.

Effect of agitation time on the extraction of BPA by amine-modified fiber was investigated for time intervals of 1, 5, 15, 30 60 and 120 min. The conditions for the extractions were; solution pH: 7.0, BPA concentration: 25.0 µg/L stirring speed: 240 rpm, solution volume: 15.0 mL. Separately, effect of the agitation speed on extraction of the BPA was studied at 160, 240, 320 and 400 rpm stirring speeds. Desorption conditions for both experiments were; desorption time: 20 min, 150 µL (8/2V) Methanol/water (pH 3.0 acetic acid).

Effect of the ionic strength on the extraction of arsenic species was investigated by addition of various amount of NaCl into arsenic containing solution. The investigated concentrations were 1.0 M, 0.10 M, 0.010 M and without addition of NaCl. Extraction conditions for the mentioned experiment were; solution pH: 7.0, BPA concentration: 25.0 μg/L extraction time: 30 min, stirring speed: 400 rpm, solution volume: 15.0 mL. Desorption conditions were; desorption time: 30 min, 150 μL (8/2V) Methanol/water (pH 3.0 acetic acid).

The one of the most important task in the SPME fiber production is preparation of stable fiber coatings. The repetitive use of the same fiber is key study for confirmation the stability of the active phase. Extraction conditions were; extraction time: 30 min, BPA concentration: 25.0 μgL^{-1}, solution pH: 7.0, stirring speed: 400 rpm, solution volume: 15 mL. Desorption conditions were; desorption time: 30 min, 150 μL (8/2V) Methanol/water (pH 3.0 asetic acid). To prevent the carry-over of the remaining analytes on the fibers to the next running, a cleaning step was applied before reuse of the fiber. The cleaning conditions were; 10 min desorption into 15 mL (8/2V) Methanol/water (pH 3.0 asetic acid), 5 min cleaning in 15 mL ultra-pure water, 2 min activation in 15 mL 0.10 M HNO_3 and 5 min conditioning at 110 ºC. The single fiber was reused for ten successful microextractions.

Interference studies were performed for TCS and β-estradiol. Each compound was examined by addition into the solution containing 25.0 μgL^{-1} bisphenol A (BPA). The added concentration for TCS and β-estradiol was 25.0 μgL^{-1}. The experiments were conducted at the optimum conditions for pH 7.0. stirring speed: 400 rpm, solution volume: 10 mL. Desorption conditions were; desorption time: 30 min, 150 μL (8/2V) Methanol/water (pH 3.0 acetic acid)

5.1.5. Method Validation

The performance of the proposed analytical method was characterized in terms of the limit of detection (LOD_{3s}) and the limit of quantification (LOQ_{10s}). Also, the regression coefficients in the calibration plot were used to prove the correlation between the extracted amount of analytes and concentrations in a typical SPME experiment. Calibration plots were constructed to relate the variation of arsenic concentration as a function of the peak area. For this purpose various amounts of BPA spiked in ultra-pure water (1.0 μg/L to 50.0 μg/L) and extraction/desorption to HPLC-DAD were performed

under optimized experimental conditions Extraction conditions were; solution pH: 7.0, extraction time: 30 min, stirring speed: 400 rpm, solution volume: 15.0 mL. Desorption conditions; desorption time: 30 min, 150 μL Methanol/water (pH 3.0 acetic acid). Validity of the developed method was tested by spike addition of BPA into bottled water and tap water. In all validation experiments extraction conditions were; extraction time: 60 min, solution pH: 7.0, stirring speed 400 rpm, solution volume: 15.0 mL. Desorption conditions; desorption time: 30 min, 150 μL Methanol/water (pH 3.0 acetic acid). Both standard addition method and aqueous calibration plots were applied to determine the species and concentration of BPA in the samples.

5.2. Results and Discussion

5.2.1. Characteriation of sol-gel based electrospun amine-modified SPME fiber Coating

. To obtain a solution appropriate for electrospinning the preparation of sol-gel solution was modified. For this purpose 2.0 mL PDMS, 3.0 mL THF, 2.0 mL APTES were mixed in a 20 mL glass vial by continuous stirring. Sol-gel process was initiated by addition of 1.0 mL TFA solution containing 5% by volume H_2O. The reaction was continued for 48 h at room temperature (RT) followed by evaporation of THF until appropriate viscosity for electrospinning was achieved. To obtain homogenous coating, coating procedure was applied ten times. When more coating was done, homogeneity appeared to disappear. Scanning electron microscopic images of the bare silica fiber and agarose coated fibers can be seen in Figure 5.1. The diameters of bare silica fiber and coated silica fiber were determined 125 μm and 135 μm, respectively. It has been observed that the coating is homogeneous.

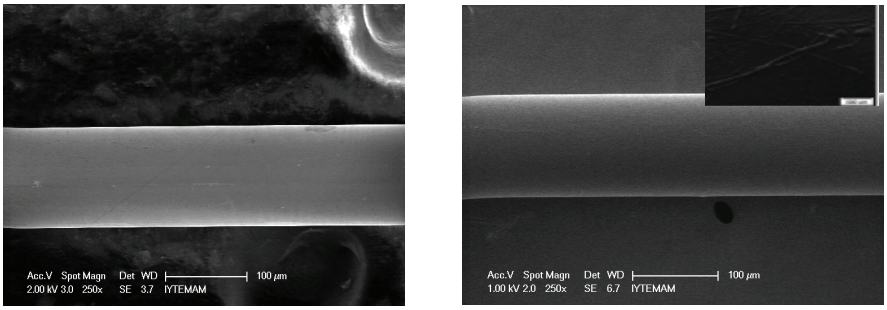

Figure 5.1. SEM images of (a) bare silica fiber (b) amine-modified SPME fiber

Moreover, EDX results of the fibers (Figure 5.2) were used for further identification of the fiber coatings. Carbon peak observed in EDX spectrum of amine-modified SPME fiber and the disappearance of Si peak on the surface were signs of successful coating of amine modified SPME fiber

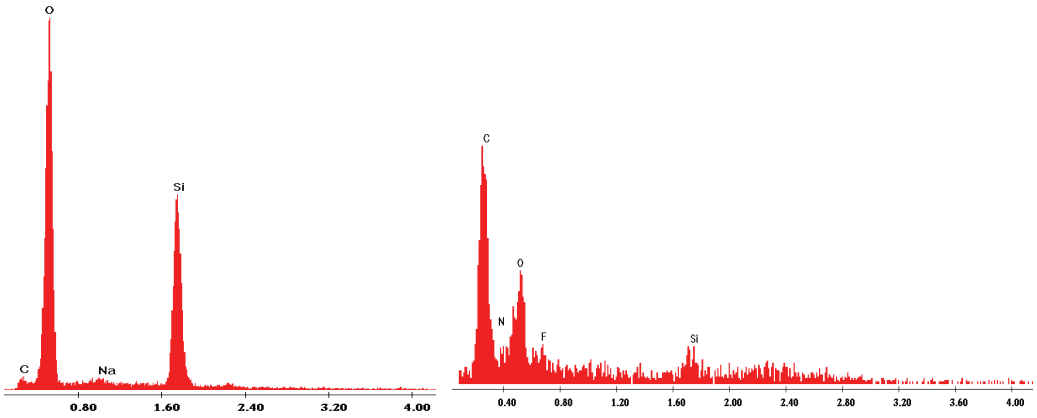

Figure 5.2. EDX spectra of (a) bare silica fiber and (b) amine modified SPME fiber

Especially, the reason of chosen of electrospinning process to obtain homogenous fiber coating, electrospining process produces more valuable fiber coatings even under same coating thicknesses obtained with different coating methods Amine functionality in the polymeric sol-gel solution is positively charged and it can be speculated that these functional groups most likely are oriented under influence of the electric field. Thus, amine groups on the electrospin coated fibers are self-oriented and more available than the functional groups randomly distributed though the matrix where no electrical field is applied. Proposed coating system for electrospinning is given figure 4.2 in previous chapter.

5.2.2. SPME Optimization Parameters

The optimization study was started with the determination of pH of the solution where the maximum extraction of BPA was obtained. As expected from the results have been obtained in SPE part of the study BPA was retained by amine-modified SPME fibers. When the pH of sample solution was 3.0, the lowest sorption efficieny was observed (Figure 5.4). This can be explained the protonation of hydroxyl groups of BPA can increase its solubility in water so the hydrogen bonding between the amine and phenolic hydroxyl groups was interfered and weakened at acidic medium. On the other hand, the reduction in sorption efficiency of BPA at pH 10.0 can be explained due to the deprotonation of BPA at high pH value. If all results were taken into consideration, the pH of solution was adjusted 7.0 for further experiments.

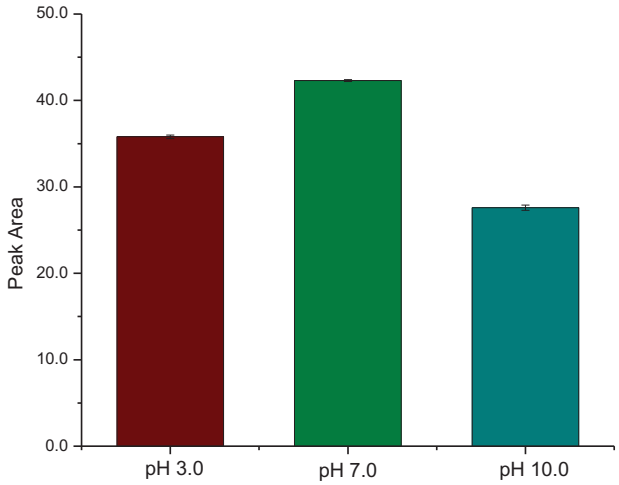

Figure 5.3. Effect of solution pH on extraction of BPA (Extraction conditions: 25.0 ng/mL BPA, extraction time: 30 min, stirring speed: 400 rpm, solution volume: 15.0 mL. Desorption conditions were; desorption time: 30 min, desorption volume: 150 µL)

Since the sorption time is very important in SPME (equilibrium method), various extraction times were investigated for prepared fiber at pH 7.0. Extraction times of 1, 5, 15, 30, 60, 90, 120 min were tried. it is clearly observed that in 60 min equilibrium is reached (Figure 5.5)

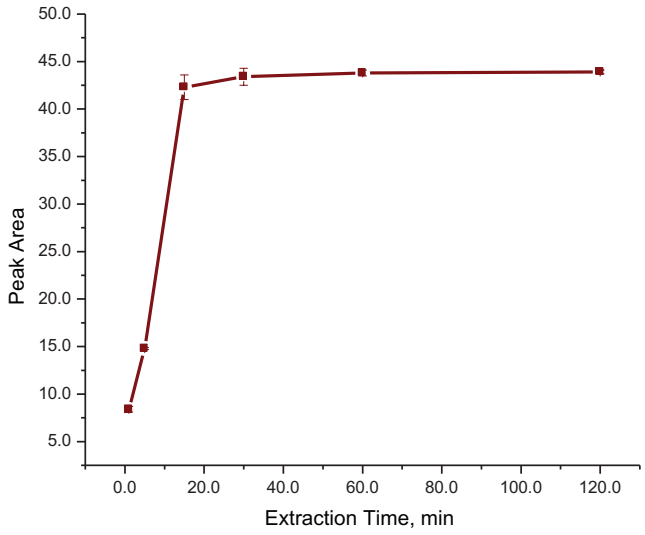

Figure 5.4. Effect of agitation time on extraction of BPA. Extraction conditions: 25.0 ng/mL BPA, extraction time: 30 min, stirring speed: 400 rpm, solution volume: 15.0 mL. Desorption conditions were; desorption time: 30 min, desorption volume: 150 µL)

The effect of stirring speed of the solution during the extraction of BPA was also investigated. The obtained results are given in Figures 5.6. Maximum BPA extraction was achieved at 400 rpm

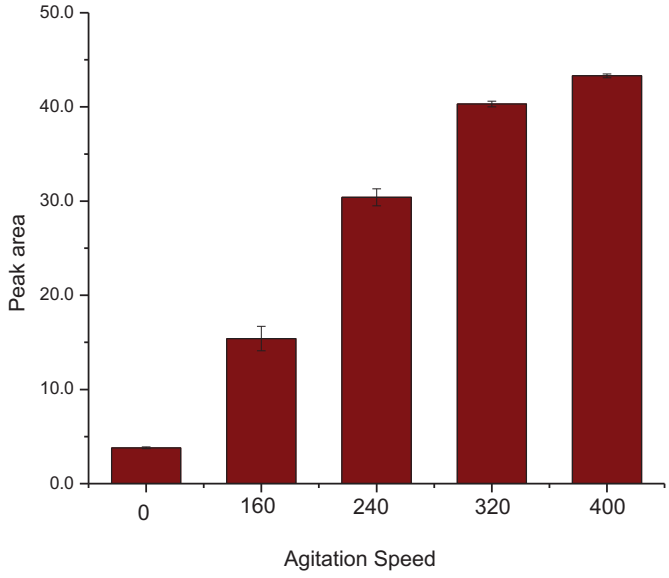

Figure 5.5. Effect of agitation speed on extraction of BPA. Extraction conditions: 25.0 ng/mL BPA, extraction time: 30 min, stirring speed: 400 rpm, solution volume: 15.0 mL. Desorption conditions were; desorption time: 30 min, desorption volume: 150 µL)

The effect of NaCl concentration on extraction of BPA was studied in 1.0 M, 0.10 M, and 0.010 M NaCl solutions. The result was given in Figures 5.7 indicate that increasing the ionic strength of the solution results in decrease in the extracted amount of BPA. It can be speculated that the decrease in the amount of the extracted BPA is related to competitive sorption of Cl^- ions by protonated amine groups.

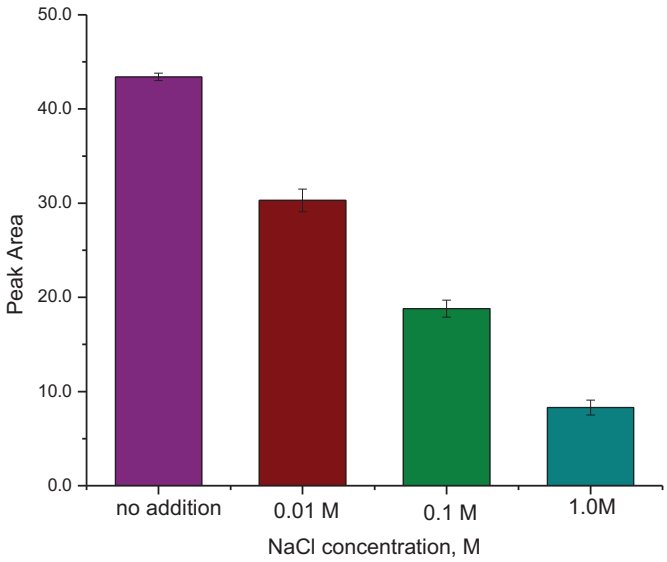

Figure 5.6. Effect of ionic strenth on extraction of BPA. Extraction conditions: 25.0 ng/mL BPA, extraction time: 30 min, stirring speed: 400 rpm, solution volume: 15.0 mL. Desorption conditions were; desorption time: 30 min, desorption volume: 150 µL)

Effect of desorption time was studied for 5, 15, 30, 45, 60 min. Figure 5.8 indicates clearly that, 30 min was chosen as the optimum desorption time among 15, 45 and 60 min

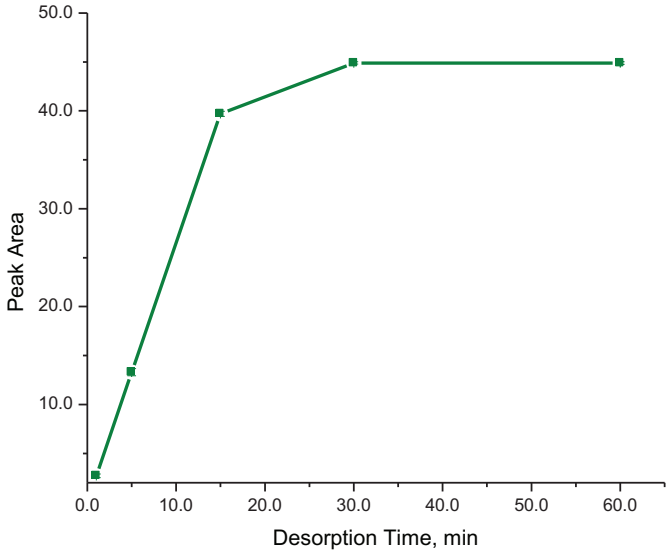

Figure 5.7. The effect of desorption time on BPA extraction Extraction conditions: 25.0 ng/mL BPA, extraction time: 30 min, stirring speed: 400 rpm, solution volume: 15.0 mL. Desorption conditions were; desorption time: 30 min, desorption volume: 150 µL

The chosen of desorption solvent is very necessary which should effectively destroy the hydrogen bonding between template molecule and active binding sites of MIP nanoparticles. Therefore, acetonitrile, methanol-acetic acid (9/1) methanol and methanol-water acetic acid (8/2) mixture were selected as desorption solvents. As seen in Figure 5.9, Methanol and acetic acid mixture exibited highest desoption ability which could make hydrogen bonding interaction with the active sites of fiber and finally methanol-water acetic acid (8/2) mixture was chosen as desorption solvent that had been used mobile phase

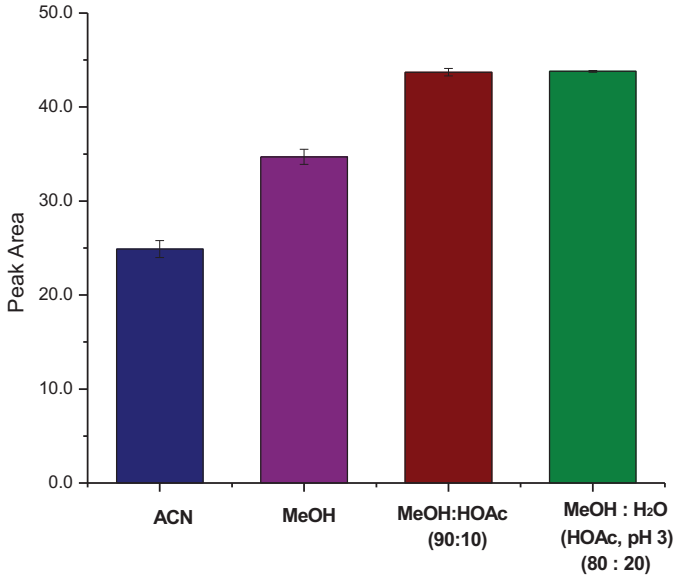

Figure 5.8. The effect of desorption solvent on BPA extraction Extraction conditions: 25.0 ng/mL BPA, extraction time: 30 min, stirring speed: 400 rpm, solution volume: 15.0 mL. Desorption conditions were; desorption time: 30 min, desorption volume: 150 µL)

Interference studies were performed for TCS and β-estradiol. Each compound was examined by addition into the solution containing 25.0 μgL^{-1} bisphenol A (BPA). The added concentration for TCS and β-estradiol was 25.0 μgL^{-1}. The experiments were conducted at the optimum conditions for pH 7.0. Figure 5.10 shows that both TCS and β-estradiol interfere with BPA

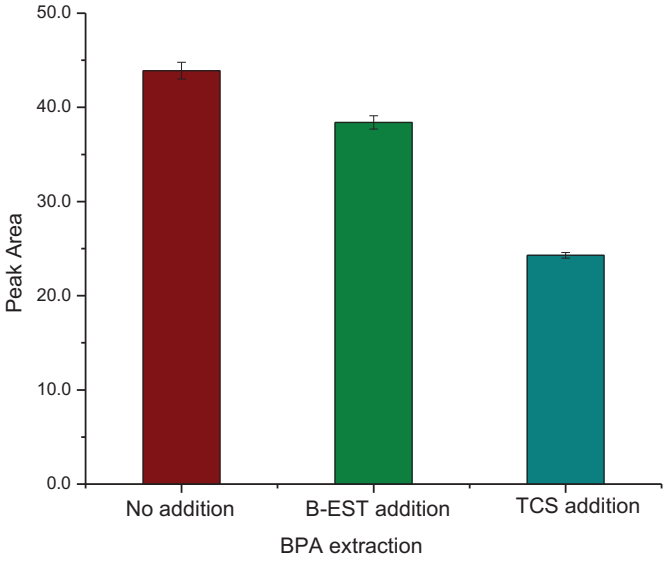

Figure 5.9. The interference study Extraction conditions: 25.0 ng/mL BPA, extraction time: 30 min, stirring speed: 400 rpm, solution volume: 15.0 mL. Desorption conditions were; desorption time: 30 min, desorption volume: 150 µL)

The one of the most important task in the SPME fiber production is to prepare stable fiber coatings. Results obtained from repetitive use of the same fiber are illustrated in Figure 5.11. the extracted BPA was not varied from one extraction to another there were significant amount of the extracted BPA until six SPME cycle. These results show potential for preparation of stable fiber coatings. On the other hand, the fiber to fiber reproducibility study which is shown in Figure 5.12 SPME method is not mainly suffers from the reproducibility in preparation of the fibers.

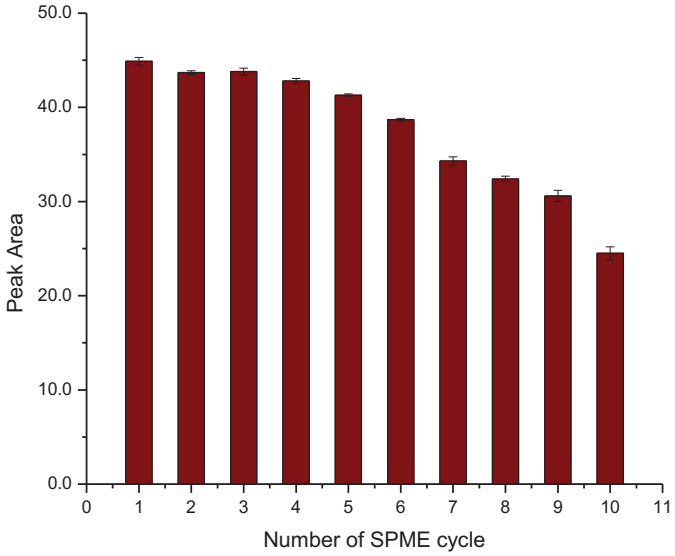

Figure 5.10. Repetitive use of the same fiber Extraction conditions: 25.0 ng/mL BPA, extraction time: 30 min, stirring speed: 400 rpm, solution volume: 15.0 mL. Desorption conditions were; desorption time: 30 min, desorption volume: 150 µL)

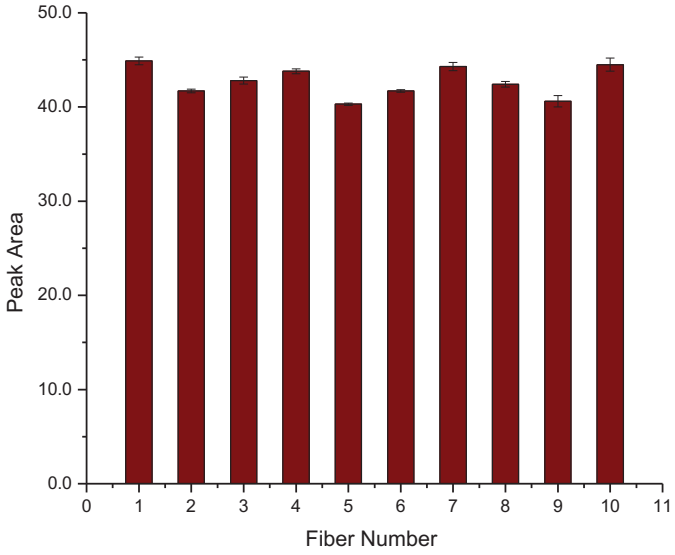

Figure 5.11. Fiber-to-fiber reproducibility Extraction conditions: 25.0 ng/mL BPA, extraction time: 30 min, stirring speed: 400 rpm, solution volume: 15.0 mL. Desorption conditions were; desorption time: 30 min, desorption volume: 150 µL

5.2.3. Method Validation

Under optimized conditions, The limit of detection (LOD), and the limit of quantification (LOQ) values for BPA were calculated. The limit of detection (LOD) based on 3.3s/m was calculated as 2.8 µg/L and limit of quantification (LOQ) based on 10s/m was 14.7 µg/L.

The method was applied to tap, bottled and sea water samples. 25.0 μgL^{-1} BPA were added and the optimum parameters were applied at pH7.0. While bottle and tap water, samples were 2-fold diluted, sea water sample was 10-fold diluted because of their complex matrix. For bottled and tap water samples, Table 5.3illustrates the determined values of both spiked and non-spiked samples. BPA could not be detected in non-spiked samples

Table 5.2. Method validation with SPME fibers (Extraction conditions; extraction pH: 7.0, extraction time: 30 min, stirring speed: 400 rpm, solution volume: 15 mL, Desorption conditions; desorption time: 30 min, desorption volume: 150 µL, eluent: 90 % MeOH – 10 % H_2O (adjusted to pH 3.0 with acetic acid)

	Bottled water (μgL^{-1})	Bottled water (25 .0 μgL^{-1})	Tap Water (μgL^{-1})	Tap Water (25.0 μgL^{-1})	Sea Water (μgL^{-1})	Sea Water (25.0μgL^{-1})
BPA	ND	28.3 ± 0.2	ND	25.6 ± 2	ND	20.4 ± 27

CHAPTER 6

CONCLUSION

The thesis progressed in two main parts, namely, solid phase extraction and solid phase microextraction. The solid phase extraction part of the study was performed with molecularly imprinted polymer for determination of Bisphenol A and amine-functionalized silica and molecularly imprinted silica for analysis of steroid hormones. The solid phase microextraction part was about the preparation of stable and selective fiber coatings and their application for the determination of parabens and bisphenol A.

A simple MISPE method was developed for the selective determination of bisphenol A prior to HPLC-DAD analysis. Bisphenol A imprinted polymers were prepared by precipitation polymerization. Non-covalent imprinted approach was applied by using bisphenol A as template molecule, AA as functional monomer and TRIM as crosslinker. The adsorption capacity and selectivity of imprinted polymers were investigated. To improve the MISPE method, the parameters including pH of sample solution, adsorption time, amount of sorbent, desorption solvent etc. were examined. In interfence studies analytes were separated with C30 reverse phase column and determined with DAD detector at 220 λ. Analytes are well min. by isocratic elution program, 0.8 mL/min as flow rate, 30 °C as column temperature and use 80 % MeOH-20 % H_2O (adjusted to pH 3.0 with acetic acid) as mobile phase. The optimized parameters for sorption were as; pH: 7.0, amount of sorbent: 10 mg, shaking time 30 min. Quantitative desorption of BPA can be achieved using 80 % MeOH-20 % H_2O (adjusted to pH 3.0 with acetic acid). Analytical parameters (Linearity, LOD) of developed method were established. The extraction efficiency of BPA imprinted polymer was investigated by using the spiked samples of ultrapure, drinking and tap water. The results show that this new material can be used selective determination of BPA in waters.

For determination of steroid hormones two synthesis routes were performed. (NH_2) silica, containing amine functionalities, was prepared by modification of silica gel with 3-(triethoxysilyl)propylamine. In addition to this synthesis, novel and simple was developed to synthesize β-Estradiol-imprinted amino-functionalized silica gel sorbent with a surface molecular imprinting technique, namely (MIP)silica . Comparison of

sorption performances of these sorbents indicates that (MIP)silica has shown superior sorption performance compared to (NH_2)silica and correspondig (NIP) silica . The reason of showing higher sorption values of (MIP)silica is to have specific binding sites in it which belong to β-est. The extraction ability of MIP and NIP silica was investigated in the presence of 17α-ethynylestradiol and estrone. The results exhibit that prepared MIP silica have recognition ability to common structure of steroid hormones. To develop the method for determination, the parameters including pH of sample solution, adsorption time, amount of sorbent, desorption solvent etc. were examined. The validity of the proposed method was checked through spike tests of ultrapure water and drinking water and results also confirmed the applicability of the method.

In SPME study, MIP encapsulated electrospun polystyrene -coated SPME fiber with benzyl paraben as template for selective extraction of parabens was prepared. Its selectivity and extraction ability were compared with the commercial fiber and the corresponding NIP coated fiber. It was found that under optimized conditions, the prepared MIP coated fiber showed better extraction ability among them. Also, the results revealed that MIP coated fiber has good recognation abilities for benzyl paraben and the other structure releated compounds,such as methyl and propyl paraben. Extraction efficiency of prepared fibers for three paraben has been tested by spiking bottled,tap and sea water samples. The recoveries were 94.8-96.8% , %92.4-97.4, % 95-99.8 for bottled, tap and sea water respectively. This MIPSPME HPLC method colud be used for selective and sensitive determination of parabens

The amine-functionalized electrospun SPME fiber produced by sol-gel method was used as novel stable selective SPME fiber coating for determination BPA. Amine-functionalized fiber coating revealed that the fibers coated by electrospinning process showed superior performance. The extracted amounts of analytes were shown to be affected from various parameters, namely, solution pH, ionic strength and extraction time. Extensive studies for extraction conditions have given the optimized parameters as; extraction pH: 7.0, agitation speed: 400 rpm, extraction time: 30 min. The fibers demonstrated reproducible extraction (< 10% rsd), good mechanical strength and good solvent resistivity. The validity of the proposed methodology was verified through spike recovery tests.

Finally, it can be said that this study incorporates various multidisciplinary fields of chemistry such as analytical chemistry, nanoscience, organic chemistry and physical chemistry with the intention of preparation novel SPE sorbents and SPME fiber coatings.

The main outcomes of the SPE study were the potential applicability of the prepared sorbents for both water remediation and analytical applications. The prepared SPE sorbents have potential for daily use as a resin for endocrine disrupting compounds from waters. These suggest new ideas for the researchers in the related area for preparation of more specific coatings.

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