

DEVELOPMENT OF MOLECULARLY IMPRINTED SOLID PHASE EXTRACTION METHOD FOR THE DETERMINATION OF VITAMIN D DERIVATIVES

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ABSTRACT

DEVELOPMENT OF MOLECULARLY IMPRINTED SOLID PHASE EXTRACTION METHOD FOR THE DETERMINATION OF VITAMIN D DERIVATIVES

Vitamin D, also known as the sunshine vitamin, is both a micronutrient and a prohormone. There are two structurally different forms of vitamin D: vitamin D2 (ergocalciferol) and vitamin D3 (cholecalciferol). Both forms can be taken into the body through food or dietary supplements. In addition, ergocalciferol and cholecalciferol can be synthesized non-enzymatically by UVB radiation of sunlight from provitamin forms, which are ergosterol and 7-dehydrocholesterol.

In this study, a methodology based on molecularly imprinted solid phase extraction (MISPE) is intended prior to the determination of D2 and D3 by HPLC-DAD. In accordance with this purpose, precipitation and sol-gel polymerization methods were used to synthesize molecularly imprinted polymers (MIPs) and non-imprinted polymers (NIPs). Also, different templates, monomers, crosslinkers, and their ratios were tried in the synthesis of MIPs/NIPs by precipitation polymerization. Among all syntheses, MIP, which was synthesized using vitamin D2 as template (T), 4-vinylpyridine (4-VP) as monomer (M), ethylene glycol dimethacrylate (EGDMA) as crosslinker (C) at a molar ratio of 1:6:30 (T:M:C), enabled the co-determination of vitamin D2 and D3. It selectively worked on the vitamin D prohormone group compared to both NIP and commercial solid phase extraction (SPE) sorbents in the presence of other structurally related compounds.

The critical experimental parameters of the MISPE method were optimized for the analyte concentration of 1.0 mgL^{-1} and determined as follows: 8 hours as the sorption time, 5.0 mg as the sorbent amount, 5.0 mL as the sample solution volume, methanol as the eluent, and 24 hours as the desorption time.

A matrix-matched calibration curve with optimized parameters was proposed. The applicability of the developed MISPE method was confirmed by analyzing the sample of vitamin D3 supplementation and overall recovery was found as 95.97% (± 1.84) for $n=2$.

ÖZET

D VİTAMİNİ TÜREVLERİNİN BELİRLENMESİ İÇİN MOLEKÜLER BASKILI KATI FAZ EKSTRAKSİYON YÖNTEMİNİN GELİŞTİRİLMESİ

Güneş ışığı vitamini olarak da bilinen D vitamini, hem bir mikro besin hem de bir prohormondur. D vitamininin yapısal olarak farklı iki formu vardır: D2 vitamini (ergokalsiferol) ve D3 vitamini (kolekalsiferol). Her iki form da vücuda gıda veya diyet takviyeleri yoluyla alınabilir. Bunun yanı sıra, ergokalsiferol ve kolekalsiferol, ergosterol ve 7-dehidrokolesterol olan provitamin formlarından güneş ışığının UVB radyasyonu ile enzimatik olmayan bir şekilde sentezlenebilir.

Bu çalışmada, HPLC-DAD ile D2 ve D3'ün belirlenmesinden önce moleküler baskılı katı faz ekstraksiyonuna (MISPE) dayalı bir metodolojinin geliştirilmesi amaçlanmaktadır. Bu amaca uygun olarak, moleküler baskılanmış polimerler (MIP'ler) ve baskılanmamış polimerler (NIP'ler) sentezlemek için çökeltme ve sol-jel polimerizasyon yöntemleri kullanılmıştır. Ayrıca çökeltme polimerizasyonu ile MIP/NIP sentezinde farklı şablonlar, monomerler, çapraz bağlayıcılar ve bunların oranları denenmiştir. Tüm sentezler arasında, şablon (T) olarak D2 vitamini, monomer (M) olarak 4-vinilpiridin (4-VP), çapraz bağlayıcı (C) olarak etilen glikol dimetakrilat (EGDMA) kullanılarak 1:6:30 (T:M:C) molar oranında sentezlenen MIP, vitamin D2 ve D3'ün birlikte belirlenmesini sağlamıştır. Ayrıca yapısal olarak ilgili diğer bileşiklerin varlığında hem NIP hem de ticari katı faz ekstraksiyon (SPE) sorbentlerine kıyasla D vitamini prohormon grubu üzerinde seçici olarak çalışmıştır.

MISPE yönteminin kritik deneysel parametreleri 1.0 mgL^{-1} analit derişimi için optimize edilmiş ve bu parametreler sorpsiyon süresi 8 saat, sorbent miktarı 5.0 mg, numune solüsyon hacmi 5.0 mL, desorpsiyon çözeltisi metanol ve desorpsiyon süresi 24 saat olarak belirlenmiştir.

Optimize edilmiş parametrelerle matris uyumlu bir kalibrasyon eğrisi önerilmiştir. Geliştirilen MISPE yönteminin uygulanabilirliği, D3 vitamini takviyesi örneği analiz edilerek doğrulanmış ve $n=2$ için toplam geri kazanım $\%95.97 (\pm 1.84)$ olarak bulunmuştur.

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LIST OF ABBREVIATIONS

Abbreviation	Explanation
BET	Brunauer-Emmett-Teller
DBP	Vitamin D Binding Protein
7-DHC	7-dehydrocholesterol
DRI	Dietary Reference Intake
ERG	Ergosterol
FTIR	Fourier Transform Infrared Spectroscopy
GC	Gas Chromatography
HPLC	High-Performance Liquid Chromatography
HPLC-DAD	High-Performance Liquid Chromatography with Diode-Array Detection
HPLC-UV	High-Performance Liquid Chromatography-Ultraviolet
HLB	Hydrophilic-Lipophilic Balance
IOM	Institute of Medicine
IUPAC	International Union of Pure and Applied Chemistry
LC-MS/MS	Liquid Chromatography Tandem Mass Spectrometry
LOD	Limit of Detection
LOQ	Limit of Quantitation
MIP	Molecularly Imprinted Polymer
MISPE	Molecularly Imprinted Solid Phase Extraction
μLC-MS/MS	Microflow Liquid Chromatography Tandem Mass Spectrometry
NIH	National Institutes of Health
NIP	Non-Imprinted Polymer
PP	Protein Precipitation
SD	Standard Deviation
SEM	Scanning Electron Microscopy
SPE	Solid Phase Extraction
UHPLC-MS/MS	Ultra-High Performance Liquid Chromatography Tandem Mass Spectrometry
VDR	Vitamin D Receptor

CHAPTER 1

INTRODUCTION

1.1. Vitamin D and Its Metabolites

Vitamin D is both a micronutrient and a prohormone. In addition to being one of the fat-soluble vitamins taken into the body through food, it is a prohormone in the seco-steroid structure synthesized in the body. Besides the well-known effect of vitamin D on bone development and maintenance, it also has a major impact on the proper functioning of the brain, heart, muscles, immune system, and skin. Vitamin D circulating in the blood binds to the vitamin D receptor (VDR) found in most cells in the body and ensures the fulfillment of biological activities with endocrine, paracrine, or autocrine actions (Mostafa and Hegazy, 2013).

There are two forms of vitamin D: vitamin D2 (ergocalciferol) and vitamin D3 (cholecalciferol). These two forms are structurally different from each other (Figure 1.1). Unlike vitamin D3, vitamin D2 contains a double bond and an additional methyl group in its side chain attached to the steroid group. Both forms can be taken through dietary supplements as ergocalciferol and cholecalciferol. While vitamin D2 usually comes from plant-based sources, animal foods are the main source of vitamin D3. In addition, ergocalciferol and cholecalciferol can be synthesized non-enzymatically from their provitamin forms, which are ergosterol and 7-dehydrocholesterol, via sunlight. Ergocalciferol is produced from ergosterol found in fungi and invertebrates in response to UVB exposure, while cholecalciferol is synthesized in the body of humans and mammals as a result of the reaction of the skin precursor 7-dehydrocholesterol with UVB radiation (Gezmish and Black, 2013). Vitamin D2 and D3 synthesis begins with the broken of the B ring of ergosterol (provitamin D2) and 7-dehydrocholesterol (provitamin D3) with the effect of UVB radiation. During exposure to sunlight, solar radiation with a wavelength of 290-315 nm causes the activation of the double bonds of provitamins, thereby opening the B ring. First, previtamins D2 and D3 are formed in seco-steroid (broken steroid) structure. These forms are then converted into

thermodynamically stable vitamin D2 (ergocalciferol) and vitamin D3 (cholecalciferol) as a result of thermal isomerization (Jäpelt and Jakobsen, 2013).

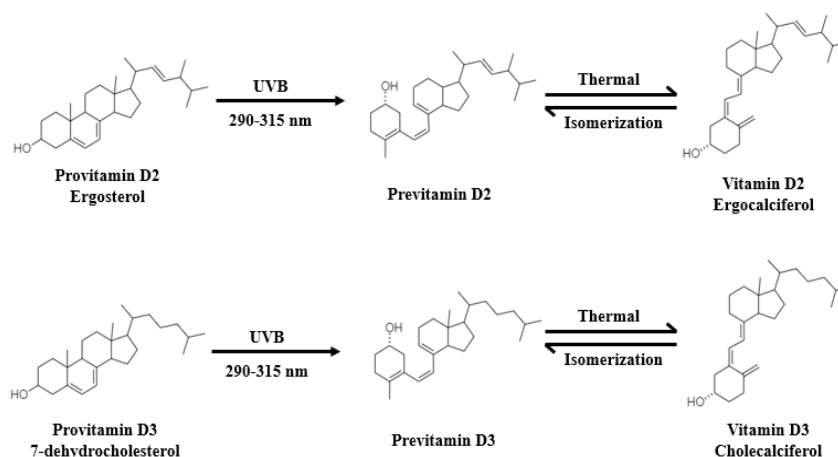


Figure 1.1. Synthesis of vitamin D2 and vitamin D3

In addition to two separate forms of vitamin D, it has various metabolites formed as a result of metabolic activities in the body. These metabolites are classified as prohormone, prehormone and hormone (Table 1.1) (Friedl and Zitt, 2017).

Table 1.1. Metabolites of Vitamin D

	Metabolites of Vitamin D2	Metabolites of Vitamin D3	Total Vitamin D
Prohormone	Vitamin D2 (Ergocalciferol)	Vitamin D3 (Cholecalciferol)	Vitamin D
Prehormone	25-Hydroxyvitamin D2 (Ergocalcidiol)	25-Hydroxyvitamin D3 (Calcidiol)	25-Hydroxyvitamin D
Hormone	1,25-Dihydroxyvitamin D2 (Ergocalcitriol)	1,25-Dihydroxyvitamin D3 (Calcitriol)	1,25-Dihydroxyvitamin D

Vitamin D can enter the human circulation both as ergocalciferol and cholecalciferol through food sources and as cholecalciferol by synthesis from the skin (Figure 1.2) (Mostafa and Hegazy, 2013). However, ergocalciferol and cholecalciferol are not biologically active. The activation of the vitamin D in the body consists of two hydroxylation steps. Although vitamin D taken into the body as two distinct forms, these forms follow the same metabolic pathway during activation. The first step

involves the conversion of prohormones to prehormones. The 25-hydroxylase enzyme in the liver leads to hydroxylation of carbon 25 of vitamins D2 and D3, resulting in the formation of 25-hydroxyvitamins D2 and D3. Prehormones are then converted to their hormone forms, 1,25-dihydroxyvitamins D3 and D2, in the kidney or locally in other organs by the enzyme 1- α hydroxylase. Transport of vitamin D metabolites is accomplished by vitamin D binding protein (DBP) and vitamin D receptor (VDR) mediates the active form of vitamin D to carry out its biological functions in the body (Ayelign et al. 2020).

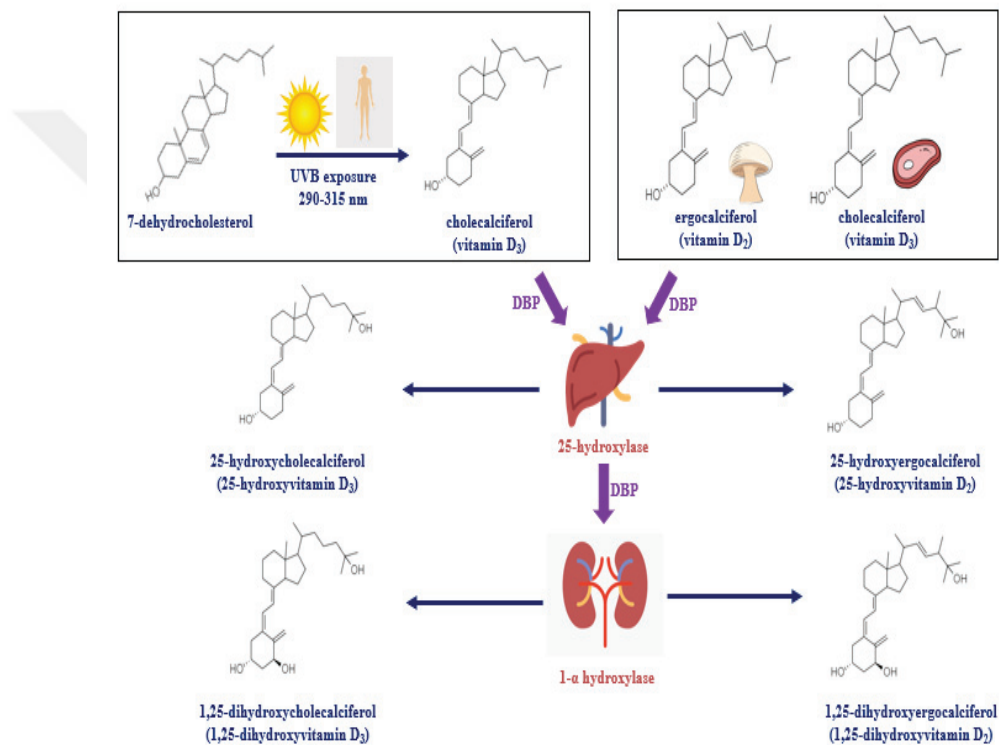


Figure 1.2. Metabolic pathway of vitamin D2 and D3 in the body

1.1.1. Vitamin D Status in the Body

Vitamin D entering the body follows a metabolic pathway in order to fulfill its functions. While the conversion of ingested or synthesized vitamin D to 25(OH)D occurs rapidly, only a portion of 25(OH)D is converted to 1,25(OH)₂D. Although these two forms of vitamin D are found in the blood as products of metabolic activities, vitamin D tests mostly measure the blood 25-hydroxyvitamin D level. The biologically

active form 1,25(OH)₂D is not suitable for ideal measurement. This is because its half-life is as short as 4-6 hours and its circulating levels are 1000 times lower than 25(OH)D (Holick 2009). Therefore, 25(OH)D, which has a half-life of 2-3 weeks, is the metabolite that best reflects vitamin D stores in the body, since it shows both vitamin D intake and endogenous production (Bendik et al. 2014). Vitamin D tests performed to assess vitamin D levels enable the detection of deficiency, adequacy, or toxicity in individuals. According to NIH Office of Dietary Supplements, vitamin D level in the blood is classified into four stages as nmol/L or ng/mL 25(OH)D. As seen in the Table 1.2, 30 nmol/L and below shows deficiency, 30-50 nmol/L potential deficiency, 50-125 nmol/L normal levels and 125 nmol/L and above high levels.

Table 1.2. 25(OH)D levels corresponding to vitamin D status in individuals

▪ Deficiency	—————→	< 30 nmol/L (12 ng/mL)
▪ Potential deficiency	—————→	30–50 nmol/L (12-20 ng/mL)
▪ Normal levels	—————→	50-125 nmol/L (20-50 ng/mL)
▪ High levels	—————→	> 125 nmol/L (50 ng/mL)

There are certain factors that affect the level of vitamin D in the human body, causing a deficiency or toxicity. These factors are related to both skin synthesis and supplementation of vitamin D. Cutaneous production of vitamin D depends on the amount of UVB reaching the skin. This amount depends on environmental factors such as season, time of day, weather conditions, air pollution, as well as personal variables such as skin type, clothing habits, lifestyle, and workplace (Mostafa and Hegazy, 2013). In addition, the amount of vitamin D taken as a nutritional supplement should be at the level determined by age. Taking vitamin D below the recommended level causes deficiency, while taking it above the recommended level can cause poisoning. The following table shows the dietary reference intake (DRI) values for vitamin D as determined by the Institute of Medicine (IOM) (Bendik et al. 2014).

Table 1.3. Dietary reference intake (DRI) values for vitamin D

▪ Dietary allowance	—————→	15 µg/day
▪ Children	—————→	10 µg/day
▪ 70 years and older	—————→	20 µg/day
▪ Upper intake	—————→	100 µg/day

In case of inadequate or excess vitamin D, various disorders occur in the body. Rickets in children, osteomalacia in adults are among the diseases that can be seen in the bones in vitamin D deficiency. It also increases the risk of breast, colon, and prostate cancer in humans by causing weakening of the immune system. Vitamin D poisoning leads to hypercalcemia, hyperphosphatemia and hypercalciuria, which manifests as nausea, dehydration, and constipation (Kennel, Drake, and Hurley 2010). As a result of studies, it has been reported that approximately 1 billion people worldwide (Siddiquee et al. 2021) and 51% of Turkey's population (Roth et al. 2018) have vitamin D deficiency. For all these reasons, it is of great importance that vitamin D analysis be performed sensitively and accurately.

1.1.2. Determination of Vitamin D

In the literature, there are studies on the determination of vitamin D and its metabolites in both human serum and plasma. In these studies, generally, the extraction and purification of vitamin D was achieved with commercial SPE sorbents, while separation and quantification were performed by techniques such as HPLC and LC-MS/MS. In a study, C18 and silica cartridges were used for the extraction of vitamin D, 25-OH-D, 24,25-(OH)₂-D and 1,25-(OH)₂-D from plasma. While C18 cartridges enabled the extraction of all tested compounds, silica cartridges were highly effective on vitamin D metabolites with increasing polarity. Separation of metabolites was achieved by HPLC-UV (Hollis and Frank, 1985). In another study, C18 sorbent was used for solid phase extraction of 25-OH-D₃ from human plasma. Kand'ár and Žáková (2009) developed a method for the determination of 25(OH)D₃ using HPLC-UV detection after protein precipitation with ethanol and purification with Discovery DSC-18 SPE tubes. The limit of detection for 25(OH)D₃ was calculated as 2.5 nmol/L. In the study of the determination of 25-(OH)-D₂ and 25-(OH)-D₃, metabolites were extracted from blood serum sample with Orochem C8 commercial cartridge and analyzed by LC-MS/MS (Knox et al. 2009). The lower limit of quantitation was determined as 7.5 nmol/L and 4.0 nmol/L for 25-(OH)-D₂ and 25-(OH)-D₃, respectively. There are also studies in which Oasis-HLB is used as the SPE sorbent for the determination of vitamin D and its derivatives. One of these studies provides simultaneous quantitative determination of

the hydroxy and dihydroxy forms of both D2 and D3 by UHPLC-MS/MS (Ding et al. 2010). The limit of quantitation (LOQ) was calculated as 20 pg/mL for 1,25-(OH)₂-D3, 10 pg/mL for 1,25-(OH)₂-D2, 25-OH-D3 and 25-OH-D2. In another study, a method couples SPE and microflow liquid chromatography tandem mass spectrometry (μ LC-MS/MS) were developed for the sensitive determination of 25-(OH)-D2, 25-(OH)-D3, 24,25-(OH)₂-D3 and 1,25-(OH)₂D3 in serum with the limit of detection of 0.5 pg/mL, 1.0 pg/mL, 1.0 pg/mL and 0.5 pg/mL, respectively. (Duan et al. 2010).

In the literature, there are very few studies on the determination of vitamin D with molecularly imprinted polymers. Hashim et al. (2016) used ergosterol, the precursor of vitamin D2, as the template molecule. First of all, ergosteryl-3-O-methacrylate template-monomer compound was synthesized, then EGDMA was used as crosslinker, and MIP synthesis was carried out with semi-covalent imprinting strategy. The analyses were performed with RP-HPLC. The synthesized MIP showed higher binding performance compared to NIP. The applicability of the developed method was evaluated with the medicinal mushroom and the recovery for ergosterol was found as 98%. Kia et al. (2016), on the other hand, used cholecalciferol (D3) as a template molecule and synthesized MIP by sol-gel method. The selectivity of the synthesized MIP was evaluated by competitive binding assay in the presence of cholecalciferol, 25-hydroxycholecalciferol, 17-beta-estradiol and betamethasone and it was observed that MIP showed higher recovery for D3 compared to other analytes. The analyses were performed with RP-HPLC and the limit of detection for cholecalciferol was calculated as 1 ng/mL.

In the quantification of Vitamin D, competitive protein binding (CPB) assays, enzyme-linked immunosorbent assays (ELISA), radioimmunoassays (RIA) and chemiluminescence immunoassays (CLIA) are widely used in clinical applications. These tests, which are available in kit form, allow hundreds of samples to be measured for hours. However, the possible affinities of other vitamin D metabolites against the antibodies used in the analyses and the use of different sample preparation procedures between test platforms for the DBP release of 25-hydroxyvitamin D may lead to variations in the results obtained (Stokes, Lammert, and Volmer 2018).

1.1.3. Stability of Vitamin D

Vitamin D is affected by many physical and chemical factors. It is known that the stability of vitamin D varies depending on conditions such as light, temperature, pH, oxygen, humidity, and storage time. Although the number of detailed studies on this critical point is few in the literature, the findings provide some clarification on the issue of maintaining stable conditions for vitamin D. In a comprehensive study by Rakuša et al. (2021), the stability of cholecalciferol under various conditions was examined by HPLC-UV analysis. In the study, it has been shown that the solvent from which the vitamin D solution is prepared has a remarkable effect on its stability. The solutions prepared as aqueous and non-aqueous. Much more stable results were obtained in non-aqueous solutions of vitamin D compared to aqueous solutions. When the aqueous solutions were evaluated, it was observed that the stability decreased in Milli-Q, tap and distilled water, respectively. This case has been explained with the hydrogen and hydroxide ions present in the water. Also, metal ions that catalyze the oxidation reaction may have caused the degradation of cholecalciferol. The temperature factor was considered at different temperatures (4, 25, 40°C), and it was seen that the increase in temperature caused a decrease in the stability of D3. A study was also conducted on the stability of vitamin D in aqueous medium under different concentration conditions. Solutions were prepared at 10, 25, 50, 100 mgL⁻¹ in water-methanol mixture (90:10 (v/v)). This experiment showed that the stability of aqueous solutions of D3 is concentration dependent and the stability decreases as the concentration decreases. The reason for this has been explained as the effect of factors that will disrupt the stability will be greater at low concentrations. In this first study on the effect of pH on D3 stability, pH values in the 1-8 range were examined. It has been demonstrated that the vitamin is stable in a neutral and slightly basic environment (pH above 5) but shows instability in an acidic environment. In addition, the effects of light and oxygen on the stability of the aqueous solution of vitamin D3 were evaluated. It has been observed that the presence of oxygen and light has an effect on reducing stability. This study demonstrated that the degradation of vitamin D solutions occurs by thermal, photochemical, and oxidative reactions.

In another study by Grady and Thakker (1980), the effect of temperature and humidity on D2 and D3 powders was investigated. Powder samples were stored in

various humid environments (dry air, 45% and 85% relative humidity, dry nitrogen) at different temperatures (25, 40°C) without light and analyzed by HPLC. It was concluded that both vitamins, especially D3, are less stable at high temperatures. While ergosterol, whose decomposition is dry and oxidative, is more stable at higher humidity, cholecalciferol showed the opposite behavior. In addition, it was observed that the color of D2, which was initially in the form of white crystalline powder, turned yellow depending on the storage conditions. In this study, it has been proven that both forms of vitamin D undergo solid-state decomposition. The decomposition reaction takes place in the side chain attached to the steroid group of the vitamins. Structurally, the only difference between the two species is the double bond in the side chain of ergocalciferol. Since this double bond makes vitamin D2 more ready for oxygen attack, vitamin D2 is more prone to degradation than D3. In addition to all these findings related to stability, complex degradation mechanisms of vitamin D are still a subject that is ongoing to be investigated, although it is not fully known.

1.2. Sample Preparation Techniques for Biological Samples

Biological samples such as plasma, serum, urine to be chemically analyzed contain a complex matrix of many components other than the analyte. Therefore, separating the analyte from this matrix is a crucial process. This process is called sample preparation and is an important step before performing analyses with various instruments (LC, GC, MS, etc.). Sample preparation has two main objectives: purification and enrichment. The purification process eliminates impurities that may affect the reliable detection of the analyte or cause contamination of the instruments. Protein precipitation (PP), liquid-liquid extraction (LLE), solid phase extraction (SPE) and dilute-and-shoot (DAS) are conventional biological sample preparation techniques. In this thesis, solid phase extraction method will be used for the determination of vitamin D derivatives namely, vitamin D2 and vitamin D3.

1.2.1. Solid Phase Extraction

In solid phase extraction, which is similar in principle to liquid-liquid extraction, one of the phases is solid and the other is liquid. This method provides purification and enrichment by retention of analyte molecules in the liquid sample onto the solid phase. The advantages offered by solid phase extraction are listed below.

- It is an easy-to-apply and fast-resulting method.
- It is a practical and economical method.
- Small amount of sample and solvent is consumed.
- Highly enriched and purified samples can be obtained with high recovery.

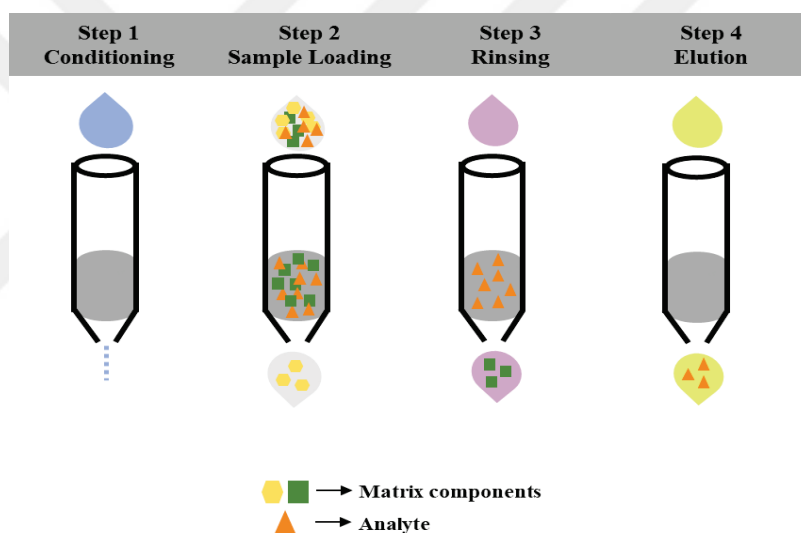


Figure 1.3. Basic steps of solid phase extraction

The SPE method is basically based on filling various adsorbents into small and disposable columns and passing the sample liquid through the column. It consists of four main steps as shown in the Figure 1.3. The first step is conditioning. The solid phase is washed with a suitable solvent. In this way, both impurities are removed, and the stationary phase material is conditioned. The adsorbent, which is activated by conditioning, provides reproducible interaction with the components in the matrix. Conditioning is carried out with nonpolar solvents for polar sorbents, and with water-miscible solvents such as methanol, tetrahydrofuran, isopropyl alcohol for non-polar sorbents. In the second step, the sample is passed through the conditioned column by

gravity or with the help of a pump. The point to be considered here is the flow rate of the sample in the column. The flow rate should be sufficiently slow to allow the interaction of the sample with the sorbent. In the third step, the solid phase is rinsed with an appropriate solvent, generally water, and the unwanted components that possibly come from the matrix are removed from the sorbent. In the final step, the analyte is eluted by passing a proper eluent through the column.

There are two general approaches used in solid phase extraction, column and batch. In the column type SPE method, the sorbent is loaded into a column. The sample solution containing the analyte is passed through the column and the analyte retained on the sorbent is recovered with an appropriate solvent (Figure 1.4 (a)). In the batch type SPE method, the sorbent is shaken with sample solution containing the analyte and then filtered. The filtered sorbent is mixed with a proper solvent for a given time. Recovery of the analyte from the sorbent is achieved by repeated agitation-filtration processes, respectively (Figure 1.4 (b)).

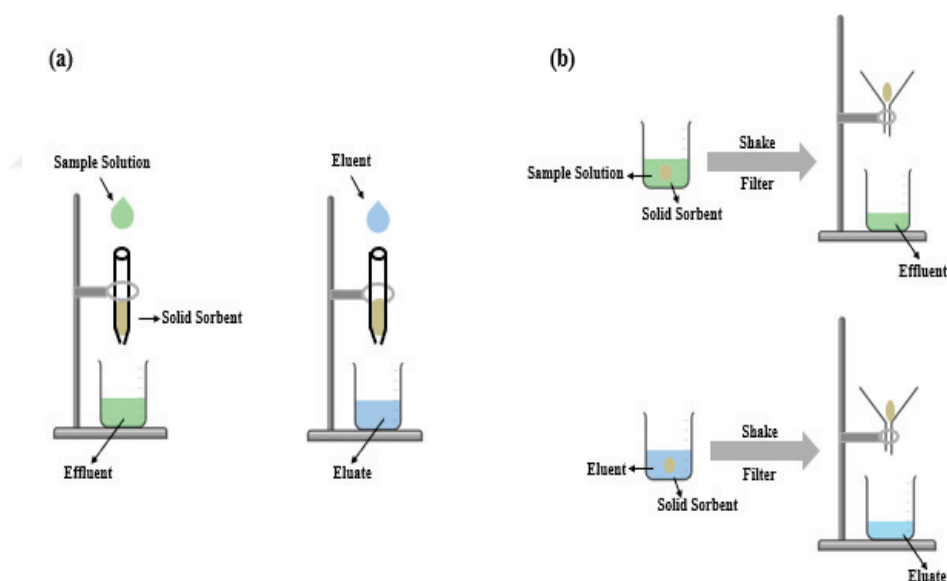


Figure 1.4. (a) Column type and (b) batch type SPE

The most critical point in the SPE method is to select the convenient adsorbent type for the target analyte. For a successful purification process, the sorbent to be used should be determined by considering the chemical properties of the analyte and should have the necessary functionality to interact with the analyte. For this purpose, different SPE sorbents have been synthesized and used, such as chemically modified polymeric

resins, highly cross-linked polymers, immunosorbents, and molecularly imprinted polymers (Masque et al., 1998). Recently, molecularly imprinted polymers have been started to utilize as sorbent in SPE method due to their high selectivity and stability. These polymers ensure the successful removal of the target molecule from the sample matrix. In this thesis, molecularly imprinted polymers will be used as the SPE sorbent for the extraction of vitamin D derivatives.

1.3. Molecularly Imprinted Polymers

Molecularly imprinted polymers (MIPs) can be defined as artificial receptors made by mimicking the enzyme-substrate mechanism that works naturally in the body based on the lock-and-key model. These polymers have become a popular topic in the field of chemistry in recent years due to their high selectivity towards the target molecule. In addition to its applicability for various analytes, low cost, long shelf life, high mechanical resistance, strength to heat and pressure, stability against extreme conditions such as acids, bases and organic solvents are among the advantages offered by molecularly imprinted polymers. Considering all these advantages and features, MIPs can be applied as artificial receptor, sensor component and solid phase extraction (SPE) sorbent.

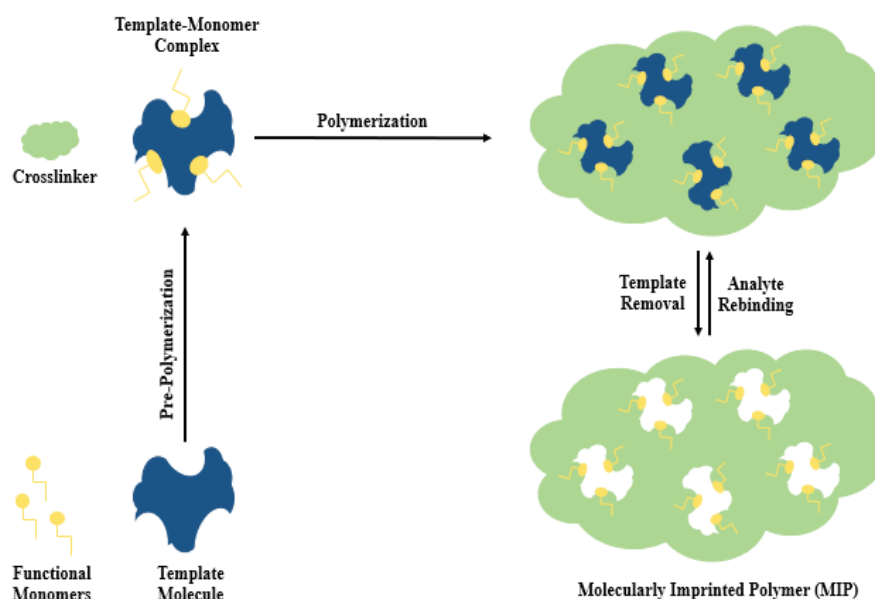


Figure 1.5. Schematic representation of the molecular imprinting process

Molecular imprinting is the process of synthesizing polymers containing cavities with template molecule recognition capabilities. Functional monomer, template molecule, crosslinker, initiator, and porogen are used for the synthesis of polymers. As shown in the Figure 1.5, the imprinting process consists of three basic steps: pre-polymerization, polymerization, and template (analyte) removal. The selection of the molecule to be used as a template that enables the formation of imprinted regions is the most critical point in MIP synthesis. The molecule to be used as a template should not contain a functional group that will stop or retard the polymerization reaction. The chemical inertness of the template is also of great importance in polymerization. Being unstable under thermal or UV polymerization conditions or entering into different reactions will reduce the success of imprinting. Besides, molecular size should be considered in imprinting. Small molecules are more suitable for use as templates since it is difficult to remove very large molecules from the cross-links of the polymer.

In the first step of imprinting, pre-polymerization, a monomer-template complex is formed. A covalent or non-covalent chemical interaction takes place between the polymerizable monomer endowed with the appropriate functional group and the analyte (target molecule/template) of interest. Therefore, it is possible to classify the molecular imprinting method into two main groups covalent and non-covalent imprinting. In covalent imprinting, the functional monomer and the template molecule are bound to each other by strong and reversible covalent bonds. In non-covalent imprinting, the bond formation between the monomer and the template molecule is based on spontaneous intermolecular interactions such as hydrogen bonding, π - π , ion-ion, ion-dipole, dipole-dipole, hydrophobic and Van der Waals interactions. Straightforward formation of the monomer-template complex, ease in the extraction of the template molecule, formation of multiple high-affinity binding sites, and rapid rebinding kinetics are the features that make non-covalent imprinting stand out.

The choice of solvent/porogen plays an important role in the type of imprinting. It should promote the interaction between template and monomer. The stronger this interaction, the greater the effect of imprinting will be. In covalent imprinting, many solvents can be used as long as the solubility of the components is ensured. In non-covalent imprinting, aprotic solvents with low polarity (e.g., chloroform, dichloromethane, toluene) are used to increase polar non-covalent interactions such as hydrogen bonding between the template and monomer. However, polar protic solvents highly reduce hydrogen bonding.

The formation of the monomer-template complex is governed by chemical equilibrium. In this case, it is an important point to increase the interaction of the functional monomer and the template molecule. Increasing the number of imprinted regions in non-covalent imprinting is achieved by having a higher mole number of monomer than the number of moles of the template molecule (the mole ratio of template to the monomer is 1:4 or above). Depending on the ratio used, many different monomer-template complexes are formed in the synthesized MIP. These complexes have different affinity constants and heterogeneous binding sites.

In the polymerization step, the formed monomer-template complex is polymerized with the use of a suitable crosslinker on the functional monomer with the help of an initiator. The crosslinker fulfills its functions such as providing mechanical strength, stabilizing and protecting the structure of the imprinted binding sites by copolymerizing on the monomer. A stoichiometric balance must be established between monomer and crosslinker for copolymerization to occur. Therefore, the monomer used in polymerization must have functional compatibility with the crosslinker as well as with the template molecule. The molar ratio of the crosslinker added to the polymer matrix has a great influence on the binding sites. A very high molar ratio results in reduced imprinting due to the crosslinker performing non-covalent interactions with the monomer or template, while a very low molar ratio results in the binding sites being too close to each other and inhibiting these sites by each other.

Free radical polymerization is the most used mechanism in MIP synthesis. In this polymerization method, heat or light-activated azo initiators are used as a radical source. Thanks to the free radical polymerization, it is possible to rapidly perform the polymerization at temperatures below 80°C and at atmospheric pressure. If the interaction between the monomer and the template molecule is weak, it will not be the right way to carry out the polymerization at high temperatures. In these cases, it is preferable to use UV-activated initiators instead of heat.

The polymerization techniques follow free radical mechanism are bulk, emulsion, suspension and precipitation polymerization depending on the reaction medium (Adumitrăchioaie et al. 2018). The precipitation and bulk polymerization techniques used in this thesis will be briefly explained.

In bulk polymerization, monolithic MIPs are synthesized. The grinding and sieving processes follow the MIP synthesis to obtain particles in the desired size range. These time-consuming and labor-intensive processes have some disadvantages. There is

material loss during grinding and particle shape control is very difficult (Cheong, Yang, and Ali 2013). In addition, the interaction regions are likely to be damaged. This leads to a decrease in the selective binding capacity of the synthesized MIP. Precipitation polymerization enables MIP particles to be obtained in a single step in a shorter time and with high efficiency. Unlike bulk polymerization, a larger volume of porogen is used in this polymerization method. Particle formation begins with the growth of the polymer chain and continues until the extended chain becomes insoluble in the mixture. Since the dilute conditions that occur with the increase of the solvent volume lead to less oligomer and nucleation formation, uniform particles with sizes as low as 100 nm and as high as a few micrometers can be obtained through this method (Haupt, Medina Rangel, and Bui 2020). It is known that the use of monolith obtained by bulk polymerization in solid-phase extraction is limited. However, MIP microspheres with homogeneous particle size distribution obtained by precipitation polymerization are widely used (Hu et al. 2021).

In the last step, the template molecule is removed from the polymer in order to create cavities in the polymer structure that will replace the target molecule. At this step, it is very important to ensure that the template molecule is completely removed from the polymer structure. When the analyte is used as the template molecule, if the template is not completely removed, it will make the analyte appear to be in excess of its actual amount. This case is called “template bleeding”. Dummy molecule which is similar to analyte in terms of size, shape and functionality is used as template to overcome this problem.

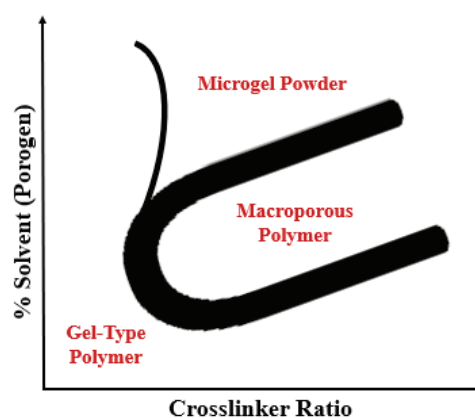


Figure 1.6. Pseudo diagram of polymer demonstrating three separate regions (e.g., gel-type polymers, macroporous polymers, microgel powders)

MIP can be synthesized in various types, such as gel type, macroporous, or microgel powder, depending on the intended use in analytical applications (Yan and Ho Row 2006). The morphology of MIP is determined by the ratio of crosslinker to total number of monomer and amount of porogen used during polymerization (Cormack and Elorza 2004). Therefore, crosslinker and porogen are effective in controlling physical form of synthesized polymer (Figure 1.6).

Gel-type polymers are synthesized at low crosslinker ratio or high crosslinker ratio at low porogen volume. In the presence of high amount of crosslinker, the specific surface areas of such polymers are low, as the growing polymer chains will be in close contact with each other. A low crosslinker ratio, on the other hand, causes poor mechanical strength of the polymer. Macroporous polymers are obtained as a result of the polymerization reaction carried out with a partially high cross-linker in more solvent medium. Compared to the gel type polymer, it contains higher specific binding sites and is more resistant due to high ratio of crosslinker. With a further increase in the solvent volume, microgel powders are synthesized. These polymers consist of spherical particles with micron size (Cormack and Elorza, 2004). In order for MIP to be synthesized as desired, these parameters must be determined and optimized simultaneously.

The cavities created in the molecularly imprinted polymer recognize the size, structure, and chemical properties of the template molecule and it ensures the analyte binds selectively to the polymer. A second polymer is synthesized under the same conditions to prove the existence of imprinted cavities in MIP. This time, no template molecule is added to the polymerization medium. Therefore, this polymer does not contain any imprinted regions and is called a “non-imprinted polymer” (NIP). By comparing the rebinding of the analyte to both MIP and NIP, it is possible to evaluate the selectivity of the synthesized MIP.

1.4. Aim of the Study

The aim of this study is to prepare a selective solid phase extraction sorbent that enables sensitive and accurate determination of vitamin D (ergocalciferol and cholecalciferol). For this purpose, various molecularly imprinted polymers were

synthesized using D2 and its provitamin form, ergosterol, as template molecules. The synthesized polymers were evaluated in terms of binding characteristics and cross-sensitivity. MIP, which has better sorption capacity and higher selectivity towards both D2 and D3, was chosen to be used in MISPE. In addition, the performance of MIP selective to the vitamin D prohormones group was compared with commercial sorbents. The parameters, which are sorption time, sorbent amount, sample solution volume, eluent type, desorption time, and reusability of the sorbent, of the developed MISPE method, were optimized through batch type SPE studies. A matrix-matched calibration curve was proposed, and the applicability of the method was confirmed by spiking a known concentration of vitamin D3 supplementation to the determined matrix.



CHAPTER 2

EXPERIMENTAL

2.1. Chemicals and Reagents

Analytical grade chemicals and reagents were used throughout the study. Ergosterol, 7-dehydrocholesterol, ergocalciferol (vitamin D₂), cholecalciferol (vitamin D₃) and vitamin K₁ were supplied from Sigma Aldrich. Monthly, 500 mgL⁻¹ stock solutions were prepared in ethanol and stored in amber bottles at -20 °C under Ar gas. Standard and sample solutions were prepared daily with proper dilutions. Double distilled ultrapure water was used in all studies. Before HPLC analysis, all solutions were filtered through 0.2 µm cellulose acetate filter paper or 0.2 µm polyamide filter paper depending on the solvent system and degassed in an ultrasonic bath for 15.0 minutes. In the synthesis of D₂ imprinted polymer, methacrylic acid (MAA, 99%, Aldrich), acrylamide (AA, ≥98.0%, Fluka) and 4-vinylpyridine (4VP, 95%, Aldrich) as functional monomer, trimethylpropane trimethacrylate (TRIM, technical grade, Aldrich), ethylene glycol dimethylacrylate (EGDMA, 98%, Aldrich) and tetraethyl orthosilicate (TEOS, 98%, Aldrich) as crosslinker, 4, 4'-Azobis(4-cyanovaleric acid) (AIVN, ≥98.0%, Aldrich) and benzoyl peroxide (BPO, 97%, Alfa Aesar) as initiator, and HPLC grade acetonitrile (Sigma Aldrich) as porogen were used. All of the other solvents used during the experiment were HPLC grade.

2.2. Instrumentation of HPLC

All analyses were performed with Agilent 1200 series HPLC with Diode Array Detector. C₁₈ column (Waters SunFire C₁₈ 5 µm 4.6 mm × 250 mm) was used as the stationary phase and 100% methanol was used as the mobile phase in order to provide chromatographic separation of vitamin D₂, vitamin D₃, ergosterol, 7-

dehydrocholesterol, and vitamin K1. It was studied at column oven temperature of 30°C and a mobile phase flow rate of 0.8 mLmin⁻¹, and wavelengths were determined as 264 and 280 nm (Table 2.1).

Table 2.1. HPLC-DAD parameters

Column	Waters SunFire C18 (particle size 5 µm, 4.6 mm x 250 mm)
Mobile phase	100% Methanol
Flow rate	0.8 mLmin ⁻¹
Thermostat temperature	30°C
Sample injection volume	20 µL

Standard solutions of 0.025, 0.05, 0.10, 0.25, 0.50 and 1.0 mgL⁻¹ were prepared in a water:methanol mixture (90:10 (v/v)) and the calibration graph was obtained. Limit of detection (LOD) and limit of quantification (LOQ) were calculated by analyzing the lowest concentration standard 10 times.

$$\text{LOD} = 3.3 \times \text{SD} / m \quad \text{Equation 2.1.}$$

$$\text{LOQ} = 10 \times \text{SD} / m \quad \text{Equation 2.2}$$

The formulas given in Equations 2.1. and 2.2., respectively, were used in the calculations of LOD and LOQ. SD given in the formula represents the standard deviation of the lowest concentration of the analyte solution and m represents the slope of the calibration curve. Other solutions were analyzed in duplicate to evaluate the standard deviation. The formula of sample standard deviation (s) is indicated in Equation 2.3. Here, x_i is each of the values of data, \bar{x} is the sample mean and N is the number of data.

$$S = \sqrt{\frac{\sum_{i=1}^N (x_i - \bar{x})^2}{N-1}} \quad \text{Equation 2.3.}$$

2.3. Solubility and Stability of Vitamin D2

The solubility of vitamin D2 in aqueous solution is restricted. Therefore, D2 solutions at concentrations of 0.50, 1.0, 5.0, 10.0 and 25.0 mgL⁻¹ were prepared in methanol and water:methanol (90:10 (v/v) medium and the solutions were analyzed by Perkin Elmer, Double beam UV-Visible Spectrophotometer (Model- Lambda 45) using 1 cm quartz cells.

In addition, the stability of the powder form (solid standard) of vitamin D2 stored at 4°C was evaluated by monitoring the change in the amount of D2 in the monthly prepared stock solutions over time.

2.4. Synthesis of Molecularly Imprinted and Non-Imprinted Polymers (MIPs and NIPs)

In this thesis, precipitation and sol-gel polymerization methods were used to synthesize MIPs and NIPs. Table 2.2 summarizes synthesized polymers.

Table 2.2. MIP/NIP components used in the syntheses

	Template (T)	Monomer (M)	Crosslinker (C)	T:M:C Molar Ratio	Solvent	Solvent Amount
MIP1	D2	MAA	TRIM	1:8:20	ACN	100 mL
NIP1	-	MAA	TRIM	1:8:20	ACN	100 mL
MIP2	ERG	AA	TRIM	1:8:20	ACN	100 mL
NIP2	-	AA	TRIM	1:8:20	ACN	100 mL
MIP3	ERG	4-VP	EGDMA	1:8:20	ACN	100 mL
NIP3	-	4-VP	EGDMA	1:8:20	ACN	100 mL
MIP4	ERG	4-VP	EGDMA	1:6:30	ACN	100 mL
NIP4	-	4-VP	EGDMA	1:6:30	ACN	100 mL
MIP5	D2	4-VP	EGDMA	1:6:30	ACN	100 mL
NIP5		4-VP	EGDMA	1:6:30	ACN	100 mL
MIP6	D2	MAA	TEOS&EGDMA	1:8:20	EtOH	4.5 mL
NIP6	-	MAA	TEOS&EGDMA	1:8:20	EtOH	4.5 mL

(MAA: Methacrylic acid, AA: Acrylamide, 4-VP: 4-vinylpyridine, TRIM: Trimethylpropane trimethacrylate, EGDMA: Ethylene glycol dimethacrylate, TEOS: Tetraethyl orthosilicate, ACN: Acetonitrile, EtOH: Ethanol)

Different templates, monomers, crosslinkers, and their ratios were tried in the synthesis of MIP/NIP numbered as 1,2,3,4 and 5 by precipitation polymerization. D2 was chosen as the template molecule to synthesize vitamin D-specific MIP. Also, in order to make the synthesis more economical, ergosterol, the provitamin form of D2, was used as the dummy template molecule due to its similar structure and size.

The experimental steps in this polymerization method were as follows: First, the template and monomer were stirred overnight in the porogen medium to achieve pre-polymerization. Then, the crosslinker was added to the polymerization solution and the copolymerization of the crosslinker was performed by adding the initiator. The initiator was used at 10% of the mole of the crosslinker and its addition to the reaction medium was carried out under Ar gas to remove dissolved oxygen. The polymerization reaction was carried out in an oil bath at 60°C, overnight.

In the synthesis of MIP6/NIP6, sol-gel method was used. First, two different solutions were prepared for the organic and inorganic part of the MIP. In one solution, MAA, EGDMA, and initiator were mixed at 50°C for 20 minutes, while D2 and TEOS were allowed to form hydrogen bonds in the other solution forming the sol. Then the organic solution was slowly added to the inorganic solution and stirred for 1 hour at 50°C. The mixture was left for 48 hours to form a gel network.

For both methods, solid MIPs were obtained after polymerization. Since MIP6 synthesized by sol-gel method is in bulk form, a grinding and sieving process was applied for this polymer. The template molecules were removed from the polymers with methanol. After the template removal process was completed, MIPs were dried in an oven at 60°C and made ready for the SPE experiments. NIPs were prepared in the same manner as the MIP synthesis procedure, except that the template was added to the polymerization medium. MIP/NIP pair, which shows the best sorption performance compared to other polymers, was characterized by Perkin Elmer BX Fourier transform infrared (FTIR) spectroscopy, FEI Quanta 250 FEG scanning electron microscopy (SEM) and Micromeritics Gemini VII Brunauer-Emmett-Teller (BET) surface area analysis.

2.5. Characterization Experiments

2.5.1. Binding Characteristic Assay

Several binding characteristic assays were performed for MIP1 and NIP1 to determine the sample preparation solvent used during the rebinding experiments.

Firstly, methanol was used as the sample solution. The D2 solutions were prepared at the concentrations of 1.0, 5.0 and 10.0 mgL⁻¹ and 10.0 mL of these solutions were added to amber vials containing 10.0 mg of MIP1 or NIP1 sorbent. Then, mixtures were shaken at 50 rpm, 24 hours. The solid/liquid mixture was filtered through cellulose acetate membranes (0.2 µm pore size) to separate the sorbents from solutions. Effluents were analyzed with HPLC-DAD at 264 nm.

Secondly, 5.0 mgL⁻¹ D2 solution was prepared in acetonitrile and the same procedure above was applied for MIP1 and NIP1.

Then, different ratios of H₂O:MeOH, which are 85:15, 90:10 and 95:5, were tried in the preparation of 5.0 mgL⁻¹ D2 solution. The same binding assay was performed also under these conditions.

As a result of these experiments, H₂O:MeOH (90:10 (v/v)) was selected as the most suitable solvent for sample preparation and binding characteristic experiments for MIP1/NIP1 were carried at D2 concentrations from 1.0 to 25.0 mgL⁻¹.

In accordance with this experiment, the highest concentration that can be worked was determined and the parameters given in Table 2.3 were used during the sorption experiments of all synthesized MIP/NIP pairs. Effluents were analyzed with HPLC-DAD at 264 and 280 nm depending on the analyte used in the rebinding experiments.

In the characterization experiments, the sorption time of 24 hours was especially chosen to allow equilibrium to be established and to evaluate the performance of sorbents under these conditions.

Table 2.3. Parameters of the binding characteristic assay (n=2)

Standard concentrations	0.05, 0.10, 0.25, 0.50, 1.0 mgL ⁻¹
Sorbent amount	10.0 mg
Sample solution volume	10.0 mL (90:10 H ₂ O:MeOH)
Sorption time	24 h
Shaking speed	50 rpm
Sample solution pH (measured)	7.0
Sorption temperature	25°C

2.5.2. Cross-Sensitivity Assay

Parameters in the cross-sensitivity assay, which was conducted to understand the behavior of synthesized MIP4/NIP4 and MIP5/NIP5 in the presence of other structurally related compounds, are given in Table 2.4. 1.0 mgL⁻¹ mixtures of D2, D3, ergosterol (ERG), 7-dehydrocholesterol (7-DHC), and vitamin K1 were prepared. 10.0 mL of this mixture was added into 10.0 mg of MIP or NIP containing vials. Sorption was realized at 50 rpm and 24 hours. Cellulose acetate membranes (0.2 µm pore size) were used to filter the mixtures. Effluents were analyzed by HPLC-DAD at 264 and 280 nm.

Table 2.4. Parameters of the cross-sensitivity assay (n=2)

Standard concentration	1.0 mgL ⁻¹
Sorbent amount	10.0 mg
Sample solution volume	10.0 mL (90:10 H ₂ O:MeOH)
Sorption time	24 h
Shaking speed	50 rpm
Sample solution pH (measured)	7.0
Sorption temperature	25°C

2.5.3. Sorption Performance Comparison with Commercial Sorbents

In order to compare the selectivity of MIP5, cross-sensitivity assay was also performed for HLB and C30 sorbents. A mixture of 10.0 mL of 1.0 mgL⁻¹ D2, D3, ERG, 7-DHC, and vitamin K1 was added to the sample vials containing 10.0 mg of HLB, C30 or MIP5. Sorption was carried out in the shaker at 50 rpm for 24 hours.

Membrane filtration system with cellulose acetate membranes (0.2 μm pore size) was used to filter the mixtures. Effluents were analyzed by HPLC-DAD at 264 and 280 nm. Table 2.5 shows the experimental parameters.

Table 2.5. Parameters used for comparison of MIP5 with commercial sorbents (n=2)

Standard concentration	1.0 mgL^{-1}
Sorbent amount	10.0 mg
Sorbent type	HLB, C30, MIP5
Sample solution volume	10.0 mL (90:10 H_2O :MeOH)
Sorption time	24 h
Shaking speed	50 rpm
Sample solution pH (measured)	7.0
Sorption temperature	25°C

2.6. Optimization Parameters

2.6.1. Effect of Sorption Time

10.0 mL of 1.0 mgL^{-1} D2-D3 solution was added into sample vials containing 10.0 mg MIP5. Sorption was achieved in the shaker at 50 rpm. Samples were taken at specific time intervals as given in Table 2.6 and the mixtures were filtered. Effluents were analyzed by HPLC-DAD at 264 nm.

Table 2.6. Studied parameters in sorption time determination (n=2)

Standard concentration	1.0 mgL^{-1}
Sorbent amount	10.0 mg
Sample solution volume	10.0 mL (90:10 H_2O :MeOH)
Sorption time	5 min, 15 min, 30 min, 1 h, 2 h, 3 h 5 h, 8 h, 24 h
Shaking speed	50 rpm
Sample solution pH (measured)	7.0
Sorption temperature	25°C

2.6.2. Effect of Sorbent Amount

Effect of sorbent amount on sorption performance of MIP was investigated as follows: MIP5 sorbents weighed as given in Table 2.7 were taken into amber vials. 10.0 mL of 1.0 mgL⁻¹ D2-D3 solution was added. Sorption was achieved in the shaker at 50 rpm for 8 hours. The filtrated effluents were analyzed by HPLC-DAD at 264 nm.

Table 2.7. Studied parameters in sorbent amount determination (n=2)

Standard concentration	1.0 mgL ⁻¹
Sorbent amount	1.0, 2.5, 5.0, 10.0, 25.0, 50.0 mg
Sample solution volume	10.0 mL (90:10 H ₂ O:MeOH)
Sorption time	8 h
Shaking speed	50 rpm
Sample solution pH (measured)	7.0
Sorption temperature	25°C

2.6.3. Effect of Sample Solution Volume

1.0 mgL⁻¹ D2-D3 solutions were prepared in different volumes as described in Table 2.8. They were added into the sample vials containing 10.0 mg MIP5. Sorption was carried out in the shaker at 50 rpm for 8 hours. The mixtures were filtered through cellulose acetate membranes (0.2 µm pore size) and effluents were analyzed by HPLC-DAD at 264 nm.

Table 2.8. Studied parameters in sample solution volume determination (n=2)

Standard concentration	1.0 mgL ⁻¹
Sorbent amount	5.0 mg
Sample solution volume	1.0, 5.0, 10.0, 15.0, 25.0, 50.0 mL (90:10 H ₂ O:MeOH)
Sorption time	8 h
Shaking speed	50 rpm
Sample solution pH (measured)	7.0
Sorption temperature	25°C

2.6.4. Effect of Eluent Type

Six different solutions given in Table 2.9 were tried for the elution of vitamin D2 and D3 from MIP5 sorbent. 5.0 mL of 1.0 mgL⁻¹ D2-D3 solutions were added into the sample vials containing 5.0 mg MIP5 and sorption was performed in the shaker at 50 rpm for 8 hours. The mixtures were then filtered through membrane filtration system by cellulose acetate membranes (0.2 µm pore size) to separate MIP5 from solutions. To carry out D2 and D3 desorption, the resulting MIP5 solids were shaken for 24 hours with 5.0 mL volume of eluents, ethanol (EtOH), ethanol:water (EtOH:H₂O) (90:10), ethanol:acetic acid (EtOH:HOAc) (90:10), methanol (MeOH), methanol:water (MeOH:H₂O) (90:10) and methanol:acetic acid (MeOH:HOAc) (90:10). After filtration, effluents were analyzed by HPLC-DAD at 264 nm.

Table 2.9. Studied parameters in eluent type determination (n=2)

Standard concentration	1.0 mgL ⁻¹
Sorbent amount	5.0 mg
Sample solution volume	5.0 mL (90:10 H ₂ O:MeOH)
Sorption time	8 h
Sample solution pH (measured)	7.0
Eluent type	EtOH, MeOH, EtOH:H ₂ O (90:10), MeOH:H ₂ O (90:10), EtOH:HOAc (90:10), MeOH:HOAc (90:10)
Eluent volume	5.0 mL
Desorption time	24 h
Shaking speed	50 rpm
Sorption and desorption temperature	25°C

2.6.5. Effect of Desorption Time

5.0 mL of 1.0 mgL⁻¹ D2-D3 solution was added into sample vials containing 5.0 mg MIP5. Sorption was achieved in the shaker at 50 rpm for 8 hours. After sorption, MIP5 solids were obtained by filtration. Desorption was performed with 5.0 mL of methanol at different time intervals as given in Table 2.10. The mixtures were filtered through cellulose acetate membranes (0.2 µm pore size) and effluents were analyzed by HPLC-DAD at 264 nm.

Table 2.10. Studied parameters in desorption time determination (n=2)

Standard concentration	1.0 mgL ⁻¹
Sorbent amount	5.0 mg
Sample solution volume	5.0 mL (90:10 H ₂ O:MeOH)
Sorption time	8 h
Sample solution pH (measured)	7.0
Eluent type	MeOH
Eluent volume	5.0 mL
Desorption time	1 h, 3 h, 5 h, 8 h, 24 h
Shaking speed	50 rpm
Sorption and desorption temperature	25°C

2.7. Applicability of the Developed MISPE Method

The applicability of the developed MISPE method was evaluated by analyzing liquid vitamin D3 supplementation which contains 0.375 mg vitamin D3 in each 1.0 mL oral drop. The supplementation was diluted to 1.0 mgL⁻¹ in H₂O:MeOH (90:10 (v/v)) and the batch-type SPE process was applied to MIP5 with optimized parameters. The parameters are given in Table 2.11.

Table 2.11. Parameters used during the analysis of vitamin D3 supplementation

Sample type	Vitamin D3 supplementation
Analyte concentration	1.0 mgL ⁻¹
Sorbent amount	5.0 mg
Sample solution volume	5.0 mL (90:10 H ₂ O:MeOH)
Eluent volume	5.0 mL (MeOH)
Sorption and desorption time	8 h and 24 h
Sample solution pH (measured)	7.0
Shaking speed	50 rpm
Sorption and desorption temperature	25°C

CHAPTER 3

RESULTS AND DISCUSSION

3.1. Instrumentation of HPLC

Instrumental parameters given in Table 2.1 were used throughout the study. The chromatogram obtained under these operating conditions is given in Figure 3.1.

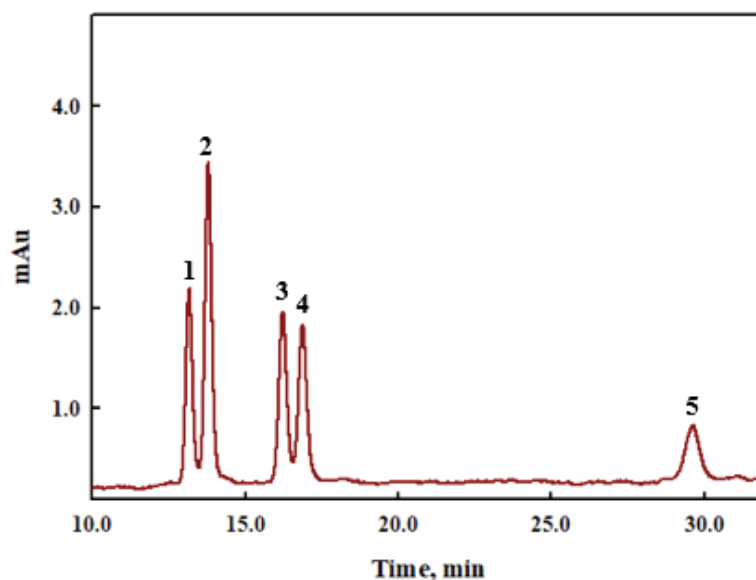


Figure 3.1 A representative chromatogram at the concentration of 1.0 mgL^{-1} obtained by the chromatographic method utilized for vitamin D derivatives and vitamin K1 (1) vitamin D2, (2) vitamin D3, (3) ergosterol, (4) 7-dehydrocholesterol, (5) vitamin K1

The resolution of the D2 and D3 peaks and the ergosterol and 7-dehydrocholesterol peaks were calculated as 1.44 and 1.10, respectively, using the formula given below.

$$R = \frac{t_{R2} - t_{R1}}{\frac{1}{2}(W_1 + W_2)} \quad \text{Equation 3.1.}$$

The calibration graph for D2, D3, ergosterol, 7-dehydrocholesterol and vitamin K1 is shown in Figure 3.2.

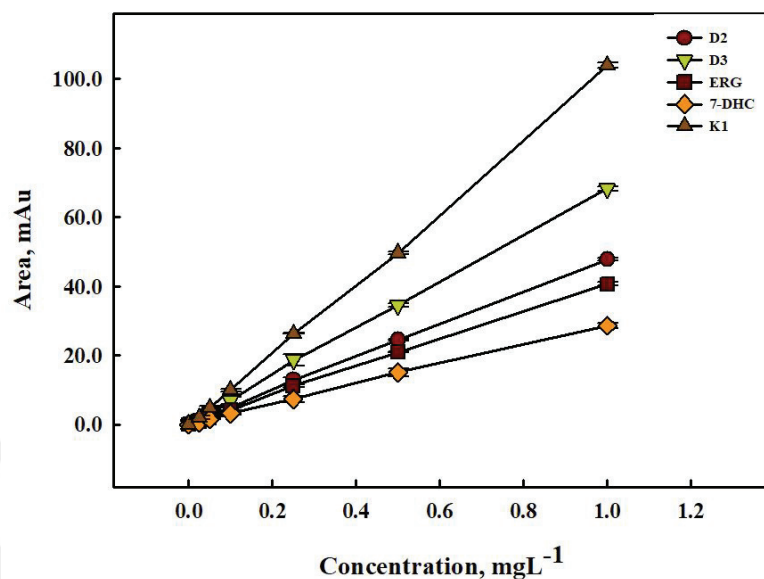


Figure 3.2. Calibration plots for vitamin D derivatives and vitamin K1 obtained by HPLC-DAD

Also, calculated LOD, LOQ, linear equation of calibration curve and R^2 values are given in Table 3.1.

Table 3.1. LOD, LOQ, linear equation, R^2 and wavelength values for vitamin D derivatives and vitamin K1

	LOD (mgL ⁻¹)	LOQ (mgL ⁻¹)	Linear Equation	R ²	Wavelength (nm)
D2	0.031	0.094	$y = 47.526x + 0.410$	0.9987	264
D3	0.018	0.055	$y = 67.869x + 0.592$	0.9980	264
ERG	0.027	0.082	$y = 40.359x + 0.414$	0.9983	280
7-DHC	0.064	0.193	$y = 28.316x + 0.440$	0.9955	280
K1	0.019	0.057	$y = 102.93x - 0.212$	0.9993	264

3.2. Solubility and Stability of Vitamin D2

The absorbances of two different vitamin D2 solutions (in methanol, and water:methanol (90:10)) prepared in the concentration range of 0.50 and 25.0 mgL⁻¹ were measured at 264 nm against the corresponding blank using UV/Vis spectrophotometer. The absorbances of the solutions were compared and the solubility of vitamin D2 was evaluated. As seen in Figure 3.3, the results obtained at concentrations below 5.0 mgL⁻¹ are in agreement with each other. However, different results were obtained from both solutions at concentrations of 5.0 mgL⁻¹ and above, and as the concentration increased, the difference between them gradually began to widen. From this, it was concluded that the solubility of vitamin D2 prepared in a 90:10 ratio of water:methanol mixture decreased at concentrations of 5.0 mgL⁻¹ and above. Therefore, 1.0 mgL⁻¹ was determined as the highest concentration to be worked in SPE experiments.

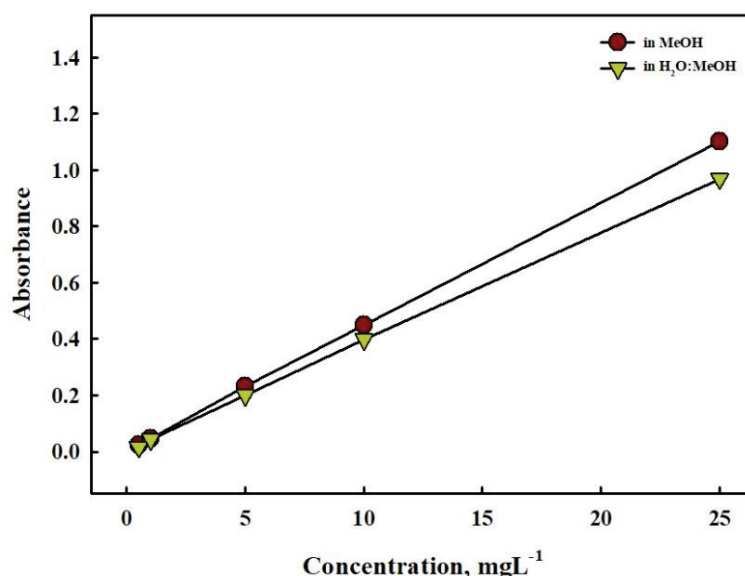


Figure 3.3. Absorbance comparison of vitamin D2 solutions at different concentrations prepared in MeOH and H₂O:MeOH (90:10 (v/v))

In addition, the stability of vitamin D2 solid standard was investigated. Figure 3.4 shows the change in concentration of 1.0 mgL⁻¹ vitamin D2 solutions diluted from monthly prepared stock solutions over time. While the amount of D2 was stable in the first two years, it started to decrease after 2 years and decreased to 0.05 mgL⁻¹ at the end

of 30 months. This can be explained by the fact that oxygen entering the bottle where the solid standard is stored attacks the double bond of vitamin D₂ and causes air oxidation.

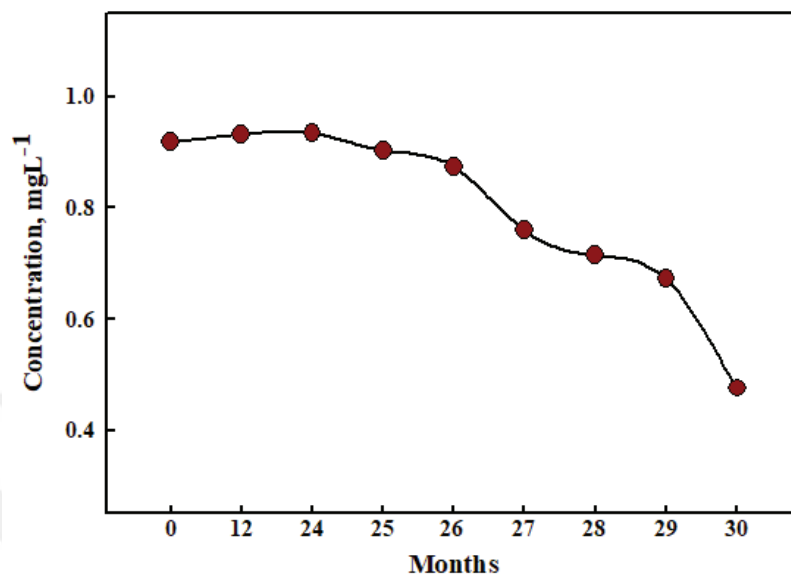


Figure 3.4. Change of D₂ amount in freshly prepared stock solutions over time

There is also a noticeable color difference between the fresh and decomposed solid standard (Figure 3.5). From all these, it was inferred that the decrease in concentration and color change are among the factors that can be followed in order to determine whether the D₂ solid standard decomposes.

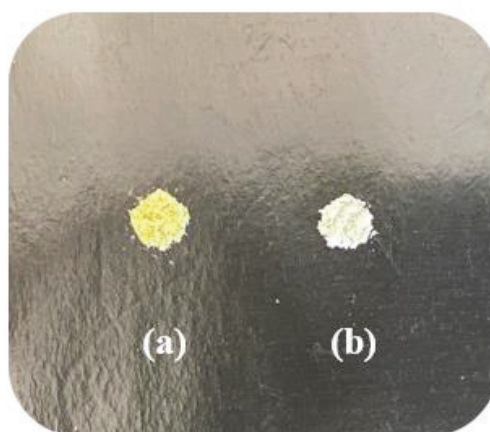


Figure 3.5. Color difference between (a) decomposed and (b) fresh vitamin D₂ solid standard

3.3. Synthesis of Molecularly Imprinted and Non-Imprinted Polymers (MIPs and NIPs)

Different types of MIPs were synthesized for the selective determination of vitamin D. Among all syntheses mentioned in Section 2.4, the highest selectivity difference in cross-sensitivity experiments was achieved with MIP5. Figure 3.6 shows the schematic illustration of MIP5 synthesis.

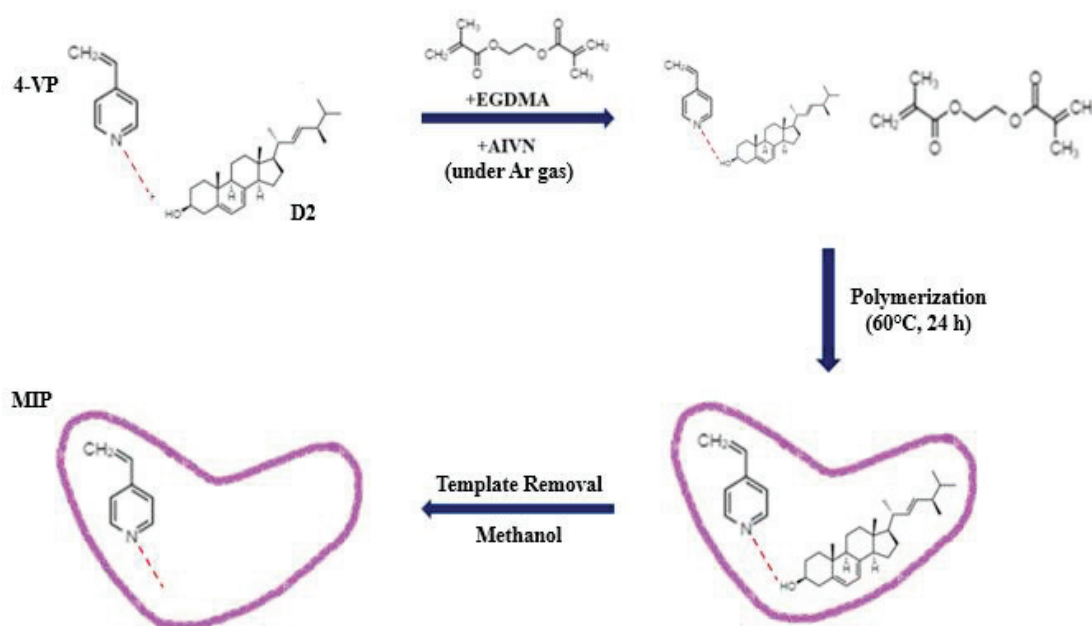


Figure 3.6. Synthesis of MIP5 by copolymerization of 4-VP and EGDMA

The most crucial point in MIP synthesis is template removal. During SPE experiments, it should be ensured that the template molecule is completely removed to prevent template bleeding. After MIP5 synthesis, washing process was applied as outlined in Section 2.4 to remove template D2 from MIP5. After washing process, the effluents were analyzed by HPLC-DAD. The chromatogram of D2 in Figure 3.7 indicates complete removal of D2 from MIP5 after the 15th wash.

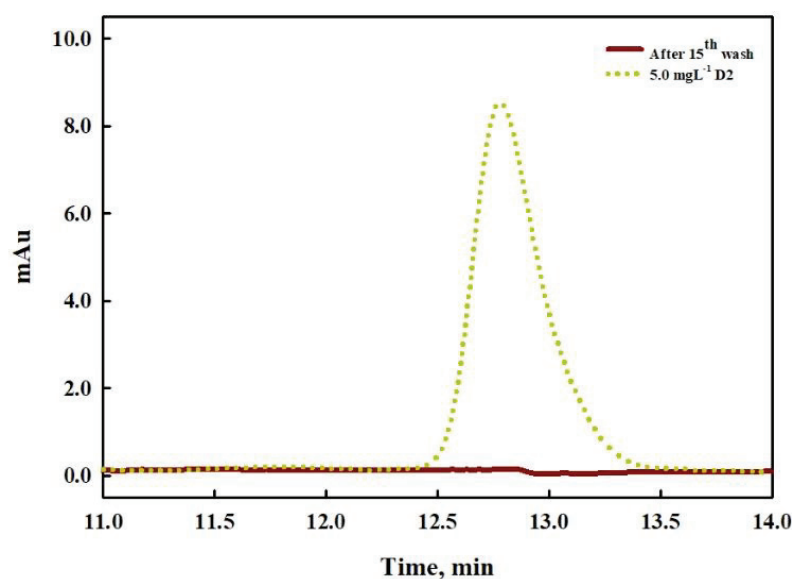


Figure 3.7. Comparison of the D2 peak obtained after the 15th wash of MIP5 with the peak belongs to 5.0 mgL⁻¹ D2

FTIR spectroscopy of MIP5/NIP5 was performed to identify functional groups present in the obtained MIP/NIP particles. In addition, FTIR spectra of MIP5 before and after washing were compared to confirm formation of template-monomer complex. The FTIR spectra of polymers as seen in Figure 3.8 were recorded using ATR method.

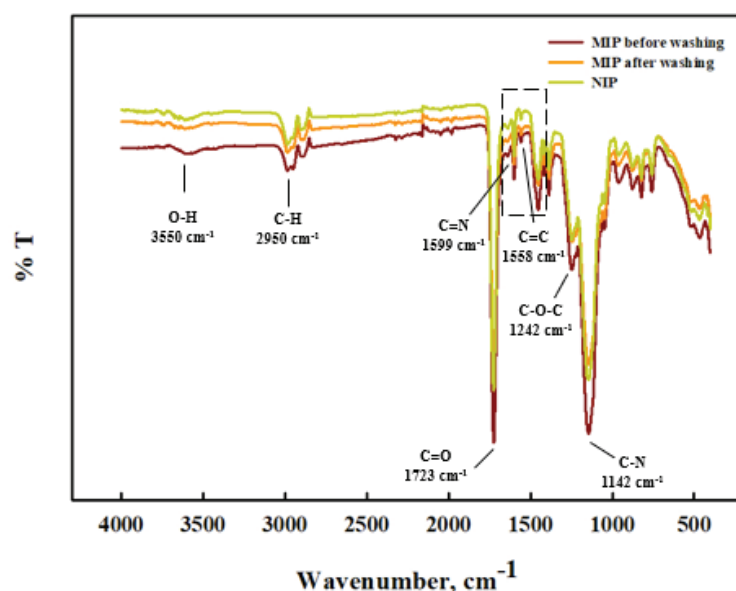


Figure 3.8. Fourier transform infrared (FTIR) spectra of MIP5 before washing, MIP5 after washing and NIP5

The region indicated by the dashed line in all spectra is thought to belong to 4-vinylpyridine (4-VP), which is used as the monomer in the synthesis. The 1599, 1558 and 1142 cm^{-1} peaks represent the C=N, C=C (aromatic) and C-N functional groups of 4-vinylpyridine, respectively. Peaks denoting C=O (ester) and C-O-C functional groups of ethylene glycol dimethacrylate (EGDMA) used as the crosslinker in the synthesis were observed at 1723 and 1242 cm^{-1} , respectively. The peak at 2950 cm^{-1} was attributed to the functional group of C-H found in the structure of both monomer and crosslinker. In line with the similarities in the FTIR spectra of MIP5 and NIP5, it can be said that these two polymers have similar backbone structures. Besides, in the spectrum of MIP before washing unlike NIP, it is possible to state that the band appearing at 3550 cm^{-1} is due to OH stretching present in ergocalciferol structure. The broad character of the peak supports that OH moiety is hydrogen bonded which possibly is formed between the monomer (4-VP) and the template molecule (ergocalciferol). This peak, which was not observed in MIP after washing, also proves that template removal was accomplished successfully.

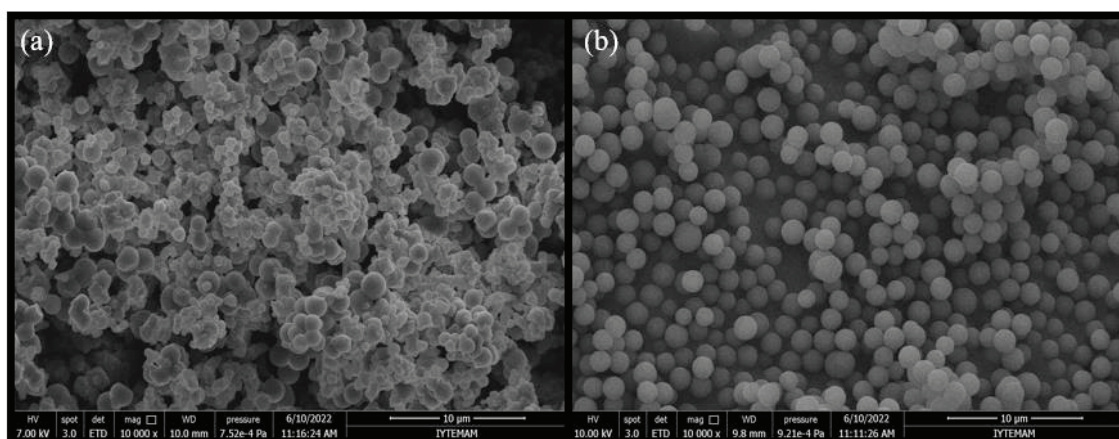


Figure 3.9. SEM images of (a) MIP5 and (b) NIP5

In the synthesis of MIP5 and NIP5, precipitation polymerization method was used since bulk polymerization requires grinding and sieving processes that damage the binding sites of the synthesized polymers and cause a decrease in sorption capacity. The type of polymerization and the morphology of polymers can be determined before synthesis as considering the ratio of total monomer to porogen (w/v %). When this ratio is less than 5%, precipitation polymerization takes place and spherical polymer particles are formed. However, over 5% ratio results in bulk polymerization and polymer

particles of varying sizes with monolithic morphology are synthesized (Olcer et al. 2017). In the case of MIP5/NIP5, the ratio of total monomer to porogen was calculated as 1.97%, so the polymer particles are expected to have a spherical morphology via precipitation polymerization. Morphological characterization of the synthesized polymers was performed by SEM. The images in Figure 3.9 show that both MIP5 and NIP5 particles are spherical in shape. Considering the size of the polymer particles, both sizes are in the micro-range. The average particle size for MIP5 and NIP5 are 1.12 (± 0.58) μm and 1.56 (± 0.24) μm , respectively. Thus, it can be said that both MIP5 and NIP5 have almost the same size distribution.

BET analysis was performed to determine the specific surface area and porosity properties of MIP5 and NIP5. The specific surface area, pore volume, and pore diameter of polymers are given in Table 3.2. The MIP5 exhibited a larger surface area and pore volume than NIP5. Considering the similarity of the MIP5/NIP5 pair in terms of surface morphology and particle size, the higher surface area and pore volume of MIP than NIP can be explained by the presence of cavities originating from the template molecule. According to the International Union of Pure and Applied Chemistry (IUPAC) classification, the pore diameter sizes of MIP5 and NIP5 in the range of 20-500 Å indicate that both polymers are in mesoporous form. Generally, the synthesized MIP is expected to have a larger pore size than NIP, since the presence of the template molecule will limit the shrinkage of the pores during the polymerization process. The fact that NIP5 has a larger pore diameter than MIP5 does not mean that binding sites could not be created in MIP. Therefore, the formation of specific binding sites on the MIP5 particles will be indeed confirmed by the binding capacity of D2 in selective and competitive binding assays.

Table 3.2. BET results of MIP5 and NIP5

	Surface Area (m^2g^{-1})	Pore Volume (cm^3g^{-1})	Pore Size (Å)
MIP5	6.49	0.032	196.89
NIP5	4.27	0.023	216.23

3.4. Characterization Experiments

3.4.1. Binding Characteristic Assay

The main purpose of this study is to provide a sensitive and accurate determination of vitamin D to guide the evaluation of its effect on serum 25(OH)D level. Before the analyzes to be carried out in the serum, the proteins that bind to vitamin D must be removed and the vitamin released. This process, called protein precipitation (PP), is usually carried out with the help of organic solvents such as acetone, ethanol, methanol and acetonitrile (Zhang et al. 2020).

Firstly, methanol was tried as the sorption solvent considering the protein precipitation process. Experiments were carried out as described in Section 2.5.1. The low sorption results indicate that D2 prefers to stay in solution rather than bind to the MIP1 or NIP1 (Figure 3.10). Therefore, it was decided that this solvent is not appropriate for the sorption of D2.

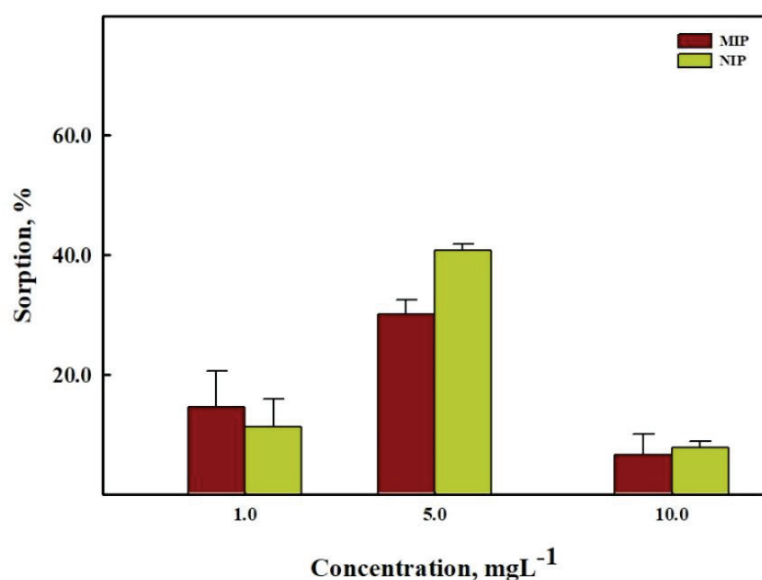


Figure 3.10. Sorption capacities of MIP1/NIP1 using methanol as sample solution (sorbent amount of 10.0 mg, sample solution volume of 10.0 mL, sorption time of 24 h)

Acetonitrile, which is the most widely used solvent in the PP process, has also been tried as the sorption solvent for MIP1/NIP1. Experiments were performed as

described in Section 2.5.1. A good sorption result could not be obtained due to the strong hydrogen bonds between the acetonitrile and D2 (Figure 3.11).

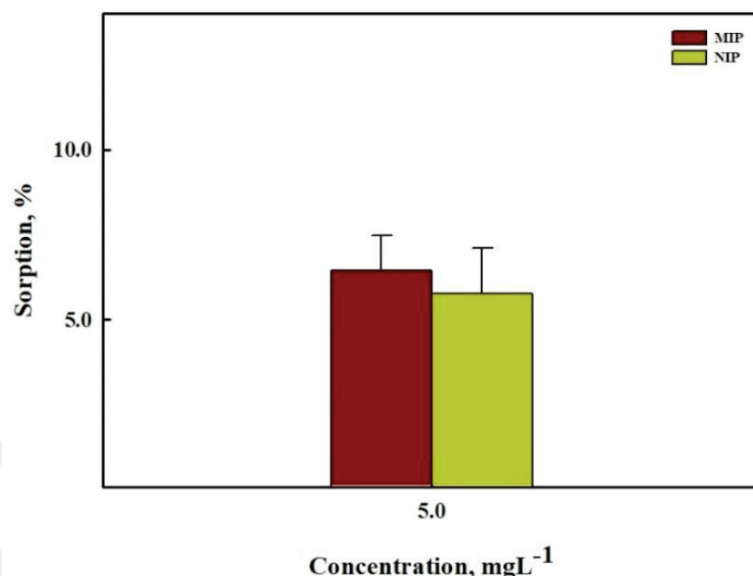


Figure 3.11. Sorption capacities of MIP1/NIP1 using acetonitrile as sample solution (sorbent amount of 10.0 mg, sample solution volume of 10.0 mL, sorption time of 24 h)

Since D2, which is in the fat-soluble vitamin class, has limited water solubility, it has been tried to promote the interaction between D2 and MIP1/NIP1 with different water:methanol ratios. These ratios were determined as 85:15, 90:10 and 95:5, considering the octanol-water partition coefficient of D2 ($\log P$: 7.4). The binding assay was applied at the concentration of 5.0 mgL⁻¹ D2. When the sorption performances of the MIP1/NIP1 couple were compared in all sorption solvents tried at this concentration, the best results were obtained with water:methanol mixtures (Figure 3.12). Among the ratios used, the highest sorption percentage (around 70%) was observed at the ratio of 90:10 although there is no difference between MIP and NIP. Therefore, H₂O:MeOH (90:10 v/v) was chosen as the sample preparation solvent.

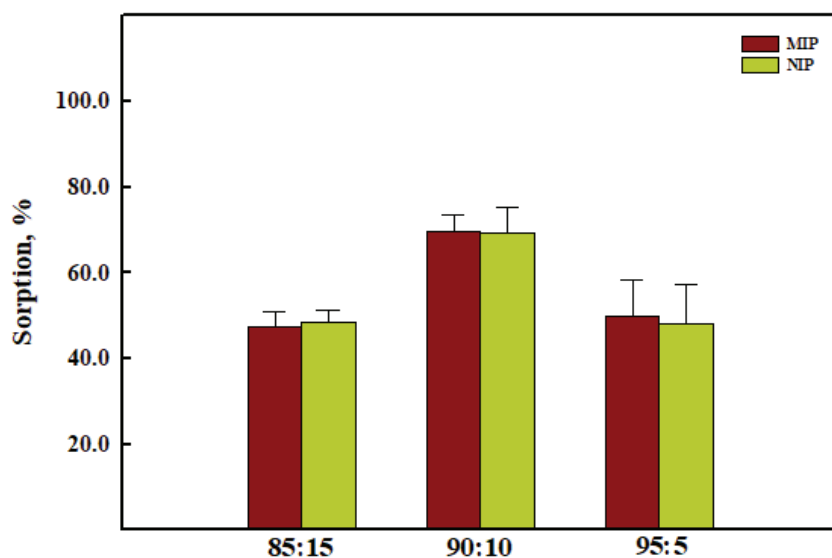


Figure 3.12. Sorption capacities of MIP1/NIP1 using different ratios of H₂O:MeOH as sample solution (sorbent amount of 10.0 mg, sample solution volume of 10.0 mL, sorption time of 24 h)

The sorption capacities of MIP1/NIP1 are shown in Figure 3.13. The experiments were carried out in H₂O:MeOH (90:10 v/v)) at different concentrations ranging between 1.0 and 25.0 mgL⁻¹ to evaluate the sorption difference between MIP1 and NIP1. The ordinate, Q, shows the ratio of mmol of D2 to 10.0 mg of MIP/NIP.

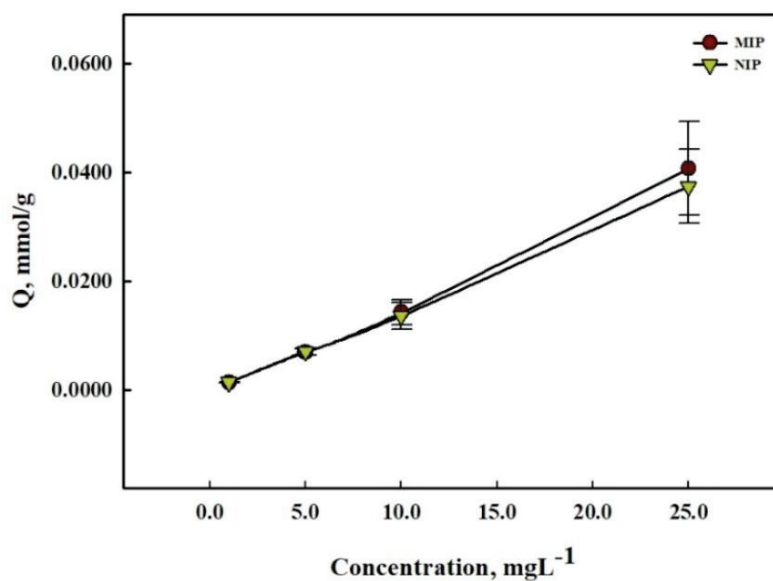


Figure 3.13. Binding characteristic assay of MIP1/NIP1 (sorbent amount of 10.0 mg, sample solution volume of 10.0 mL, sorption time of 24 h)

The difference in the sorption capacities of MIP1 and NIP1 is clearly seen at 25.0 mgL⁻¹. The higher sorption performance of MIP1 can be explained as the presence of created D2 cavities. However, it has been found that the solubility of fat-soluble vitamin D2 in 90:10 H₂O:MeOH solution decreased at concentrations above 1.0 mgL⁻¹. The high standard deviation of MIP1/NIP1 at these concentrations shows that the results obtained are not reliable and it is possible to explain this with the solubility problem encountered at high concentrations explained in Section 3.2.

Polymers (MIP4/NIP4 and MIP5/NIP5) synthesized with 4-vinylpyridine monomer at a 1:6:30 (T:M:C) molar ratio showed almost 100% sorption at all analyte concentrations from 0.05 mgL⁻¹ to 1.0 mgL⁻¹. Figure 3.14 represents the ratio of ergosterol or D2 mmol bound to 10.0 mg of MIP4 or MIP5 and NIP4 or NIP5 at different concentrations. There is no difference in sorption performance between MIPs and NIPs, although this is to be expected at low analyte concentrations. At low concentrations, firstly, non-specific binding sites fill up. The difference in sorption performance between MIP and NIP is usually evident at concentrations above 25 mgL⁻¹. However, these concentrations, where the created selective binding sites in MIP come into play, are not suitable for work due to the limited solubility of vitamin D metabolites in aqueous media.

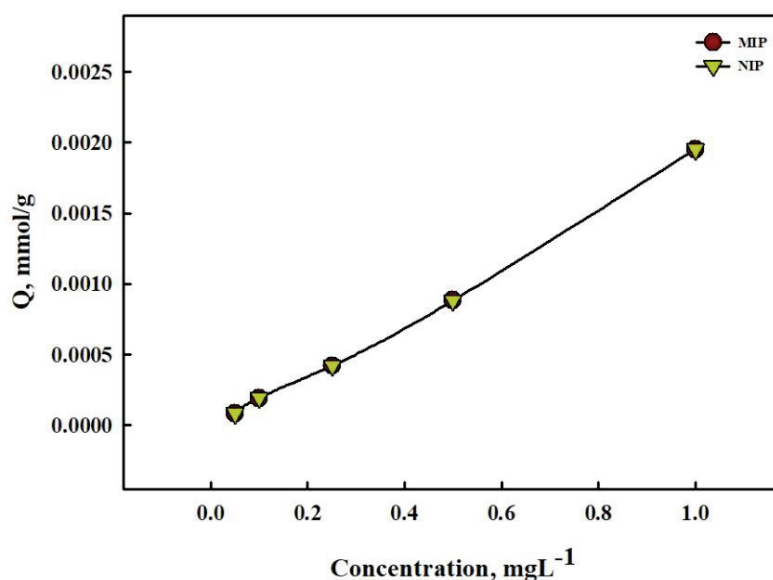


Figure 3.14. Binding characteristic assay of MIP4/NIP4 and MIP5/NIP5 (sorbent amount of 10.0 mg, sample solution volume of 10.0 mL, sorption time of 24 h)

3.4.2. Cross-Sensitivity Assay

Vitamins D and K are both fat-soluble vitamins and are known to play an important role in calcium metabolism. Calcium, the level of which increases in the body with vitamin D, is used by being transported to the bones through vitamin K. Increasing vitamin D intake without adequate vitamin K causes the body to not be able to use the increased calcium effectively. This increases the risk of calcium deposition in arteries and soft tissue (van Ballegooijen et al. 2017). Considering this relationship between vitamin D and K, vitamin K1 was also used in cross-sensitivity experiments. The structures of vitamin D derivatives and vitamin K1 are shown in Figure 3.15.

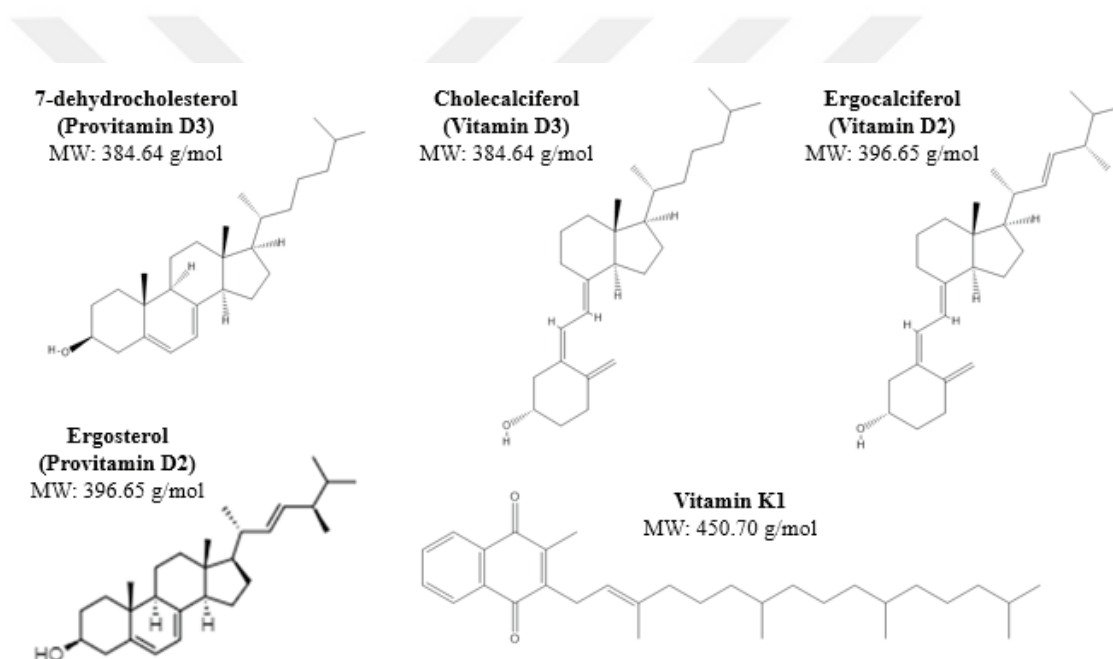


Figure 3.15. Structures of vitamin D derivatives and vitamin K1

As any difference was not observed between MIP4/NIP4 and MIP5/NIP5 with regard to sorption capacity at the concentrations worked, the selectivity performance of these polymer pairs was compared by cross-sensitivity assay. The results for MIP4/NIP4 and MIP5/NIP5 are given in Figure 3.16 and Figure 3.17, respectively.

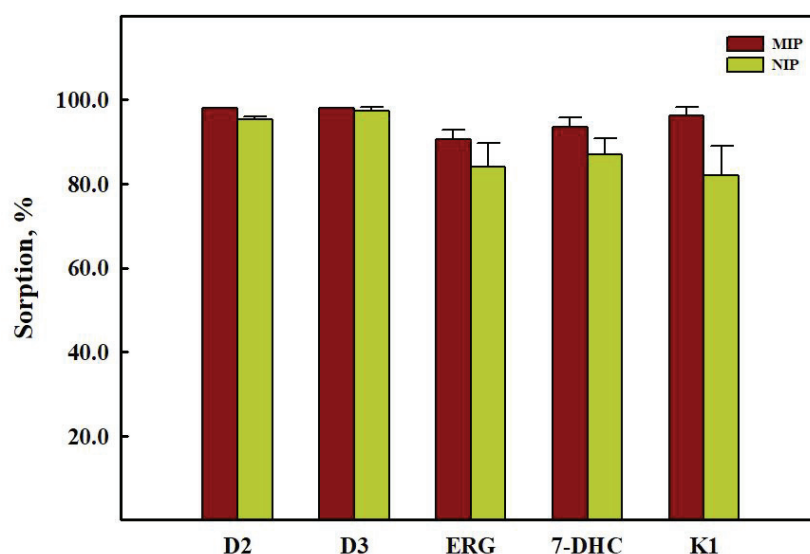


Figure 3.16. Cross-sensitivity assay of MIP4/NIP4 (standard concentration of 1.0 mgL^{-1} , sorbent amount of 10.0 mg , sample solution volume of 10.0 mL , sorption time of 24 h)

As seen in Figure 3.16, the synthesized MIP did not show selectivity against D2 and D3 or even against its template molecule, ergosterol. Besides the synthesized MIP4 did not have a high selectivity over NIP4, both showed high sorption performance towards the fat-soluble vitamin group. This is due to the presence of non-specific binding sites and especially being easily accessible on the surface and being filled first. When the sorption percentages of MIP4 and NIP4 were compared, the highest difference (14%) was observed for vitamin K1. While this difference is not significant, it indicates that the synthesized MIP was not for the purpose of the study.

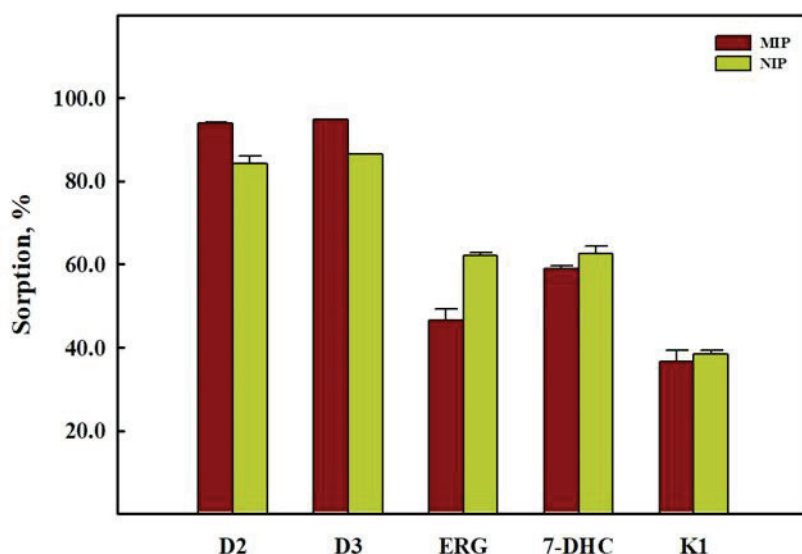


Figure 3.17. Cross-sensitivity assay of MIP5/NIP5 (standard concentration of 1.0 mgL^{-1} , sorbent amount of 10.0 mg, sample solution volume of 10.0 mL, sorption time of 24 h)

When Figure 3.17 was examined, it was seen that the sorption capacity of the MIP5/NIP5 pair was quite high for D2 and D3 compared to other analytes. Also, NIP outperformed MIP against ergosterol and 7-dehydrocholesterol. Based on these points, it can be concluded that the synthesized MIP is selective against the prohormone forms of vitamin D.

The lowest percentage of sorption for both MIP and NIP belongs to vitamin K1. Vitamin K1 is the largest of the molecules used in cross-sensitivity experiments. Therefore, its binding to MIP or NIP may occur with a slower kinetics than others, as its size restricts its movement in a crowded environment.

MIP5 allowed all analytes to bind to it, albeit in a low percentage. The reason for this situation can be explained as follows: In this synthesis, in which D2 is used as a template, the created binding sites are expected to be either specific to D2 or selective to D2 and D3. However, it is not possible for this molecule or molecules to close all binding sites at the analyte concentration worked (1.0 mgL^{-1}). Considering the similarity of structure and size of the fat-soluble vitamin groups, especially vitamin D metabolites, it is very likely that they are sorbed into MIP at this concentration. The selectivity of MIP might be seen more clearly if it could be studied at higher concentrations.

As a result, MIP5, working selectively to a group of molecules, enabled the co-determination of D2 and D3. Therefore, the parameters (sorption time, sorbent amount, sample solution volume, etc.) of the method using MIP5 as sorbent were simultaneously optimized for D2 and D3.

3.4.3. Sorption Performance Comparison with Commercial Sorbents

In Section 3.4.2, it was demonstrated that MIP5 has selectivity over D2 and D3 in the presence of structurally related compounds compared to NIP5. The selective sorption performance of MIP5 was also compared with two different commercial SPE sorbents, HLB and C30, in an additional cross-sensitivity experiment. Figure 3.18 indicates that HLB and C30 show no selectivity for vitamin D derivatives and vitamin K1. This can be cited as evidence for the evaluation of synthesized MIP5 as a selective sorbent for D2 and D3.

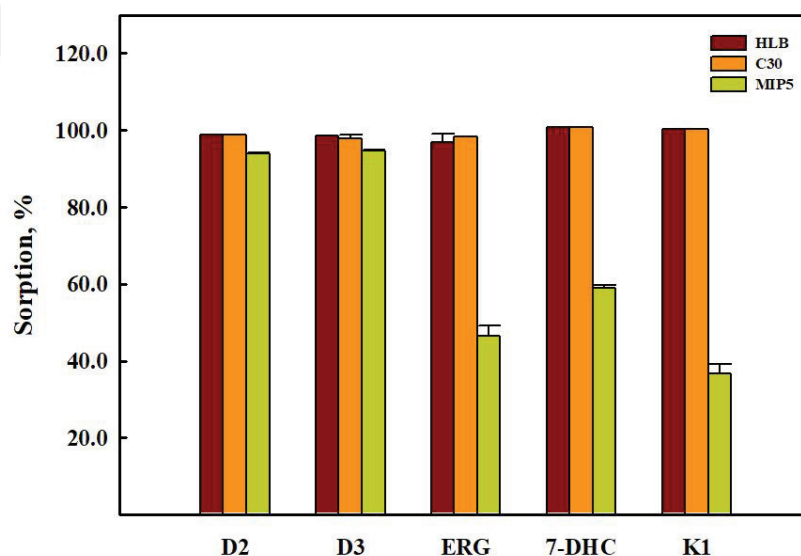


Figure 3.18. Comparison of selective sorption performance of MIP5 with commercial HLB and C18 sorbents (standard concentration of 1.0 mgL⁻¹, sorbent amount of 10.0 mg, sample solution volume of 10.0 mL, sorption time of 24 h)

3.5. Optimization Parameters

3.5.1. Determination of Sorption Time

Since the binding of the analyte to the sorbent is dependent on the contact time, the first parameter to be optimized in SPE experiments is the sorption time. The 24-hour sorption time used in characterization studies is quite long and impractical. Therefore, different time intervals given in Table 2.6 were tried and it was evaluated whether sorption would take place in a shorter period of time.

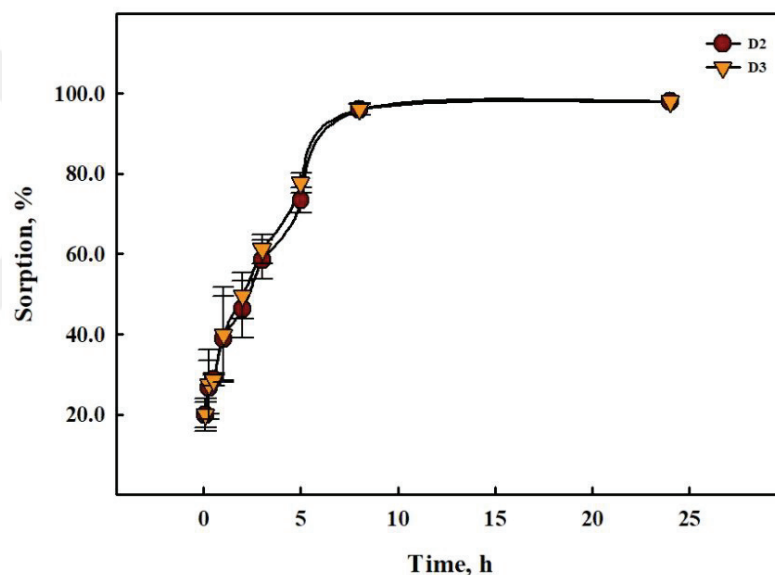


Figure 3.19. Effect of sorption time (standard concentration of 1.0 mgL^{-1} , sorbent amount of 10.0 mg , sample solution volume of 10.0 mL)

Figure 3.19 shows the effect of the interaction time between the synthesized MIP and vitamins D2-D3 on sorption. Based on this graph, it can be said that MIP has reached its maximum sorption at 8 hours. SPE sorption studies to be carried out after that will be continued with a sorption time of 8 hours.

3.5.2. Determination of Sorbent Amount

In characterization experiments, the sorption of D2 and D3 to 10.0 mg MIP was achieved with approximately 100%. The different amounts of sorbent given in Table 2.7 were studied both to evaluate the effect of the sorbent amount on the sorption performance and to control the attainability of maximum sorption with an amount of MIP less than 10.0 mg.

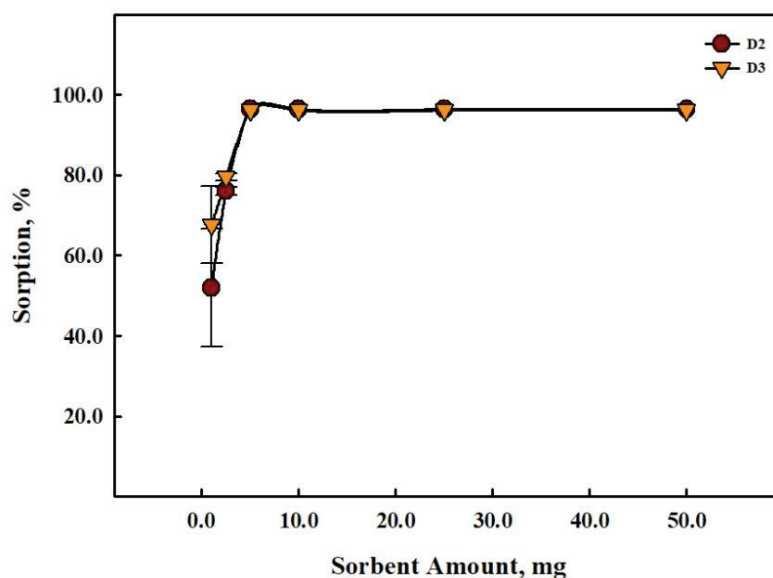


Figure 3.20. Effect of sorbent amount (standard concentration of 1.0 mgL^{-1} , sample solution volume of 10.0 mL, sorption time of 8 h)

Increasing the amount of sorbent allows for an increase in both the surface area and the binding sites. This ensures the sorption percentage to increase gradually and reach a constant value after a certain amount. In the case of MIP5, the percentage of sorption increased almost linearly up to 5.0 mg, maxing out at this point, and remained unchanged at higher amounts (Figure 3.20). Therefore, it was decided to use MIP5 as 5.0 mg, which shows maximum performance in amounts between 5.0 and 50.0 mg, to save sorbent in other optimization studies.

3.5.3. Determination of Sample Solution Volume

The purpose of this experiment is to determine the maximum volume of sample solution that can be applied to the selected amount of the MIP. Various volumes of solutions containing D2 and D3 were loaded into the MIP5 and SPE performed as explained in Table 2.8.

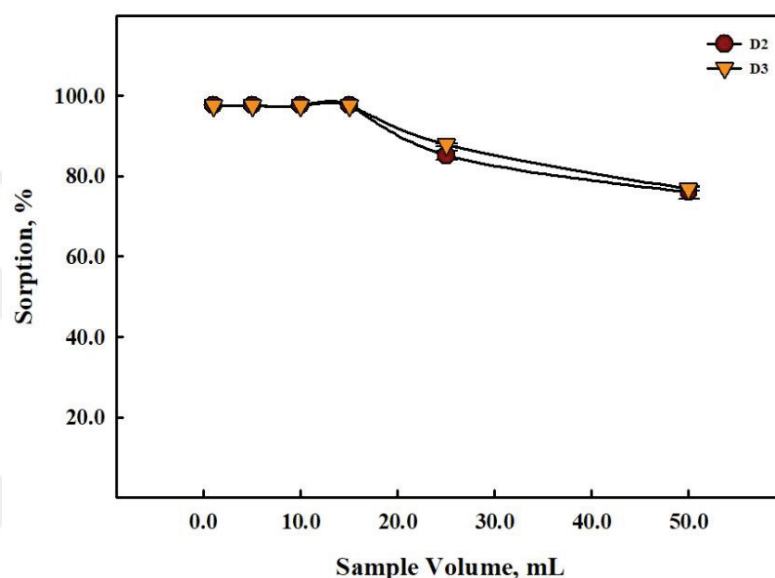


Figure 3.21. Effect of sample solution volume (standard concentration of 1.0 mgL^{-1} , sorbent amount of 5.0 mg , sorption time of 8 h)

As seen in Figure 3.21, MIP5 showed a sorption performance nearly 98% in sample volumes of 1.0 , 5.0 , 10.0 and 15.0 mL . However, it has been observed that this performance decreases as it exceeds 15.0 mL . From this, it is possible to conclude that the capacity and sorption ability of MIP5 is reduced at these volumes. Therefore, 15.0 mL can be considered as the breakthrough volume. As a result of this experiment, the sample solution volume was chosen as 5.0 mL in order to avoid extravagance and to stay in the safe range.

3.5.4. Determination of Eluent Type

In the SPE method, desorption plays an important role as well as sorption. It is therefore required to determine the most suitable eluent for quantitative recovery of the analyte. As can be seen in Figure 3.22, a recovery of over 75% was obtained with all eluent types, but the highest recovery was obtained with methanol (about 90%). In line with these results, the recovery solvent was selected as methanol in the developed method.

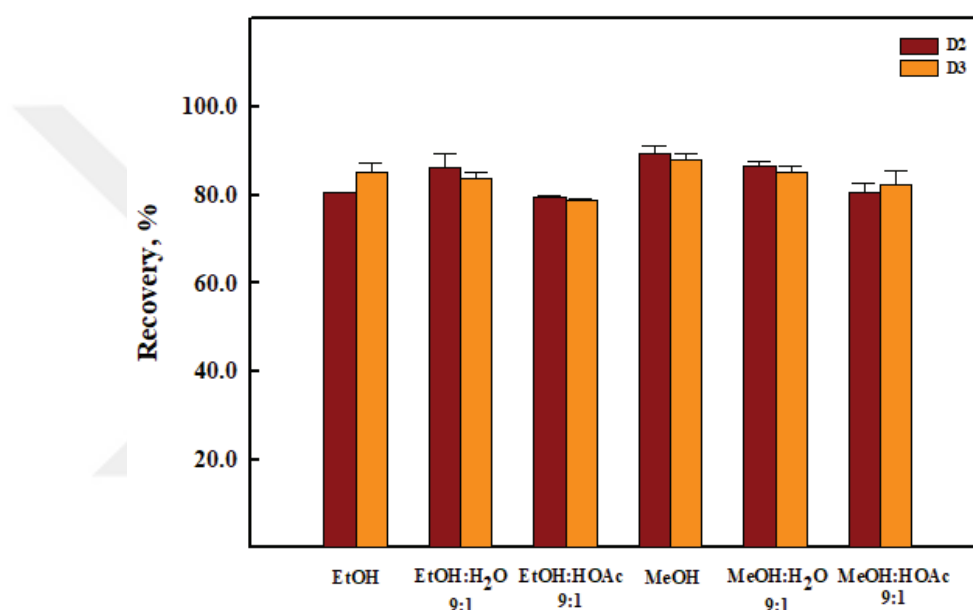


Figure 3.22. Effect of eluent type (standard concentration of 1.0 mgL^{-1} , sorbent amount of 5.0 mg, sample solution volume of 5.0 mL, sorption time of 8 h)

3.5.5. Determination of Desorption Time

The effect of shaking time on desorption was investigated at 1, 3, 5, 8 and 24 hours. According to the results shown in Figure 3.23, increasing the shaking time improved the effect of the eluent on the analyte extraction. However, for both D2 and D3, 24 hours are required for desorption to occur with high recovery.

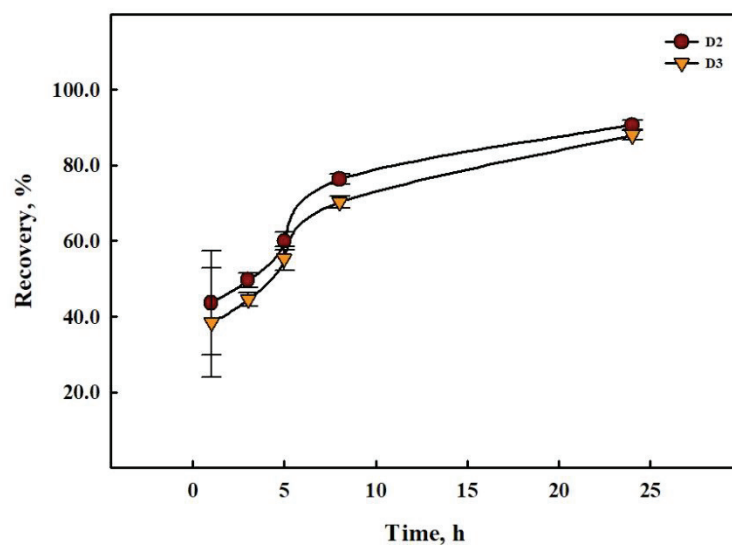


Figure 3.23. Effect of desorption time (standard concentration of 1.0 mgL^{-1} , sorbent amount of 5.0 mg , sample solution volume of 5.0 mL , sorption time of 8 h , eluent type of methanol)

3.6. Applicability of the Developed MISPE Method

In this study, the MISPE method was developed for the simultaneous determination of vitamins D2 and D3. A matrix-matched calibration curve using the optimized parameters of the developed method is proposed for vitamins D2 and D3 as seen in Figure 3.24.

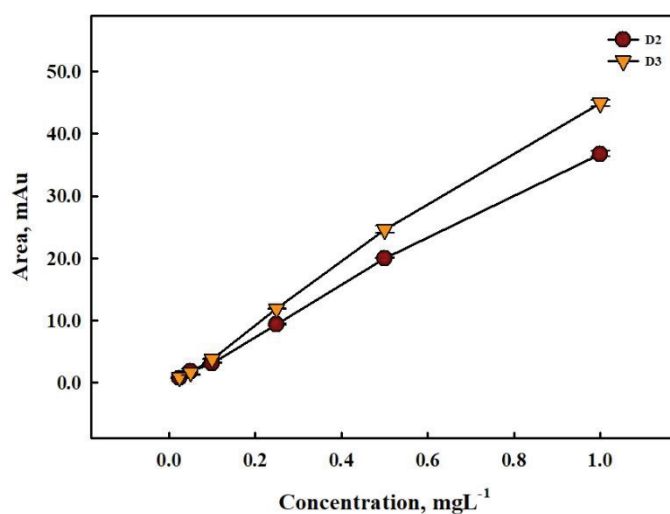


Figure 3.24. Matrix-matched calibration plot for vitamin D2 and D3

Also, calculated LOD, LOQ, linear equation of calibration curve and R^2 values are given in Table 3.3. It is obvious that the limit of quantification (LOQ) values obtained by the developed MISPE method reach the expected vitamin D concentrations in biological samples.

Table 3.3. LOD, LOQ, linear equation and R^2 values of matrix-matched calibration curve for vitamin D2 and D3

	LOD mgL⁻¹	LOQ mgL⁻¹	Linear Equation	R²
Vitamin D2	0.008	0.023	$y = 37.474x - 0.0746$	0.9974
Vitamin D3	0.006	0.018	$y = 45.968x - 0.1505$	0.9967

The applicability of the method was evaluated by analyzing the sample of vitamin D3 supplementation with the procedure described in Section 2.7. The overall recovery of spiked 1.0 mgL⁻¹ vitamin D3 was calculated as 95.97% (± 1.84) for n=2.

CHAPTER 4

CONCLUSION

In this study, molecularly imprinted polymers (MIPs) were prepared for selective determination of vitamin D derivatives for use in solid phase extraction prior to HPLC-DAD. Precipitation and sol-gel polymerization methods were used in MIP synthesis and various templates, monomers, crosslinkers and their ratios were tried in precipitation polymerization. Binding characteristic and cross-sensitivity experiments were performed to evaluate whether the desired vitamin D-specific cavities were formed in the synthesized MIPs. Among all MIPs, MIP5, showing selective behavior towards the vitamin D prohormone group, was characterized by SEM, BET and FTIR.

After the characterization experiments, the critical experimental parameters of the MISPE method, which was intended to be developed, were optimized. In the presence of ergosterol, 7-dehydrocholesterol and vitamin K1, the highest selectivity for D2 and D3 ($94.01\% \pm 0.13$ and $94.74\% \pm 0.14$, respectively) was obtained at the analyte concentration of 1.0 mgL^{-1} , therefore optimization studies were performed at this D2 and D3 concentration.

The optimized sorption time, sorbent amount and solvent volume were determined as 8 hours, 5.0 mg and 5.0 mL, respectively. Desorption was carried out with six different polar protic solutions. Recovery over 75% was reached in all eluent types, but maximum recovery was achieved with methanol. ($89.36\% \pm 1.82$ for D2 and $87.98\% \pm 1.37$ for D3). In addition, the time of desorption with methanol was optimized and it was observed that 24 hours were required to elute the maximum amount of both D2 and D3.

The applicability of the developed method was checked via analyzing the sample of vitamin D3 supplementation. 1.0 mgL^{-1} D3 was spiked to the used matrix in the MISPE method, and the total recovery was calculated as $95.97\% (\pm 1.84)$ for $n=2$ from the proposed matrix-matched calibration.

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