

Separation of STIGMA STEROL using magnetic molecularly imprinted nanopolymer fabricated by sol-gel method

Ehsan Moghaddas Kia¹, Mohammad Alizadeh^{2}, Mohammad Reza Vardast³, Mahmoud Rezazad⁴*

Received: 19 Feb, 2017; **Accepted:** 17 Apr, 2017

Abstract

Background & Aims: Magnetically molecularly imprinted polymers (MMIPs) are assumed as kind of sorbent polymers which can separate or determine bioactive compounds from environment fast and specifically. Magnetic properties, stability at various conditions (temperature, ionic strength and pH) and selective function are among the advantages of these polymers in determination of nutraceutical compound. In current research, a molecularly imprinted polymer synthesized by application of Fe₃O₄@SiO₂ nanoparticles and its functional properties were evaluated.

Materials & Methods: In order to fabricate polymer, firstly Co-precipitation method was used for manufacturing of magnetic Fe₃O₄ nanoparticles coated by silica. Then stigmasterol imprinted polymer were prepared by grafting sol-gel procedure. Finally obtained polymer were eluted by mixture of ethanol-water-chloroform to create specific sorbent cavities for stigmasterol. Polymer was incubated with stigmasterol stock solution and binding capacity was determined through high performance liquid chromatography.

Results: Structure and morphology of samples were evaluated by FT-IR spectroscopy and scanning electron microscopy and Zeta sizer was used for determination of their zeta potential. MMIPs could separate 78 % of stigmasterol from stock solution during 60 minutes and its binding capacity was 19.5 mg/g. FT-IR spectrometry and zeta potential data revealed well-designed coating of silica around magnetic Fe₃O₄ cores. Adsorbent silica layers were reinforced through sol-gel polymerization method. Polymer morphology was porous, coarse and particle dimensions were less than 50 nanometers.

Conclusion: So regarding separation and structural characteristics of properties these sorbents, the produced magnetically imprinted nanopolymer can be used for detection of stigmasterol as a nutraceutical compound.

Keywords: Stigmasterol, Molecularly imprinted polymer, magnetic Fe₃O₄@SiO₂

Address: Food science and technology department, Urmia University, Urmia, Iran

Tel: (+98)9143474341

Email: malizadeh@outlook.com

Introduction

Sterols are one of the main components of cell membranes eukaryotic and play an important role in the

growth and physiology of the cell. Plant sterols known as "phytosterols" reduce the absorption of cholesterol through competing with cholesterol in the intestine. On

¹ PhD of Food Science and technology, Urmia University, Urmia, Iran

² Associate Professor, Food Science and technology department, Urmia University, Urmia, Iran (Corresponding Author)

³ Assistant Professor, Medicinal chemistry department, Pharmacy faculty, Urmia University of Medical Sciences, Urmia, Iran

⁴ Associate Professor, Food Science and technology department, Urmia University, Urmia, Iran

the other hand, it reduces the risk of cardiovascular diseases. Phytosterols also reduce the risk of colon cancer and prevent the growth of tumors. It is worth noting that daily consumption of 1.5 to 2 grams of phytosterols resolves the need of the body. According to the consumption amount of 200 to 500 mg per day in the diet, it requires the use of foods enriched with phytosterols to create health-giving properties (3-1). Phytosterols can be found in various food sources such as vegetable oils, seeds and nuts. For example, grapeseed has 570 mg / 100g phytosterol (4). Phytosterols are derived four-ring system of ciclo-pentano perhydro phenanthrene with side chain of 17th carbon atom and hydroxyl group at carbon 3. Most phytosterols contain 28 or 29 carbon atoms and one or two double bonds, usually one in the sterol core and sometimes the latter is in the side chain. Methyl-Free sterols are the most common sterol groups in vegetable oil which contain beta-sitosterol, stigma sterol, campesterol, brassica sterol and Δ -5 avenasterol (2). The components remain in the oil after refining process and the profile of these compounds is different depending on the type of plant sources (oil grain or oil seed). The identification of these compounds is an important marker in food fraud measurement methods and identification of food source (5).

Mixing vegetable oils with animal fats (addition of Palm to milk) and cheap vegetable oil with other oils (soya and palm to olive, palm seed to grape seed) are measurable by measuring phytosterols profiles through HPLC and GC device methods. Due to increased demand for vegetable oil sources in recent years, palm oil and palm kernel have been replaced in the incorporation of more expensive oils (olive, grape seed) and animal oils (milk) due to the cheapness and desirable organoleptic characteristics which the process has been declared a fraud by regulatory bodies (6). Stigma sterol (24 α -ethyl cholest-5,22E-dien-3 β -ol) is the most dominant plant phytosterols and is more in

oilseeds and oilseed kernels (palm kernel) than vegetables and fruits. Given that it is not available in all sources of oil, monitoring the composition can be a suitable solution for fraud detection among food stuffs adding palm oil and identification of the food origin. The stigma sterol is a Nutraceutical combination and several studies have shown the health benefits, such as reducing the absorption of cholesterol in the body, anti-inflammatory properties, immune system regulators, acetylcholinesterase inhibitor and anti-dementia compounds. High levels of cholesterol has long been as one of the reasons for the increased risk of Alzheimer. Studies have shown that cholesterol increases old plaque formation. The plaques consisting of proteins particularly beta-amyloid proteins, deposit in the brain neurons and are a major cause of Alzheimer. Stigma sterol synergistically reduces Beta-amyloid proteins' production (23). Molecularly imprinted polymers today are the most advanced separation, condensation and identification methods of minor compounds in a variety of biological environments. The synthetic compounds have appropriate stability against changes in temperature, pH and ionic environments and can maintain long-term properties of their detector. Therefore, they have advantages for critical systems detection of biological molecules such as ELISA. The most striking feature of molecularly imprinted polymers is their exclusive ability to link with a target molecule or mold. Molecularly imprinted polymers are actually the systems that, functional monomers are polymerized by transverse connectors around a template molecule during their synthesis and then the mold has been taken out from the external structure by washing with solvent and ultimately cause the formation of a specific three-dimensional structure for mold molecules. Considering specific function and high physicochemical stability, the synthetic polymers can be used in the processes of identification, extraction and sensor design. Low detection limits, low cost and the ability to be reused are

other advantages of these polymers. The most common method of molecularly imprinted polymers' synthesis is through bulk polymerization in which an integrated polymer is produced and finally milled into smaller particles and the template molecule are separated. Heterogeneous large polymer components, the degradation of active sites and thus reduction in the load capacity are the main disadvantages of this method. So, other polymerization techniques such as improved sediment, suspension and snap surface have been used (9-7). One of the modern methods of imprinted molecular synthesis is through sol-gel technique on the bases of core-shell containing silane groups (Si-O). Synthetic of molecularly imprinted polymers through magnetic iron nanoparticles with silica capsule ($\text{Fe}_3\text{O}_4 @ \text{SiO}_2$) is one of these new methods which causes the isolation of both polymer and the target molecule through induction of super magnetic properties to polymer. The use of silica capsule for magnetic iron nanoparticles is due to better dispersion in the aquatic environment and to prevent the outflow of magnetic iron nanoparticles from the matrix and prevent the accumulation of Super magnetic particles and their oxidation. In this technique, silica coated nanoparticle was a magnetic core of iron and has an absorbent layer of silica (shell) that target molecule active sites is imprinted on it. This method increases the absorption kinetics of analyte due to the high surface to volume ratio of the polymer, improves selectivity and finally increases separation efficiency in a variety of aqueous and organic solvents in a wide temperature range (11-10).

For the first time in this study, hybrid molecularly imprinted polymer with nanoparticles of magnetite ($\text{Fe}_3\text{O}_4 @ \text{SiO}_2\text{-TEOS}$) has been produced for the separation of stigma sterol molecules and its functional, separation and structure are investigated.

Materials and Methods

Tetra-Ortho-silicate (TEOS) 98%, ammonia 25%, iron chloride with 6 water molecules, iron sulfate with 7 water molecules, hexane from Merck (Germany) and absolute ethanol from Charleroi (Spain) have been prepared. Stigma sterol was prepared from Sigma (England) with the purity of 95%. Mobile phase high-pressure chromatography solutions (2-propanol and acetonitrile with a purity of 99% LiChrosolv®) were prepared from Merck (Germany). Deionized water (DirectQV UV-3, Millipore, France) with water hardness of $\mu\text{S}18$ was used to prepare the aqueous solution.

Preparation of nanoparticle of Fe_3O_4 :

5 mL of 1 M aqueous solution of iron chloride 6 hydrate and 10 ml of 0.5 M of iron sulfate 7 hydrate were mixed together. The above mixture was slowly added into 20 ml of 5.3 M of ammonium hydroxide at 60°C in Ultrasonic (Parasonic 2600S, Iran) over 30 minutes. The magnetic iron nanoparticles formed by a neodymium magnet were separated from the solution and washed successively with water - diluted acid and finally were dried at 55°C in an oven (Mettler EFB 400, Germany) (15).

Magnetic Iron Core Coating:

300 mg of Fe_3O_4 in 200 ml of mixed absolute ethanol: deionized water (2: 8) was dissolved under sonication. Then, 0.7 ml of TEOS and 5 ml of ammonia was added to the solution under high stirring (Heidolph MR -Hei, Germany) and the reaction continued for 12 hours at a temperature of 40°C . The final product was isolated by magnetic field of neodymium magnet and washed with deionized water for 5 times and then dried in an oven temperature of 55°C .

Cholesterol Absorbent Molecular Nanopolymer synthesis on the bases of $\text{Fe}_3\text{O}_4 @ \text{SiO}_2$:

First, 10 ml of Tetra ortho silicate was dissolved within the absolute ethanol under nitrogen flow. Then 40 μ l of hydrochloric acid 37% in 1700 ml was diluted and added to deionized water (diluted acid chain is opening up the possibility of exposure to silica and provides a template molecule). Then, 170 mg $\text{Fe}_3\text{O}_4 @ \text{SiO}_2\text{-TEOS}$ was added to a mixture and was located in a bath sonicator for 6 minutes and was well stirred for 90 minutes at room temperature at a speed of 600 rpm. After this time, 170 mg stigma sterol (template molecules) dissolved in a mixture of absolute ethanol: chloroform (1: 3) and was added to $\text{Fe}_3\text{O}_4 @ \text{SiO}_2\text{-TEOS}$. Finally, polymer crystallization began by adding 300 ml of ammonia. After a 30-minute break, homogeneous mixture became solid and was transferred

to the refrigerator. After 18 hours of refrigerate storage, the polymer was obtained. Washing polymer was washed 5 consecutive times using a mixture of ethanol: deionized water: chloroform (1: 2: 7) to take out stigma sterol from polymer structure. Centrifuge was performed at 4000 rpm for 10 minutes (MiniSpin $\text{\textcircled{R}}$ Eppendorf Germany) per wash to remove the supernatant. Finally, after the fifth washing, the remnants of the stigma sterol were measured in the polymer. Wet polymers were placed on the glassy plate and were dried at 55 $^{\circ}$ C in an oven for 24 hours. The polymers were crushed in the mortar and then sample was used for testing with a mesh200. Polymer synthesis processes schematic is shown in Figure 1 (14 and 20).

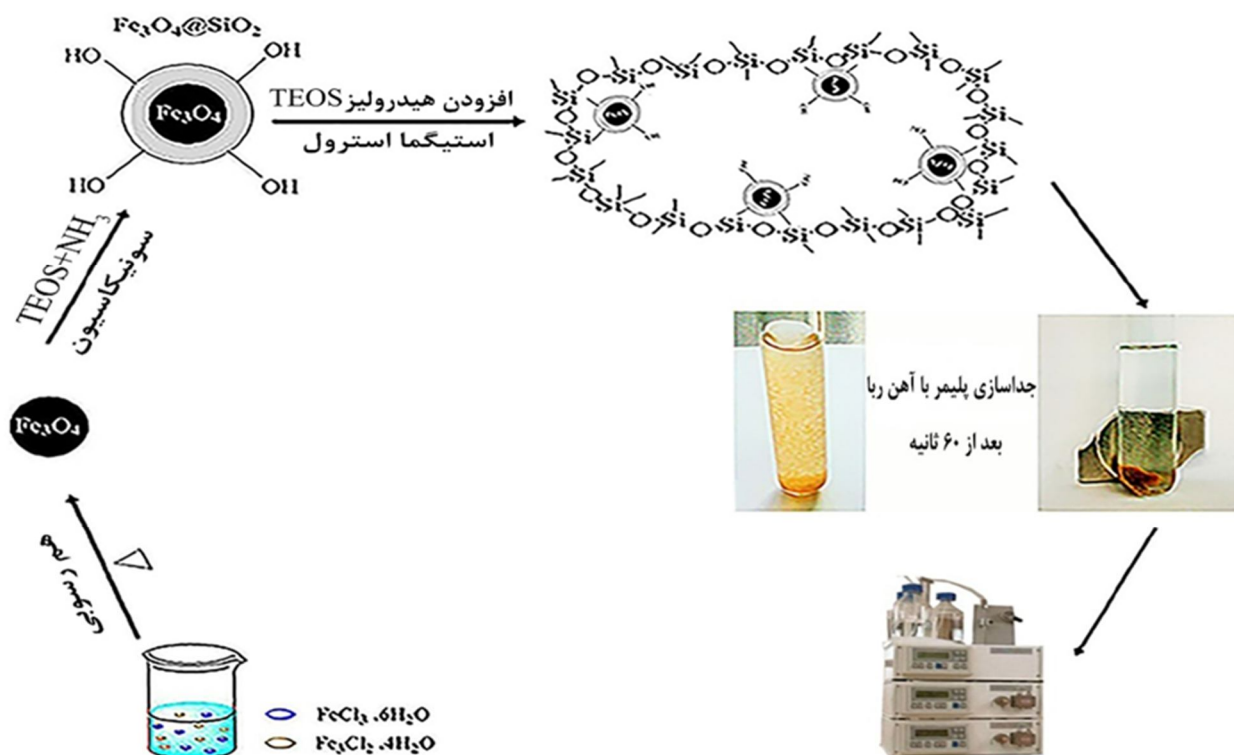


Figure 1: Schematic of the production of magnetic molecular nano-polymer stigma sterol by ChemBioDrawUltra12.0 BioDrawUltra12.0)

Stigma Sterol Absorption Test by the Polymer:

Standard Stigma sterol 100 mg solution in the hexane was prepared on a daily basis. Optimization

studies for Stigma sterol absorption had been performed at 0 to 120 minutes, with the polymer amount of 10 to 50 mg and 2 to 20 mL of the 100 mg/l solution. The results showed that the maximum absorption at 60 minutes had been obtained as 5 ml of stock solution and 20 mg of polymer. 20 mg of polymer with 5 ml of stock solution was placed in the Falcon tube for an hour on a shaker at 250 rpm. The samples have been isolated after one-hour incubation by neodymium magnet. Supernatant was smoothed by PTFE Syringe filter with a diameter of 0.22 Micron and then was ready to be injected into the HPLC system. HPLC device (Cecil Adept CE 4200, England) was used for measurement of Stigma sterol. Stigma sterol was measured by HPLC-UV at a wavelength of 202 nm and acetonitrile mobile phase (70): 2-propanol (30) under the current flow of 1.5ml / min in a period of 15 minutes with a C18 column.

Polymer absorption efficiency is calculated based on the following ratio: (Equation 1)

$$R = (C_0 - C_e) / C_0$$

Polymer absorption capacity Q (mg / g) is obtained from Equation 2:

$$Q = (C_0 - C_e) V / m$$

where C_0 and C_e , respectively, are concentration in equilibrium and initial concentration of solution. V is the volume of the solution incubated in mL, and m is the weight of the polymer in mg.

Fourier transform infrared spectroscopy:

Different types of samples (coated polymers or nanoparticles) have been mixed with dry potassium bromide and grounded for infrared spectroscopy to obtain a thin tablet with a thickness of about 60 kPa or

less under pressure for 10 minutes. Transmission spectrum of the samples has been analyzed in the range of wave number 4000 cm^{-1} - 400 cm^{-1} and Resolution of 0.5 cm^{-1} in FT-IR (SpectrumTwo, Perkin Elmer, USA).

Dimensional characteristics of the polymer:

Dry and crushed polymer samples' morphology was measured by scanning electron microscopy SEM-MIRA3 (Tescan, Czech Republic) with maximum 100,000 times magnification and magnetic field of 15 kV by In Beam. The samples were covered with a thin layer of gold before measuring.

2.8. Zeta potential measurement:

Zeta potential of the samples was solved by a light scattering diffraction (Worcestershire, UK) Nano- ZS Malvern Instrument in deionized water with the ratio of 1 to 50 and sonicated for 10 minutes to obtain uniform distribution of the sample, then measurement of samples was performed with 3 repetitions (16).

Results

Infrared spectroscopy features of polymer samples:

FT-IR spectra represents appropriate information on the structure, functional groups and the coating method of molecularly imprinted polymers. The infrared spectra of Fe_3O_4 and $\text{Fe}_3\text{O}_4 @ \text{SiO}_2$ are shown in Figure 2. The existence of link bond in 574 cm^{-1} shows vibration-bending links of Fe-O. In $\text{Fe}_3\text{O}_4 @ \text{SiO}_2$, broadband in 1096 cm^{-1} represents strong asymmetric stretching Si-O-Si bonds, the bond in 938 cm^{-1} represents stretching symmetric Si-O bonds and in 465 cm^{-1} represents Si-O-Fe bonds.

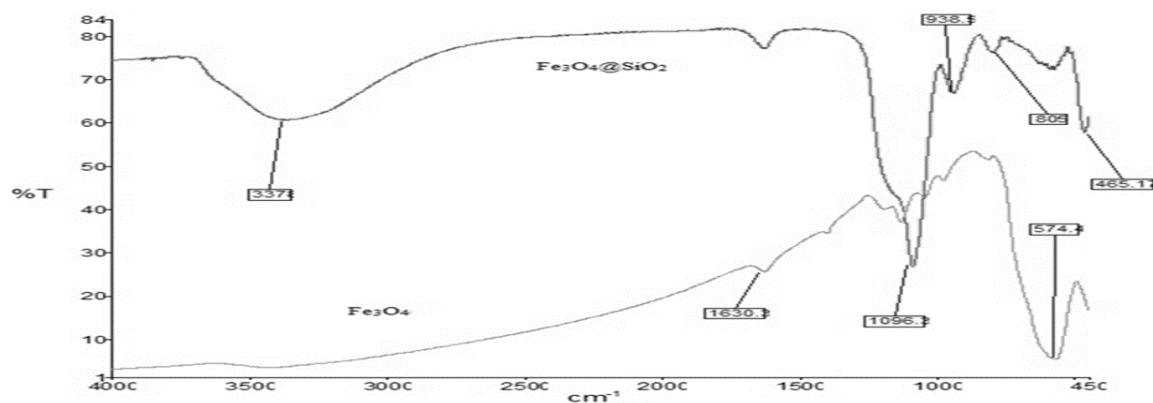


Figure 2. The infrared spectra of iron nanoparticle and iron nanoparticle with silica capsule

The existence of an important broadband in 3435 cm^{-1} in Molecularly Imprinted NanoPolymers of stigma sterol represents O-H absorbent bonds in the silica coating of the polymer and existence of a band in 1636 cm^{-1} provides possible absorption site for carbon-carbon double bond in the molecule. The band in 1092 cm^{-1}

supports Si-O-Si links in the sample polymer and also shows intensifying of silica absorbent links due to adding magnetic iron nanoparticles with Silica capsule. Si-O-Si broad bands can be due to the increasing amount of TEOS in polymer synthesis and O-H bonds in silica surface (17-19).

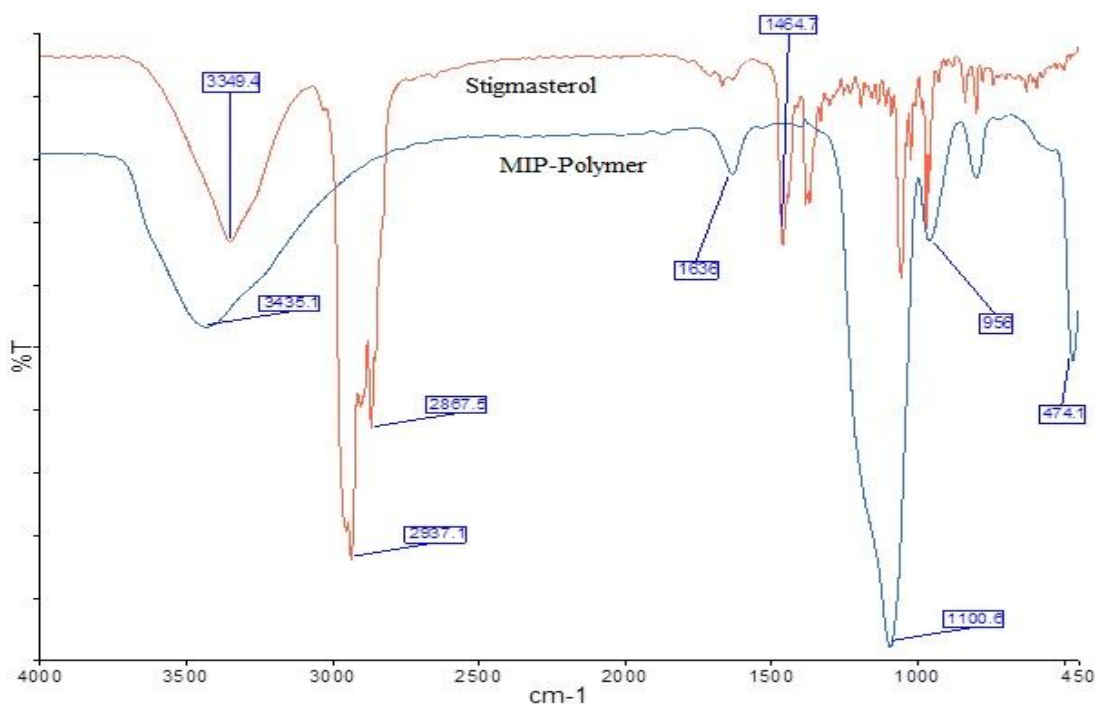


Figure 3. The infrared spectra of stigma sterol and molecularly imprinted nano-polymers of sterol stigma

Scanning electron microscope images:

Scanning electron microscope images is one way to estimate the size of polymer molecular framework.

Scanning electron microscopic image of molecular nano-polymer stigma sterol is shown in Figure 4. Polymer particles and pore size was between 20-30 nm

in the photos. The structure of the polymer is porous with morphology spherical. Spherical structure increases the ratio of the surface to volume and also

increases kinetics of molecular absorption. The uniform distribution of silica-coated magnetic iron nanoparticles can be seen in the polymer.

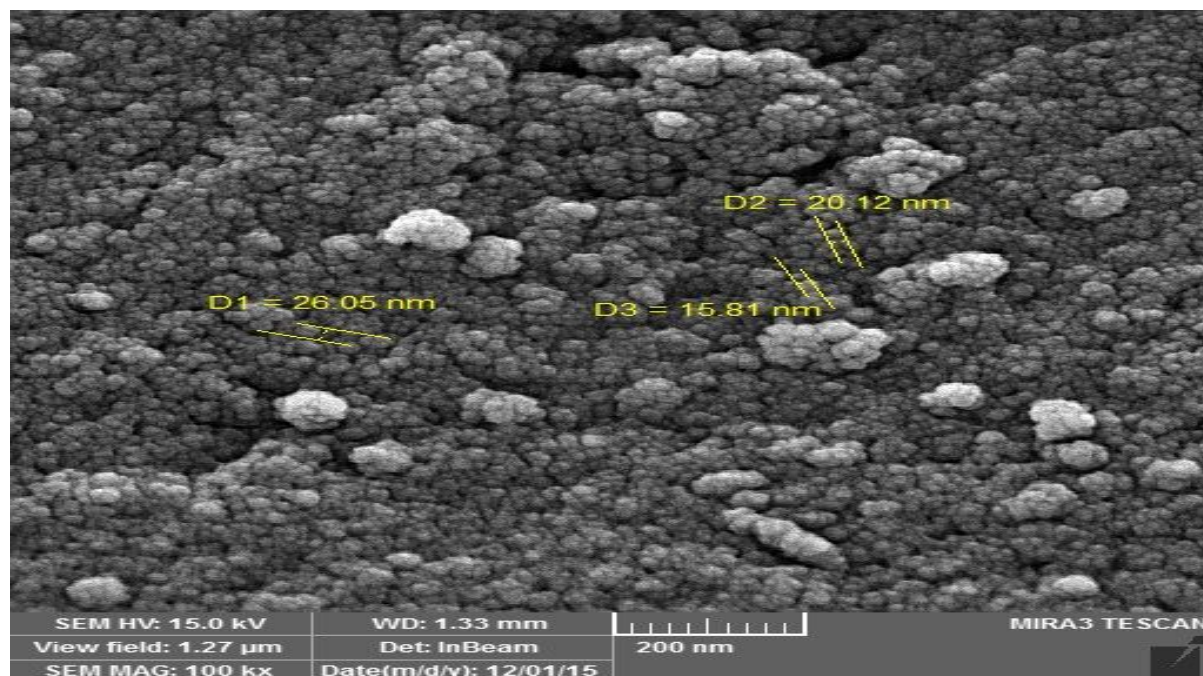


Figure 4. Scanning electron microscope image of Magnetically Molecularly Imprinted Nano-Polymer of stigma sterol

Stigma sterol absorption tests:

According to equation (1), mother stigma sterol absorption polymer solution equals to 78%. Moreover, the absorption capacity (Equation 2) for polymer of 100 mg Stigma sterol mother solution is 19.5 mg per each gram of polymer. The reason for this absorption rate is possibly related to the ratio of polymer surface to volume due to silica coated magnetic iron nanoparticles and high surface absorption of silane monomers (20). Prevention of the accumulation of magnetic iron particles by the silica coating, better dispersion of iron nanoparticles, prevention of the magnetic iron core release in a variety of acid environments and finding optimum absorption condition of Molecularly Imprinted Polymer are among the maximum separation efficiencies. The main advantage of the use of magnetic iron nanoparticles is high performance and ease of isolation.

Size of zeta potential (ζ):

Zeta electric potential represents the electrostatic load of outer surface of particles and factors such as van der Waals forces and hydrophobic interactions and electrostatic repulsion are effective on it. The zeta potential of the molecularly imprinted polymer molecule samples suggests Silica absorber coating of the outer layers on the magnetic core of iron. Zeta potential values is -45 mV for Fe₃O₄ core which is a result of the multiplicity of surface ionized carboxyl groups, but in the case of Fe₃O₄ @ SiO₂-TEOS-STG the score equals to -16.9 mV. Higher values of zeta potential in polymer samples demonstrates the replacement of absorbent hydroxyl groups in the silica shell (16).

Conclusion

Hashem et al. (2014) synthesized covalent molecularly imprinted polymer through simultaneous

incorporating of both acrylic acid monomers and ferulic acid for stigma sterol and assessed the absorption capacity in environments of hexane, ethanol and acetonitrile. The results showed that the maximum absorption (2.5 mol per gram of polymer) was obtained in hexane that the results indicate a better absorption of Stigma sterol. Hsu (2008) argued the high absorption in



Figure 5. Stigma sterol molecule structure

Oliveira (2015) used a column containing a silica-adsorbent modified polymer in extraction, filled with solid phase, for isolation of animal sterols of milk (20). Results showed that the efficiency of collecting this column is approximately 74% to 78% which are in line with the results of this study.

Moghadas Kia and colleagues synthesized cholesterol by the sol-gel method on the basis of magnetic iron-silica particles of magnetically molecularly imprinted polymers. The results showed that the dimensions of the polymer were below 100 nanometers and collected 94% cholesterol from the environment (14). They also acquired the ratio of optimal amount of absorbent polymer to the stock solution model.

The study of Zangin(2013) which used the iron nanoparticles as the core and dimethyl acrylamide monomer in ligand substitution method as shell of molecularly imprinted polymers, is the first separation application of combination by using Fe₃O₄@ SiO₂. He achieved the separation scores of 91.6%, 93.6% and 92.4% in the isolation of cholesterol from the blood

hexane loosely related to steroid compounds in polar environments such as methanol and water. Also, the ratio of testosterone and cholecalciferol (Figure 5) showed a higher separation in non-polar environments among the mixed steroidal compounds that are less hydrophobic with a relatively linear structure such as stigma sterol (21,22).

serum, milk and egg yolk. This research confirms the data of the effectiveness of Fe₃O₄ in superficial molecularly imprinted polymer. The study of Klavzen (2014) about optimization showed that the use of tetra-ortho-silicate in methacrylic molecularly imprinted polymer is achieved the maximum absorption in a ratio of 1: 5. With a separate review of the Langmuir absorption isotherm, Freundlich knew the reason due to adequate silica coating of selected holes as a result of meta acrylic cavities (15). Studies Gupta (2011) also showed that the use of compounds such as APTES (3-aminopropyl triethoxy silane) and MPS (3-propyl-methyl trimethoxysilane Ceylon) due to (Amin) organic connectors groups result in better interaction between core of Fe₃O₄@ SiO₂ and Methacrylic- silica shell (16). Absorption tests' reviewing show the ability to separate stigma sterol from the environment which is due to silica coated magnetite nanoparticles and high surface absorption desire of surface silane monomers. Due to the absorbent dimension sizes less than 50 nm, high surface to volume ratio that accelerates kinetics uptake also improves the separation properties of the

polymer. Therefore, due to special properties of stigma sterol steroid compounds, the polymers can be used in manufacturing stigma sterols biosensors on different chemical measurements bases (chromatographic, electrochemical) to be used in the pharmaceutical and food environments (19,20). Optimizing the production of the polymer through the use of other monomers with the aim of increasing its physical resistance in isolation

environments could be considered. The results of the study showed that magnetically molecularly imprinted polymers prepared by sol-gel method can be used to isolate and identify stigma sterol as a food- medicine component used in a variety of complex biological environments and improve the propriety in selectivity of these absorbers.

References

1. Santos R, Limas E, Sousa M, da Conceição Castilho M, Ramos F, da Silveira MIN. Optimization of analytical procedures for GC-MS determination of phytosterols and phytostanols in enriched milk and yoghurt. *Food chem* 2007;102(1):113-7.
2. Dutta PC. Phytosterols as functional food components and nutraceuticals. CRC Press; 2003.
3. Kritchevsky D, Chen SC. Phytosterols—health benefits and potential concerns: a review. *Nutrition Res* 2005;25(5):413-28.
4. Belitz HD, Grosch W, Schieberle P. *Food chemistry*, 4th ed. Berlin (DE): Springer; 2009.
5. Azadmard-Damirchi S. Review of the use of phytosterols as a detection tool for adulteration of olive oil with hazelnut oil. *Food Addit Contam Part A Chem Anal Control Expo Risk Assess* 2010;27(1):1-10.
6. Singhal RS, Kulkarni PK, Reg DV. *Handbook of indices of food quality and authenticity*. Elsevier; 1997.
7. Cheong WJ, Yang SH, Ali F. Molecular imprinted polymers for separation science: a review of reviews. *J Sep Sci* 2013;36(3):609-28.
8. Martín-Esteban A. Molecularly-imprinted polymers as a versatile, highly selective tool in sample preparation. *TrAC Trend Anal Chem* 2013;45:169-181.
9. Wackerlig J, Lieberzeit PA. Molecularly imprinted polymer nanoparticles in chemical sensing—Synthesis, characterisation and application. *Sensor Actuat B- Chem* 2015; 207: 144-57.
10. Uzuriaga-Sánchez RJ, Khan S, Wong A, Picasso G, Pividori MI, Sotomayor MDPT. Magnetically separable polymer (Mag-MIP) for selective analysis of biotin in food samples. *Food chem* 2016; 190: 460-7.
11. Niu M, Pham-Huy C, He H. Core-shell nanoparticles coated with molecularly imprinted polymers: a review. *Microchim Acta* 2016; 183: 2677-95.
12. Zengin A, Yildirim E, Tamer U, Caykara T. Molecularly imprinted superparamagnetic iron oxide nanoparticles for rapid enrichment and separation of cholesterol. *Analyst* 2013; 138: 7238-45.
13. Clausen DN, Visentainer JV, Tarley CRT. Development of molecularly imprinted poly(methacrylic acid)/silica for clean-up and selective extraction of cholesterol in milk prior to analysis by HPLC-UV. *Analyst* 2014;139(19):5021-7.
14. Moghaddas Kia E, Alizadeh M, Vardast MR, Rezaad M. Synthesis of cholesterol grafted molecularly imprinted polymer by means of the silanation of magnetic particles and evaluation of functional characteristics. *J Urmia Univ Med Sci* 2016; 27 (3): 231-9.
15. Zeng H, Wang Y, Nie C, Kong J, Liu X. Preparation of magnetic molecularly imprinted polymers for separating rutin from Chinese medicinal plants. *Analyst* 2012; 137: 2503-12.
16. Wang Y, Chai Z, Sun Y, Gao M, Fu G. Preparation of lysozyme imprinted magnetic nanoparticles via surface graft copolymerization. *J Biomat Sci-Polym E* 2015; 26: 644-56.

17. Ahangaran F, Hassanzadeh A, Nouri S. Surface modification of Fe₃O₄@ SiO₂ microsphere by silane coupling agent. *Int Nano Lett* 2013; 3: 1-5.
18. Uzuriaga-Sánchez RJ, Khan S, Wong A, Picasso G, Pividori MI, Sotomayor MDPT. Magnetically separable polymer (Mag-MIP) for selective analysis of biotin in food samples. *Food Chem* 2016;190:460–7.
19. Clausen DN, Pires I.M.R, Tarley C.R.T. Improved selective cholesterol adsorption by molecularly imprinted poly (methacrylic acid)/silica (PMAA–SiO₂) hybrid material synthesized with different molar ratios. *Mater Sci Eng* 2014; 44: 99-108.
20. Oliveira GR, Santos AV, Lima AS, Soares CMF, Leite MS. Neural modelling in adsorption column of cholesterol-removal efficiency from milk. *Food Sci Technol* 2015;64(2):632–8.
21. Hashim SN, Boysen RI Schwarz, LJ, Danylec B, Hearn MT. A comparison of covalent and non-covalent imprinting strategies for the synthesis of stigmasterol imprinted polymers. *J Chromatogr A* 2014; 1359: 35-43.
22. Hsu CW, Yang MC. Enhancement of the imprinting effect in cholesterol-imprinted microporous silica. *J Non-Cryst Solids* 2008; 354: 4037-42.
23. Park SJ, Kim DH, Jung JM, Kim JM, Cai M, Liu X, et al. The ameliorating effects of stigmasterol on scopolamine-induced memory impairments in mice. *Eur J Pharmacol* 2012; 676(1): 64-70.