ORIGINAL ARTICLE

Eastern Journal of Medicine

East J Med 30 (2): 295-301, 2025
DOI: 10.5505/ejm.2025. 55632

Synthesis and Characterisation of Molecularly Imprinted Microspheres for The Determination of Kanamycin

Cebrail Buran[1], Mustafa Bilici[2,3*]

[1]*Department of Nursing, Faculty of Health Sciences, Hasan Kalyoncu University, Gaziantep, Turkey*
[2]*Department of Basic Medical Sciences, Faculty of Medicine, Van Yuzuncu Yil University, Van, Turkey*
[3]*Van Security Collage, Van Yuzuncu Yil University, Van, Turkey*

ABSTRACT

This study aims to propose a methodology based on molecularly imprinted polymer solid phase extraction (MISPE) prior to instrumental analysis of kanamycin, a type of antibiotic. Solid phase extraction of kanamycin antibiotic, which is in the aminoglycoside class of antibiotics, from commercially purchased milk samples was carried out by molecular imprinting technique. After this process, the amount of kanamycin was determined by UV-visible spectrophotometry. This antibiotic in milk was removed by molecular imprinting method. The molecular imprinting technique was aimed to form complexes of functional monomers around a mould molecule with covalent or non-covalent interactions and then to form imprinted polymers with chemical function through an appropriate processing process. By removing the imprinted molecule after the process, a hollow cavity specific to the imprinted molecule was formed in the polymeric structure. These molecularly imprinted polymers were used as an ideal material for processes such as separation, chemical determination and catalysis. Molecularly imprinted microspheres were prepared for the determination of kanamycin antibiotic, the active ingredient of the drug in milk. The results obtained show that the proposed method can determine kanamycin in milk with high recovery rates and low relative standard deviation.

Keywords: Antibiotic, Kanamycin Determination, Microsphere, Milk

Introduction

Today, antibiotics are used for the treatment of many diseases. However, antibiotics used in the treatment of these diseases can bring many problems as well as treating diseases (1). Unconscious use of antibiotics may cause damage to the liver and kidneys, fungal infections, economic losses, loss of probiotics in the intestines and development of resistance to antibiotics (2). Beneficial probiotics in our intestines facilitate digestion, produce vitamins B and K, support the immune system, provide resistance to cancer and help us protect against diseases by preventing the proliferation of harmful microbes (3). However, with the unconscious use of antibiotics, these probiotics die at a rate of 20% and this lost rate can only be replaced within 2 years even in the case of a completely balanced and organic diet. Similarly, the use of antibiotics in infants causes the loss of probiotics and makes them susceptible to infections later in life (4).

Antibiotics are also used in dairy and livestock farming as in humans, and antibiotics used for therapeutic purposes in animals, just like in humans, can be used unconsciously, just like in humans (5). Antibiotics in animals are generally used for many reasons such as herd treatment, birth diseases, preventive measures, wounds or skin diseases. However, the producer should generally use these antibiotics according to official criteria and observe the legal purification process (6). If care is not taken, resistance to antibiotics develops and the effectiveness of the antibiotic used gradually decreases. This can affect the whole society. In the same way, antibiotic residues can pass into the milk after the use of antibiotics in animals using antibiotics (7). For example, antibiotics applied for the prevention of mastitis reach the milk ducts and are excreted with milk at a rate of 30% to 80% and therefore the residue of antibiotics can be very high, especially in the first milk taken from the animals, and the legal purification process recommends waiting between 2 and 6 days (6).

***Corresponding Author:** Mustafa Bilici, Department of Basic Medical Sciences, Faculty of Medicine, Van Yuzuncu Yil University, Van, Turkey
E-mail: mustafabilici@yyu.edu.tr, Phone: 0533 451 21 99
ORCID ID: Cebrail Buran: 0000-0001-9372-4494, **Mustafa Bilici:** 0000-0002-8689-6463
Received: 08.11.2024, **Accepted:** 09.03.2025

However, sometimes these rules are not followed and antibiotics are used unconsciously. In our study, we tried to remove the kanamycin antibiotic used for various reasons in fattening animals and the kanamycin antibiotic without separating the beneficial compounds in the milk by molecular imprinting method, which is the favourite method of recent years. Molecular imprinting method is used in biosensor applications, food industry, environmental and waste applications, therapeutic purposes and many similar areas (8). The molecular imprinting method aims to form a complex with a covalent or non-covalent interaction of a functional monomer around a template molecule and then to form a group of imprinted polymers with chemical functions following a suitable sequence of processes (9). In other words, molecular suppression is called molecular suppression, as in the antigen or enzyme-substrate relationship, in which structures recognize each other and interact with each other and can be structurally differentiated. Molecular imprinting is based on the relationship between substrates and receptors (10).

These tiny plastic prints were first published in 1931. However, the exact term molecular imprinting was not used. In 1930 Polyakov developed silica gels with special binding sites for dicney ethyl and methyl orange dye. Molecularly imprinted plastics are one of the most popular research topics in recent years because they are easier to prepare than other methods, much more stable, inexpensive and have recognition capabilities (11). With this method, several types of molecularly imprinted plastics can be used to identify several polymers at once with a single sample (12). This multifunctional aspect makes it much more preferable because it can be used to perform many operations for individual sensors at one time, which saves time, money and labor (13). While the suppression method is based on the capture of molecules that fit into a certain cavity, it is based on the creation of plastic molecular suppression structures by mimicking a natural molecule such as antibodies (13, 14). For example, just as our body's immune system naturally produces antibodies when it detects foreign molecules entering our body, and these antibodies gradually bind to a foreign molecule like a key-lock match, so researchers identify and exploit this foreign molecule. For example, some antibodies in the blood bind to viruses or bacteria if they are present in the environment, indicating the presence of infection in the blood (14). This method is used not only to identify foreign molecules but also to determine the amount of the desired protein or to diagnose it. However, these methods require a lot of processing and experimental animals are used. With molecular suppression, it is possible to do similar work to what antibodies do, and molecular suppression is easier than antibody production and minimizes the use of experimental animals. In addition, antibodies require a longer process because they bind to a single molecule, whereas molecular suppression is much more preferred than antibodies because many processes can be performed in one go (15). Molecules and monomers are covalently bonded to each other and the structures maintain their stability during the polymerization process. The bonding of monomers and molds is not proportional, so molecular imprinting processes are usually uniform. The bonding process occurs by covalent bonding and has a stable structure within high or low values of temperature or pH. The bonding state between non-covalent imprints is weaker than that between covalent ones (16, 20, 21). These bonds include hydrogen bonding, hydrophobic bonding, Van der Waals interactions with ionic groups and electrostatic forces, charge transfer, metal chelation and complexing between molecules and monomers. Whitcombe et al. developed semi-covalent imprinting in 1990. This different method makes it possible to combine the advantages of covalent and non-covalent methods. In this way, the monomer complex of the target molecule is formed by covalent bonding (1, 17). The surface imprinting method can be applied in two ways: first, the molecule is imprinted on a polymer by synthesizing a polymer with a thin film layer, or second, the protein molecule is bound to the surface of a flat or spherical substrate and a polymerization occurs around these molecules. This method can help to solve some of the problems of mass transfer or separation of cavities from the target molecules, as the binding sites to the target molecule are located very close to the polymer layer (8,11,21). In this study, firstly poly (2-hydroxyethyl methacrylate) microspheres were prepared by suspension polymerisation and then molecular imprinting was performed with dopamine (monomer), kanamycin (mould molecule or analyte). After detailed surface characterisation, molecular imprinting parameters were investigated. As the last step, the usability of the prepared molecularly imprinted spheres for the determination of kanamycin in milk was investigated. The results obtained showed that the proposed method has high sensitivity and selectivity.

Material and Methods

Given the use of commercially available milk in the study, ethical committee approval was deemed unnecessary. The methodology employed in this research adhered to established standards, with known methodologies appropriately referenced. This section provides a comprehensive overview of the application and relevance of each method to the research objectives. Sub-headings within this section facilitate a structured presentation of the diverse techniques employed throughout the study. Each sub-heading delineates a distinct aspect of the methodology, ensuring clarity and coherence in the exposition.

Preparation of phema microspheres: 1.125 g polyvinyl alcohol was dissolved in 70 mL deionised water and 11.2 mmol HEMA and 0.26 mmol potassium persulfate were added to the medium. The resulting mixture was stirred with an orbital shaker at 70 °C for 5 hours. At the end of this time, the microspheres formed were removed by filtration and washed first with plenty of ethanol and then with deionised water. Finally, the microspheres were dried in a vacuum oven and stored in a vacuum desiccator until use (18, 20).

Preparation of molecularly imprinted microspheres: PHEMA spheres were synthesised using spontaneous polymerisation of dopamine in basic medium for the preparation of molecularly imprinted particles. 100 mg PHEMA sphere was dispersed in 50 mL Tris-HCl buffer (10 mM, pH8.5), 50 mg dopamine and 0.365 mmol kanamycin (template molecule) were added and the mixture was stirred for 16 h at room temperature. Molecularly imprinted PHEMA spheres (MIP@PHEMA) were removed from the solution by filtration, washed with plenty of water and then dried in a vacuum drying-oven. Non-molecularly imprinted PHEMA spheres (NIP@PHEMA) were prepared by the same method but in the absence of kanamycin (18, 19).

Determination of bonding characteristics: In order to investigate the back-bonding properties of kanamycin on MIP spheres, 10 mg imprinted spheres were dispersed in phosphate buffer (0.1 M, pH 7.4) containing kanamycin at different concentrations (0.2 - 1.4 mg/mL) and incubated at different time intervals (10-120 min). The concentration of kanamycin remaining in the supernatant after different concentrations and incubation times was determined by UV-visible spectrophotometry. The selectivity of molecularly imprinted spheres was investigated using streptomycin and erythromycin similar to the kanamycin molecule. For this purpose, 10 mg of imprinted sphere was added to phosphate buffer containing 0.8 mg/mL initial concentration of each antibiotic separately and the mixture was stirred at room temperature for 60 minutes and the concentration of antibiotic remaining in the supernatant at the end of this period was determined by Uv-visible spectrophotometry (18, 19).

Results

Characterisation of Microspheres: The morphology and average diameters of the PHEMA particles were characterised by scanning electron microscopy. Figure 1.a shows the SEM image of PHEMA particles. As can be seen from the figure, the particles formed were spherical and their diameters were approximately 20 μm. The SEM image obtained after MIP on PHEMA spheres is shown in Figure 1.b. As a result of imprinting, the particles retained their spherical structure and it was determined that the spheres were homogeneously covered with a polymer layer.

The imprinted PHEMA spheres were also characterised by SEM-elemental mapping. Figure 2 shows the SEM image showing the mapped area and the map patterns of carbon, oxygen and nitrogen atoms. As can be seen from the figure, the homogeneity of carbon, oxygen and especially nitrogen atom patterns indicates that the polydopamine layer is homogeneously coated on the spheres.

The chemical composition of the prepared microspheres was analysed by x-ray photoelectron spectroscopy (XPS). Figure 3.a. shows the XPS overall spectrum of PHEMA spheres. O 1s peaks at 532.1 eV and C 1s peaks at 285.1 eV were determined in the spectrum and the same elements were determined at almost the same bonding energies in the XPS spectrum of the imprinted PHEMA spheres Figure 3.b. However, the peak of the N 1s atom at 400.0eV was observed in the spectrum of the imprinted PHEMA spheres and can be shown as the strongest evidence that the spheres are coated with a polydopamine layer.

Back-Bonding Characteristics of Kanamycin To Imprinted Spheres: The back-bonding characteristics of kanamycin on molecularly imprinted PHEMA spheres were investigated. 20 mg particles were dispersed in different

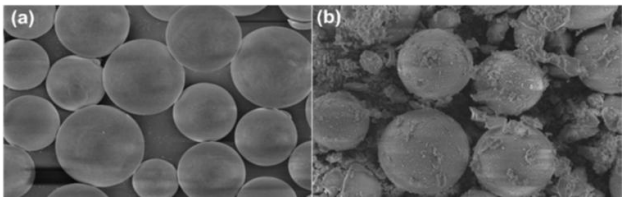

Fig. 1. SEM images of PHEMA spheres (a) PHEMA spheres and (b) molecularly imprinted PHEMA spheres

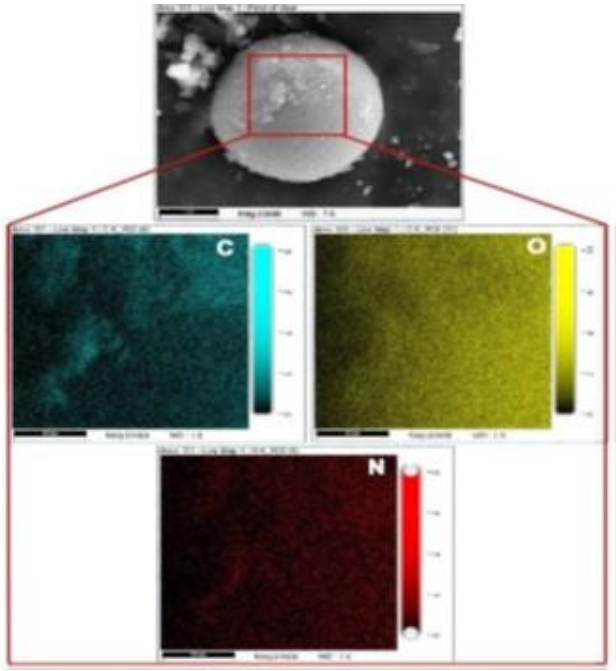

Fig. 2. SEM image of imprinted PHEMA spheres and SEM-elemental maps of carbon, oxygen and nitrogen atoms

concentrations of kanamycin aqueous solution, incubated at room temperature for different times and then the amount of kanamycin remaining from bonding in the supernatant was determined by UV-visible spectrophotometry. Figure 4. shows the change in kanamycin adsorption capacity of PHEMA spheres for different initial concentrations of kanamycin. Figure 4. shows that as the initial concentration of kanamycin increased, the molecularly imprinted (MIP) and non-imprinted (NIP) adsorption capacity increased and this increase reached a maximum value at 0.8 mg/mL. There was no change in the adsorption capacity of MIP and NIP when the initial concentration was more than 0.8 mg/mL. However, the fact that the adsorption capacity of MIP is considerably higher than the adsorption capacity of NIP indicates that there are kanamycin-specific voids on the MIP particle surfaces. The imprinting factor, which is a ratio of MIP and NIP adsorption capacities, was determined as 3.56.

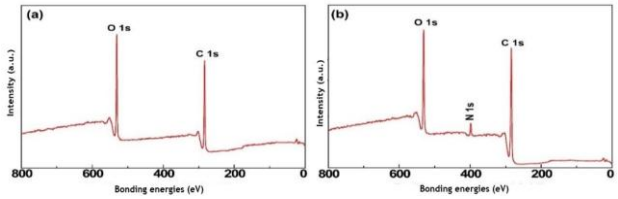

Fig. 3. XPS overall spectra of PHEMA spheres and (b) imprinted PHEMA spheres

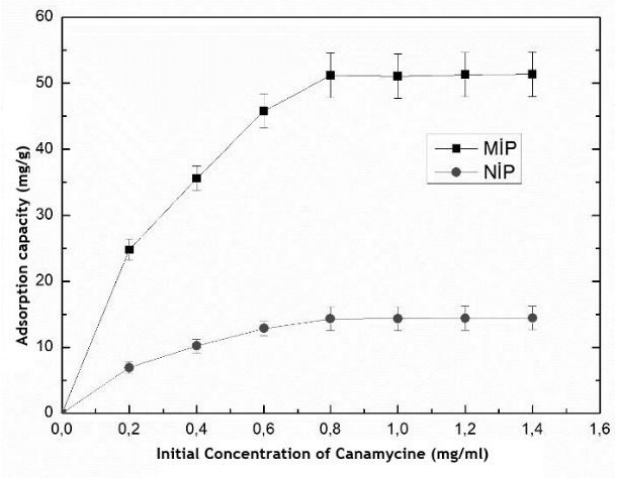

Fig. 4. Effect of initial kanamycin concentration on adsorption capacity (incubation time: 60 minutes)

The effect of adsorption time of kanamycin on MIP/NIP spheres is given in Figure 5. The graph shows that when the adsorption time reached 60 min, the adsorption capacities of MIP and NIP spheres increased and there was no significant change at the end of this period. However, as expected, the adsorption capacity of MIP particles is considerably higher than that of NIP particles. The presence of a certain adsorption value in NIP particles is due to non-specific interactions. As a result, it was determined that the initial concentration of kanamycin 0.8 mg/mL and the adsorption time of 60 minutes were sufficient for the MIP particles to reach the maximum adsorption capacity.

The most important feature of MIP materials is their selectivity. In this study, streptomycin and erythromycin, which are molecularly similar to kanamycin, were preferred for selectivity. Figure 6. shows the adsorption capacities of MIP/NIP particles for each molecule. When the graph is analysed, the fact that the adsorption of kanamycin on MIP spheres is much higher than other molecules can be said that the prepared spheres are kanamycin specific. A certain adsorption capacity in other molecules is due to non-specific bonding on surfaces.

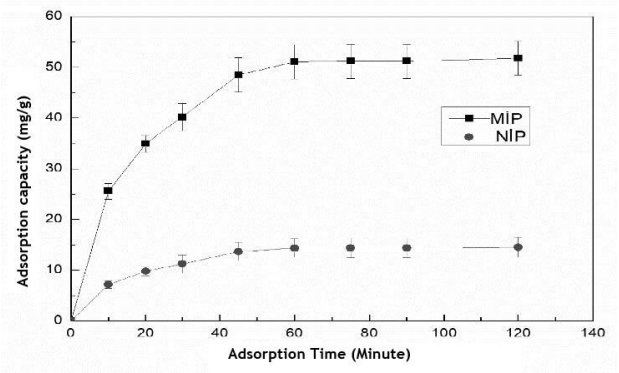

Fig. 5. Variation of adsorption capacity on MIP/NIP spheres with adsorption time (Initial kanamycin concentration: 0.8 mg/mL)

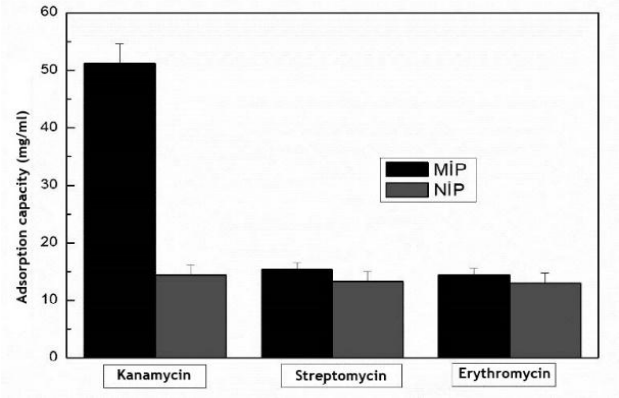

Fig. 6. Adsorption capacities of kanamycin and kanamycin-like molecules (concentration: 0.8 mg/mL and adsorption time: 60 minutes)

Another feature of molecularly imprinted polymers is that they have multiple uses. The prepared MIP PHEMA spheres were used in a number of consecutive adsorption-desorption cycles. Figure 7. shows the change in the adsorption capacity of MIP spheres in response to the number of uses. When the graph is analysed, the adsorption capacity of the prepared spheres did not change even after 6 conversions. In other words, the prepared spheres have at least 6 uses. When the number of conversions exceeded 6, the adsorption capacities decreased. This may be due to the deformation of the polymer layer with the high number of cycles.

Determination of Kanamycin In Milk Samples: To determine kanamycin in milk samples purchased from local markets, milk samples were first filtered and then diluted 10 times with phosphate buffer (0.1M PBS, pH7.4). After homogenising the diluted milk samples with the help of vortex, kanamycin concentration of 2-10 µg/L was added into the milk samples by standard addition method and 10 mg imprinted PHEMA spheres were added immediately

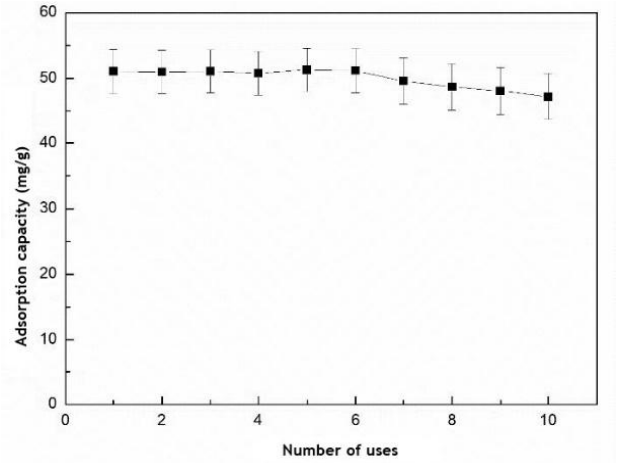

Fig. 7. Change in adsorption capacity of MIP spheres in response to the number of uses

afterwards. The mixture was stirred at room temperature for 60 min and the amount of kanamycin remaining in the supernatant was determined by UV-visible spectrophotometry. As a result of the analysis, % recovery and % relative standard deviation were calculated and the results are provided in Table 1. As can be seen from the table, low relative standard deviation values were obtained with high recovery rate. The results obtained show that the proposed method is a suitable analytical method for the determination of kanamycin in milk samples.

Discussion

In conclusion, a new spectrophotometric method was established for the selective separation and determination of kanamycin form milk samples based on molecularly-imprinted microspheres. The as-synthesized microspheres characterized by a combination of several analytical method and the results revealed the existence of an imprinting layer which disturbed homogeneously on the microspheres. Then, re-binding properties of kanamycin on the imprinted microspheres were evaluated in detail and the results implied that the imprinted microspheres had satisfactory adsorption capacity (52.2 mg/g), high imprinting factor (3.56) and high selectivity towards kanamycin. In addition, the imprinted microspheres can be easily regenerated and used 10 times without significantly decreasing its initial adsorption capacity. The analytical performance of the proposed method was also checked by using commercial milk samples and the proposed method showed high recovery percentages (97.5-99.5) with a low relative standard deviations (less than 4.2%).

Table 1: Recovery of Kanamycin In Milk By The Proposed Method

Kanamycin concentration added to milk (µg/L)	Kanamycin concentration found (µg/L)	% Recoverya	% Relative standard deviationb
0	-	-	-
2	1.95 ± 0.08	97.5	4.1
4	3.94 ± 0.16	98.5	4.06
6	5.95 ± 0.21	99.2	3.53
8	7.96 ± 0.31	99.5	3.89
10	19.98 ± 0.35	9.8	3.51

a It is the average result of five different readings for each concentration
b Relative standard deviation = (standard deviation/mean value) X 100

As a result of all studies, molecularly imprinted paper surfaces have been shown to be a good method for detecting kanamycin antibiotic in milk. By removing the antibiotic in milk with this method, public health will improve further. This method can give very good results in many fields such as biosensor applications, food industry, environment and waste applications. Indeed, this method not only gives good results, but it is also cheaper than other methods, easier in terms of accessibility and more advantageous in terms of simpler methods and tools in laboratory work.

When the MIP method is developed and used more effectively, it will reduce the need for experimental animals and will be very advantageous both in terms of cost and accessibility and because we will use animals less. In our opinion, the most important aspect of the studies carried out with the MIP method is the shorter duration and longer effect compared to other methods.

Acknowledgements: This study was supported by Van Yuzuncu Yil University Scientific Research Projects Unit (FYL-2019-8069).

References

1. Murray Christopher J. L.,"Global Burden of Bacterial Antimicrobial Resistance in 2019: A Systematic Analysis". The Lancet 399(10325):629-55. doi: 10.1016/S0140-6736(21)02724-0.
2. Ercan N, Tıraş S. 2022. "Analysıs Methods Of Antıbıotıc Resıdue In Mılk".
3. Mazziotta C., Mauro T., Fernanda M., Elena T. and Charles R. 2023. "Probiotics mechanism of action on immune cells and beneficial effects on human health". Cells 12(1):184.
4. Hızlısoy H., 2018. "The Investigation of Antibiotic Residues in Milk". Erciyes University Veterinary Faculty Journal 15(2):169-78.
5. Tang Karen L., Niamh P. Caffrey Diego B. Nóbrega, Susan C., Cork Paul E., Ronksley Herman W. Barkema Alicia J., Polachek Heather Ganshorn, Nishan Sharma, and James D. Kellner. 2017. "Restricting the use of antibiotics in food-producing animals and its associations with antibiotic resistance in food-producing animals and human beings: a systematic review and meta-analysis". The Lancet Planetary Health 1(8):e316-27.
6. Chowdhury S., Sumon G., Mohammad A. A., Shahana P., Md Ariful I., Md Mahbubur R., Zubair A., and Fahmida C. 2021. "Antibiotic usage and resistance in food animal production: what have we learned from Bangladesh?" Antibiotics 10(9):1032.
7. Xu C., Lingqiang K., Hanfang G., Xiyu C. and Xiumin W. 2022. "A review of current bacterial resistance to antibiotics in food animals". Frontiers in Microbiology 13:822689.
8. Sevindik Y., 2018. "Molecularly imprinted polymers and molecular modelling of aspartame based on artificial receptors". Master's Thesis, Namık Kemal Namik Kemal University.
9. Saylan Y., Fatma Y., Erdoğan Ö., Ali D., Handan Y. and Adil D. 2017. "Molecular imprinting of macromolecules for sensor applications". Sensors 17(4):898.
10. Zhang J., Yixiang W. and Xiaonan L. 2021. "Molecular imprinting technology for sensing foodborne pathogenic bacteria". Analytical and Bioanalytical Chemistry 413:4581-98.
11. Nawaz N., Nor K., Abu B., Habibun N. M. E. M. and Nazzatush S. J. 2021. "Molecularly imprinted polymers-based DNA biosensors".

Analytical Biochemistry 630:114328. doi: 10.1016/j.ab.2021.114328.

12. Zhao G., Yue Z., Dani S., Shili Y., Yuhao W., Yixiao W., Guisheng L., Huitao L., Jinhua L. and Zhihua S. 2023. "Recent advances in molecularly imprinted polymers for antibiotic analysis". Molecules 28(1):335
13. Xu L., Yun-An H., Qiu-Jin Z. and Chun Y. 2015. "Chitosan in molecularly-imprinted polymers: Current and future prospects". International Journal of Molecular Sciences 16(8):18328-47.
14. Derz W., Melita F. and Paul W. E. 2021. "Guiding molecularly imprinted polymer design by pharmacophore modeling". Molecules 26(16):5101.
15. Karagöz F.,. 2015. "Synthesis of fluorescent materials sensitive to target chemicals and their use in chemical sensors". PhD Thesis, Institute of Science and Technology.
16. Shumyantseva V. V., T. V. Bulko, I. H. Baychorov, ve A. I. Archakov. 2016. "Molecularly imprinted polymers (MIP) in electroanalysis of proteins". Biochemistry (Moscow) Supplement Series B: Biomedical Chemistry 10:145-51.
17. Erol K. 2014. "Preparation, Characterisation and Chromatographic Applications of Selective Adsorbents by Surface Imprinting".
18. Kodama Y., Murat B. and Olgun G. 2014. "Radiation-induced and RAFT-mediated grafting of poly (hydroxyethyl methacrylate) (PHEMA) from cellulose surfaces". Radiation Physics and Chemistry 94:98-104.
19. Akbulut Y., Zengin A., 2020. "A molecularly imprinted whatman paper for clinical detection of propranolol". Sensors and Actuators B: Chemical 304:127276.
20. Yavuz B., Kondolot Solak E. and Oktar C. 2023. "Preparation of Biocompatible Microsphere-Cryogel Composite System and Controlled Release of Mupirocin". International Journal of Polymeric Materials and Polymeric Biomaterials 1-8. doi: 10.1080/00914037.2022.2163638.
21. Meydan İ., Et Al. , "Selective Extraction and Determination of Citrinin in Rye Samples by a Molecularly Imprinted Polymer (MIP) Using Reversible Addition Fragmentation Chain Transfer Precipitation Polymerization (RAFTPP) with High-Performance Liquid Chromatography (HPLC) Detection," Analytical Letters , Vol.54, No.10, PP.1697-1708, 2021