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Molecularly Imprinted Polymer Chitosan-Sodium Tripolyphosphate: Synthesis and Applications for the Extraction of Antibiotic Residue from Agricultural Products

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Abstract: *Analysis of antibiotic residues in livestock products is challenging due to the low concentration of antibiotic residues and the complexity of the sample matrix. In this study, selective adsorbents from molecular imprinted polymer (MIP) materials were successfully used for sample preparation. An adsorbent made of chitosan-based molecular imprinted polymer (Chi-MIP) was used to extract tetracycline residues from egg and milk samples. The adsorbent was made by preparing 0.1 grams of Chi-MIP in a 2 × 2 cm cellulose filter paper bag. Next, an adsorbent was added to the sample containing tetracycline. The extraction process was carried out using a hotplate stirrer. At the end of the extraction process, the extracted antibiotics are removed from the adsorbent through a desorption process in an organic solvent via an ultrasonicator. After that, the desorbed analytes were analyzed via a UV–Vis spectrophotometer at a wavelength of 267 nm. To obtain optimum results, the extraction conditions were optimized such that the following results were obtained: extraction time of 12 minutes, desorption time of 3 minutes, and ethyl acetate as the desorbing organic solvent. Using the standard addition method, method validation results were obtained for each milk and egg sample matrix, which showed a linearity range of 1–5 mg/L, a correlation coefficient (R^2) of 0.99, accuracy values of 98.22% and 88.10%, precisions of 2.74% and 1.06%, LoDs of 0.46 mg/L and 0.51 mg/L, LoQs of 1.52 mg/L and 1.70 mg/L, and enrichment factors of 3.27.*

Keywords*: Antibiotic residues, Chitosan, Milk, Molecularly imprinted polymer, Tetracycline*

INTRODUCTION

One of the most widely prescribed medications for the management of bacterial infections in people and animals raised for food is antibiotics (Nemati et al., 2021). Antibiotics are introduced into animal diets with the intent of lowering animal mortality and improving the health of the animals. In agriculture and veterinary care, antibiotics play a significant role in promoting and enhancing animal growth. However, to prevent the misuse of antibiotics in animals raised for food, which could result in residues in agricultural products, the Ministry of Agriculture issued Regulation No. 14/PERMENTAN/PK.350/5/2017 Concerning the Classification of Veterinary Drugs, which prohibits the use of veterinary drugs as feed additives for antibiotics, either as feed additives in their finished form or as raw materials for feed additives (Nadzifah et al., 2019).

One of the most common antibiotics commonly found in agriculture is tetracycline and its derivatives (Nadzifah et al., 2019). Tetracyclines are effective against a broad spectrum of microbes in the body, such as gram (+) bacteria, gram (-) bacteria, rickettsiae,

mycoplasma, amoebae, and chlamydia bacteria (Vuran et al., 2021). However, the use of drugs, antibiotics, feed additives, or animal growth-promoting hormones that are not as recommended and do not follow the prescribed doses can cause residues in agricultural products. The misuse of antibiotics through the consumption of contaminated animal foods, such as milk, honey, eggs, and meat, can lead to the transfer of antibiotics to the human body. Tetracycline residues have many negative effects on health, such as allergic reactions, toxicity, gastrointestinal disturbance, and bacterial resistance (Etikaningrum & Iwantoro, 2017; Nemati et al., 2021).

Because of the negative effects of nonprescribed tetracycline in food-producing animals, several countries have established maximum residue limits (MRLs) to reduce its negative effects on consumer health. The World Health Organization and Food and Agriculture Organization have set the MRLs for tetracycline to 100 µg/kg cow milk, 400 µg/kg poultry eggs, 200 µg/kg beef muscle, and 200 µg/kg giant prawn muscle (International Food Standard, 2023). Considering the low concentration of antibiotic residue in complex matrices such as agricultural products, the development of an analytical method that is selective and sensitive for quantitative analysis is important (Wang et al., 2017). In a previous study of feed quality and broiler thigh meat quality from several broiler breeders in Blitar Regency using high-performance liquid chromatography (HPLC), no antibiotic residues were detected in broiler chicken meat even when the animal feed used contained tetracycline (Nadzifah et al., 2019). These findings indicate that low levels of antibiotics in food-producing animals are very difficult to analyze.

Several analytical methods have been reported for the determination of tetracycline residue in food-producing animals, namely, high-performance liquid chromatography (HPLC) (Lai et al., 2020; Moreno-González & García-Campaña, 2017), immunoassays (Bu et al., 2020), fluorescence spectrometry (Liu et al., 2020), and electrochemical sensors (Jalalian et al., 2018). Among these methods, HPLC has been reported as the preferred method because of its ability to determine different types of tetracyclines simultaneously, accurately, and sensitively. However, the presence of interfering compounds in complex matrix samples limits their application for direct analysis. Furthermore, a sample preparation step based on an extraction technique with the aim of cleaning, preconcentrating, and increasing the detection limit is suggested before analysis (Pérez-Rodríguez et al., 2018).

SPE is a well-established technique that uses a solid sorbent to isolate and preconcentrate analytes from complex matrix samples through four steps, namely, conditioning, retention, washing, and elution. The solid sorbent is filled into the SPE cartridge, and the sample solution is passed through the solid sorbent inside the cartridge (Khan et al., 2020). The analytes will be adsorbed in the solid sorbent during the retention stage. After that, the analyte is separated from other interfering substances through the washing stage. To obtain a pure analyte, the elution stage was carried out. However, it has several drawbacks, such as being time-consuming and having the possibility of an interfering matrix blocking the cartridge during the extraction procedure, resulting in the analyte being carried over in the solid sorbent (Nemati et al., 2021). Therefore, to overcome the previous drawbacks of SPE, another format of SPE derivatives, namely, dispersive solid-phase extraction (DSPE), has been proposed. In DSPE, the adsorbent is dispersed in the sample solution with the aid of stirring, shaking, or vortexing. At the end of the extraction process, the solid sorbent was collected by centrifugation and separated from the sample mixture. After the sorbent has been collected, the adsorbed analyte is eluted with an appropriate solvent with the aim of vortexing or sonicating and continues with instrumental analysis

(Büyüktiryaki et al., 2020; Nemati et al., 2020). All these stages of SDPE are quite time-consuming, with a loss of selectivity of the solid sorbent and low recovery achieved (Amanda, Rosmawati, et al., 2022).

Recently, molecularly imprinted polymer (MIP)-based solid sorbents have been proposed as selective sorbents in DSPE, which can overcome this limitation. MIPs are widely used as SPE sorbents in sample preparation for the determination of antibiotic residues (Mohsenzadeh et al., 2018). MIPs have several advantages, such as acceptable selectivity for suitable target analytes, reusability, increased stability, easy preparation, and low production cost (Soledad-Rodríguez et al., 2017). To shorten the time and avoid clogging the pores of MIPs, the MIP sorbent is placed in a tea bag made of cellulose paper. In this study, the chitosan (Chi) biopolymer was selected as the base polymer crosslinked by sodium tripolyphosphate (NPP). Chitosan is a bulk biopolymer in nature. This combination of Chi and NaTPP produces MIP materials with high stability and environmental friendliness (Alvarez-lorenzo et al., 2013). The Chi-NaTPP polymer was previously used as a drug carrier (Arozal et al., 2021), as a biosorption pollutant and as a membrane for simulating human skin (Guerle-Cavero et al., 2021). In this study, Chi-NaTPP was prepared, characterized, and applied to DSPE solid sorbent by evaluating the optimum extraction conditions before being applied in the extraction of real samples. The aim of this research is to propose new materials for the purification of antibiotic residues from complex matrices such as agricultural products. The proposed material can improve the selectivity of adsorption capacity by optimizing the interaction between analytes and specific pores of MIPs.

RESEARCH METHODS

Materials and Tools

The materials used in this study were chitosan with deacetylation at 85–90% (biochitosan, Indonesia), tetracycline p.a. (Asian Chemical), glacial acetic acid, sodium tripolyphosphate, and hydrochloric acid (Merck, Germany), distilled water, ethyl acetate, and n-hexane (Brataco). The instruments used in this study included a UV–Vis spectrophotometer (Thermo Scientific Genesys 10S), an FTIR spectrophotometer (Shimadzu), a hot plate and magnetic stirrer (Kartel), an ultrasonicator (Local Brand), an analytical balance (Ohaus PAZ14), a vortex (Thermo Scientific), and laboratory glassware.

Methods

Synthesis and characterization of molecularly imprinted polymers (MIPs) and nonimprinted polymers (NIPs)

The MIP and NIP polymer synthesis process used in this study is a modification of the method used by Haryanto (2017). Modifications of the method carried out in this study included the use of tetracycline as a template and Na-TPP as a cross-linker and the manufacture of membrane bags from Whatman filter paper, which contain MIP or NIP. First, 5.0 g of 2% chitosan dissolved in 250 mL of 2% acetic acid solution was weighed while stirring until it was dissolved via a magnetic stirrer for 24 hours. The tetracycline solution dissolved in 0.1 N HCl was subsequently added to the chitosan solution, and the mixture was stirred with a magnetic stirrer for 24 hours. Furthermore, the mixture of chitosan and tetracycline was poured into 25 mL of 1% NaTPP solution to form a small round gel, which was allowed to stand for 2 × 24 hours. The gel was subsequently washed with ethanol:water at a 50:50 ratio 10 times. The gel was subsequently blown into the air at room temperature for 24 hours, after which it was dried, and this process was repeated periodically ± 10 times.

The resulting MIP solids were ground and sieved through 60- and 90-mesh sieves. The NIP membrane synthesis process was the same as the MIP synthesis process but did not use a tetracycline template on the membrane.

The synthesized NIP and MIP materials were characterized via FTIR (Shimadzu IRSpirit-T) in the range of 4000-4750 cm^{-1}. This characterization was used to determine whether there was a change in the characteristics of the chitosan, NIP, and MIP functional groups. SEM characterization was carried out using a Hitachi SEM (Hitachi SU3500, Tokyo, Japan) to determine the morphological differences between NIP and MIP.

Dispersive solid-phase extraction (DSPE)

The DSPE method begins by preparing a solid sorbent. The solid sorbent to be used was placed in a paper bag. The paper bag was made of 1 sheet of Whatman filter paper no. 41 and was cut to a size of 2 cm × 2 cm. The edge of the bag was tightly closed to a size of approximately 0.1 cm. The finished bag is then filled with 0.1 grams of solid sorbent. The extraction process was carried out by inserting the MIP bag into a beaker containing 10 mL of spiked sample solution and stirring with a magnetic stirrer at 300 rpm for a certain time. After the extraction process was completed, the paper bag containing the solid sorbent was put into a desorbing organic solvent and desorbed using an appropriate desorption instrument for a certain time. The desorption filtrate was measured via a UV–Vis spectrophotometer at a wavelength of 276 nm. To determine the optimum conditions for DSPE, several important parameters, such as sorbent type, desorption instrument type, desorption solvent type, extraction time, and desorption time, were optimized in triplicate for each parameter. A sketch of the DSPE method is shown in Figure 1.

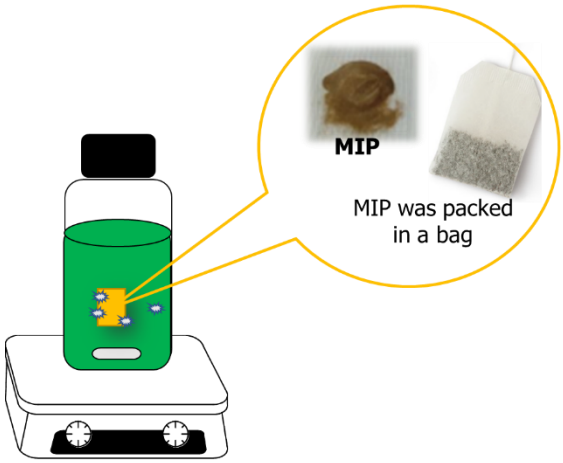


Figure 1. Setup of the dispersive solid-phase extraction (DSPE) technique

Sample Pretreatment Protocol for Matrix Match Calibration

Samples of chicken eggs and cow milk were obtained from eggs and cow breeders in the Krian area, Sidoarjo, East Java. Before extraction and analysis via the DSPE method with a combination of UV–Vis spectrophotometers, samples of chicken eggs were prepared by weighing 10 grams of egg white. Next, 20.0 ml of phosphate buffer (pH 7.4) was added to the egg whites, which were homogenized by vortexing. The homogeneous mixture was then centrifuged at 3000 rpm for 10 minutes to separate the precipitated proteins and the supernatant. The supernatant was spiked with a certain volume of tetracycline standard until

a serial concentration of tetracycline standard in the sample solution was obtained. The spiked sample solution was then taken and extracted via the DSPE method under optimum conditions. For samples of cow milk, the sample pretreatment protocol involves obtaining fresh cow milk from farmers. The milk sample was subsequently centrifuged to obtain the supernatant. The supernatant was then spiked with a certain volume of tetracycline standard and extracted via the DSPE method under optimum conditions in triplicate at each spiked concentration. The extracted series concentrations of standard solutions from the matrix sample were determined to investigate the validation performance of DSPE combined with a UV–Vis spectrophotometer, i.e., the MIP sorbent under optimum parameters.

RESULTS AND DISCUSSION

Synthesis and characterization of molecularly imprinted polymers (MIPs) and nonimprinted polymers (NIPs)

The results of the synthesis of MIP and NIP were then analyzed via FTIR and are shown in Figure 3. The results of the synthesis were also compared with those of the base material of chitosan. The third spectrum shows almost the same characteristics, namely, the peak shape in almost all the wavenumbers of the third material. The peak at wavenumber 1641 cm^{-1} indicates the presence of a carbonyl functional group (C=O), the peak at wavenumber 1416 cm^{-1} indicates the presence of a C-H functional group, the peak at wavenumber 1093 cm^{-1} indicates the presence of a C-O functional group, and the peak at wavenumber 3455 cm^{-1} indicates the presence of an amine functional group (N-H). However, a significant difference was observed in the absorption intensity of each peak, indicating that chitosan had the lowest peak absorption, whereas NIP had the highest peak absorption. This indicates that there is a polymerization process, resulting in the addition of functional groups to NIP and MIP (Haryanto, 2017).

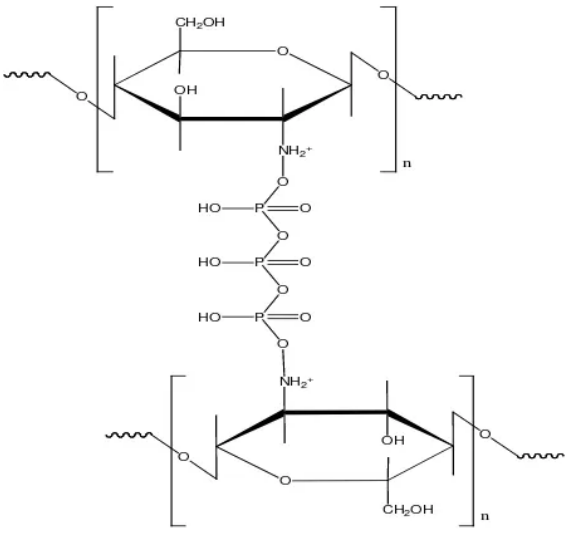


Figure 2. The chemical structure of Chi-NaTPP

The synthesis of MIP and NIP from chitosan by crosslinking NaTPP was successfully carried out via the ionic gelation method. The ionic gelation method is generally used to make

chitosan micro- to nanometers in size. The polymerization reaction that occurs during the manufacturing of MIP and NIP starts from the protonation of the NH_2 group of the chitosan molecule to NH_3^+ in 2% acetic acid. Positive ions in the amino functional group react with negative ions from sodium tripolyphosphate (NaTPP) at room temperature. The result of this reaction is the formation of a gel polymer, which is then printed in sheet form on a glass sheet, dried, and ground (Setiawan et al., 2015). The chemical structure of the Chi-NaTPP polymer is shown in Figure 2.

For MIP, the number of functional groups at the same peak as that of NIP was lower. This happens because of washing to remove the molecular template (tetracycline), so it is possible that some of the polymers are also wasted (Soledad-Rodríguez et al., 2017). This is also supported by the SEM characterization results shown in Figure 4. The polymerization process increases the viscosity of the polymer solution. The increase in viscosity causes the energy of the cavity given to the chitosan molecules to decrease so that the size of the particles formed becomes even greater. At the time of washing to release the tetracycline template on the MIP, some of the polymers and crosslinker are carried away, making the formation of cavities difficult to maintain. The high number of crosslinks allows the cavities to maintain a three-dimensional structure that is complementary in both form and chemical function to the template after template removal; thus, the functional groups are maintained in the optimal configuration for template rebinding, allowing the receptor to 'recognize' the native substrate (Soledad-Rodríguez et al., 2017)

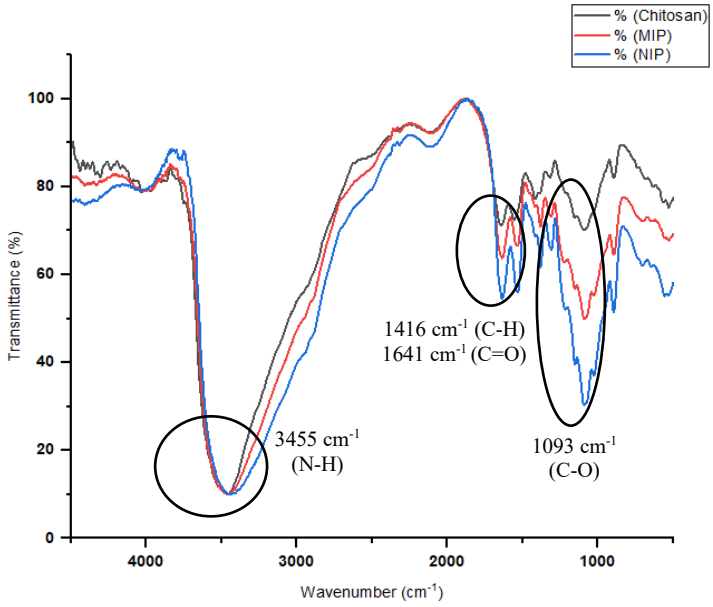
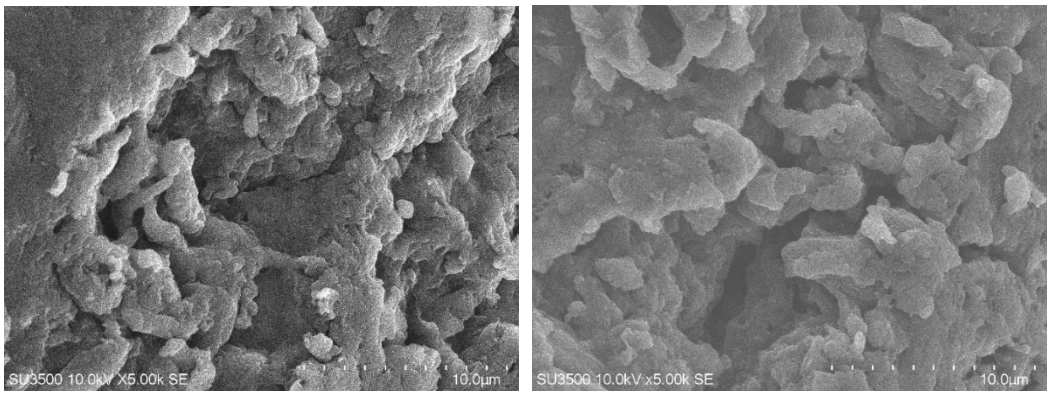


Figure 3. FTIR spectra of chitosan, MIP, and NIP



(a) (b)

Figure 4. SEM images of the MIP (a) and NIP (b)

Optimization parameters of the DSPE method

Sorbent Type

The extraction performances of the MIPs and NIPs were evaluated to investigate their extraction performance. As shown in Figure 5, the extraction efficiency of the MIP was greater than that of the NIP for all three extraction replications. MIPs and NIPs were synthesized using a crosslinker at the same concentration so that at the time of synthesis, both materials had the same ability to maintain the 3-dimensional structure formed. However, MIP has more mold cavities according to the chemical structure of the analyte because it undergoes a washing process to remove the tetracycline template, and there is no effect on the stability of the 3-dimensional structure of the polymer during template removal (Soledad-Rodríguez et al., 2017). This provides a greater opportunity for the analyte to bind to the MIP. MIP was then selected for further optimization.

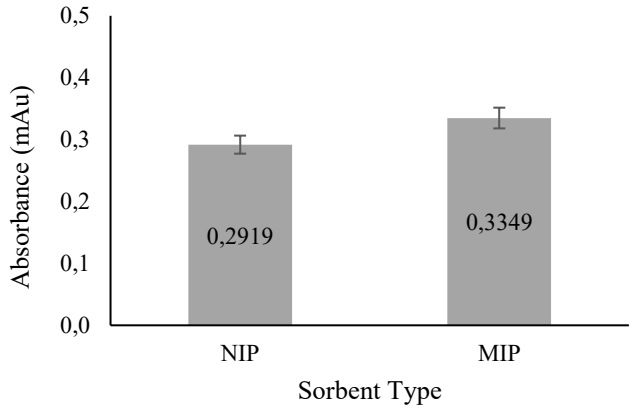


Figure 5. Optimization of sorbent type

Desorption instrument type

The instruments used for the variations were a vortex and an ultrasonicator. Figure 6 shows that the ultrasonicator has a better desorption ability than the vortex does. This happens because the ultrasonicator has intensive stirring at constant vibrations and can cause local heating of the liquid to facilitate the penetration of the analyte into the solvent according to

its solubility and will increase material transfer compared with less constant vortex vibrations, which can affect the absorbance results; thus, at the same time, the ultrasonicator was able to more quickly desorb tetracycline from the MIP surface. Ultrasonicator sonication is a liquid–liquid extraction method that uses ultrasonic waves with a frequency of 42 kHz, which can accelerate the contact time between the sample and the solvent even at room temperature. Sonication relies on wave energy, which causes a cavitation process, namely, the process of forming small bubbles due to the transmission of ultrasonic waves to assist the diffusion of solvents into the cell walls of the MIP (Dalimunthe et al., 2019). An ultrasonicator was then selected for further optimization.

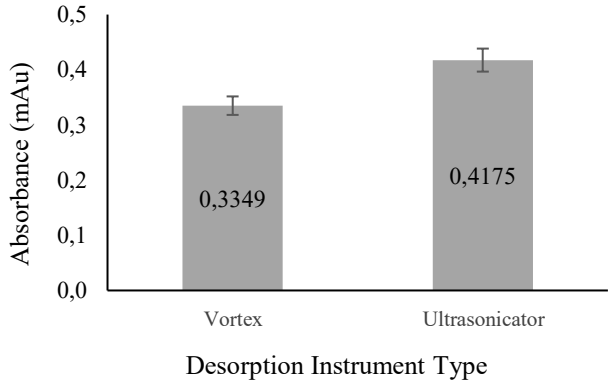


Figure 6. Optimization of the desorption instrument type

Desorption Solvent Type

The selection of a suitable organic solvent to desorb the analyte after extraction is important. The stability of the sorbent in organic solvents and the solubility of the analyte in organic solvents must also be considered to obtain better extraction efficiency (Amanda, Sanagi, et al., 2022). Desorption solvent optimization was carried out with four variations, namely, glacial acetic acid, ethanol, ethyl acetate, and n-hexane. The results of the optimization of the absorbance of the desorbing solvent are shown in Figure 7. The results revealed that ethyl acetate had the highest absorbance of 0.5539, followed by ethanol (0.4674), acetic acid (0.1826) and N-hexane (0.1690). This occurs because the solvent ethyl acetate (semipolar) has a greater solubility of tetracycline than ethanol, acetic acid and N-hexane do. Solubility can also be affected by polarity; ethanol and acetic acid are polar, ethyl acetate is semipolar, and N-hexane is nonpolar. The polarity of the solvent increases the polarity of the MIP surface so that the more polar analyte will be relatively retained in the adsorbent due to the electrostatic interaction of the MIP polar surface. Moreover, nonpolar analytes are carried away by solvent flow without experiencing electrostatic resistance to the MIP surface (Susanti et al., 2017). Ethyl acetate was then selected for further optimization.

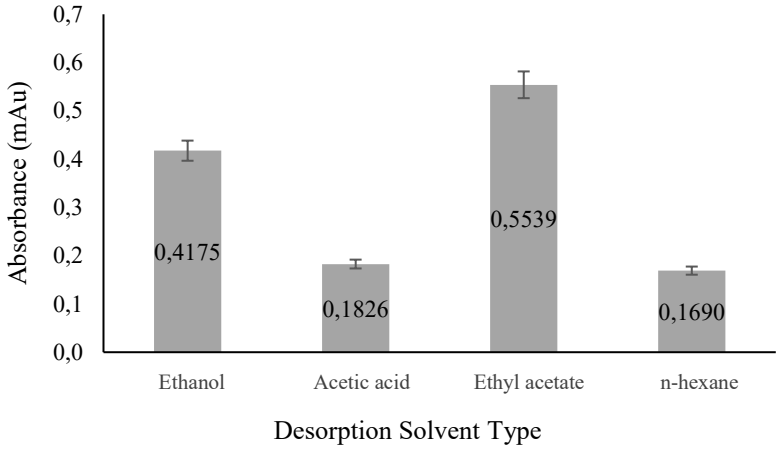


Figure 7. Optimization of the desorption solvent type

Extraction time

The extraction time is the time required for the analyte to migrate from the sample solution in the solid sorbent until equilibrium is reached. Optimization of the extraction time was carried out with four durations, namely, 5 minutes, 10 minutes, 12 minutes and 15 minutes. The extraction process was carried out with three replications. As shown in Figure 8, the absorbance peak was obtained at the 12th minute. This indicates that at an adsorption time of 12 min, the MIP sorbent was at the maximum adsorption level (saturated), so it was no longer able to absorb tetracycline from the solution. The MIP sorbents require an adsorption time of up to 12 min to achieve the best or maximum adsorption capacity results.

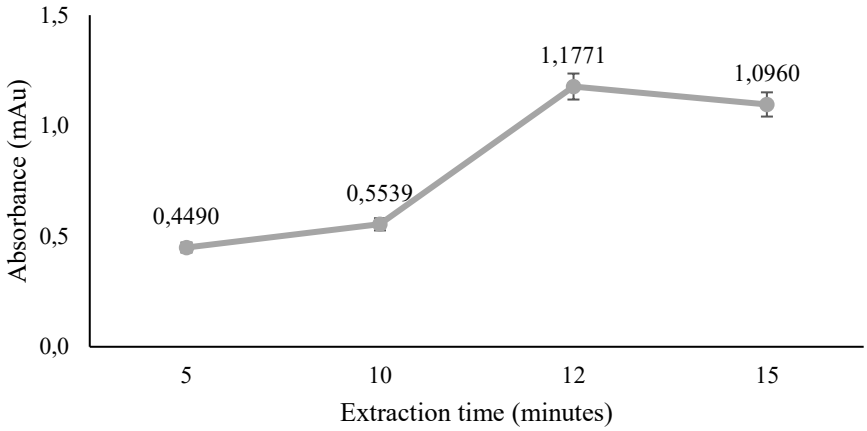


Figure 8. Optimization of extraction time

The formation of NaTPP cross-links can strengthen the material, and the pores of the material become tighter so that the ability of the MIP membrane to absorb tetracycline solutions decreases, so the extraction time required for the MIP adsorbent reaches 12 minutes to reach the maximum adsorption level. Moreover, at 15 minutes of extraction, the absorbance decreased, possibly because the longer the contact time between the adsorbent

and the analyte was, the higher the adsorption capacity and the possibility of a desorption process, namely, the release of the adsorbent after binding to the analyte due to the saturated adsorbent. The equilibrium distribution of analytes in the MIP sorbent is also affected by the number/thickness of the sorbent (Amanda, Sanagi, et al., 2022). An extraction time of 12 minutes was selected for further optimization.

Desorption Time

The desorption times were 1 minute, 2 minutes, 3 minutes, 4 minutes and 5 minutes. On the basis of the results of the optimization of the desorption time shown in Figure 9, at 3 min, the optimum absorbance results were obtained with an ultrasonicator. The desorption ability increases gradually with increasing time. From 1 minute to 3 minutes, the desorption ability increases because the tetracycline absorbed by the MIP membrane dissolves in ethyl acetate with the help of microwaves for penetration. Thus, at 3 min, the analytes were completely extracted. However, when the desorption time was extended to 4 to 5 min, the extraction efficiency decreased. This was probably due to the longer sonication time, which produced heat that caused analyte degradation (Amanda, Sanagi, et al., 2022). Three minutes of desorption time was selected for match calibration.

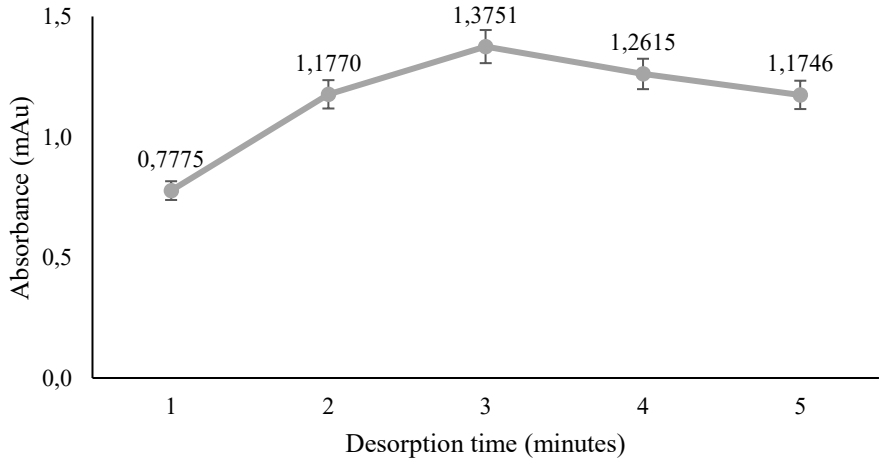


Figure 9. Optimization of desorption time

Validation Method using Matrix Match Calibration

Two different samples matching the calibration curve were prepared. These samples were prepared by plotting serial numbers of spiked concentrations in the sample solution. The validation results were then compared among spiked samples without extraction and spiked samples with the DSPE method. The comparison results of the validation are shown in Table 1. Sample preparation via DSPE resulted in better performance in terms of the linearity range, LoD, and LoQ due to the preconcentration of analytes during the extraction process. It can be seen in the column of the enrichment factor. However, the values of LoD and LoQ have not reached the MRL value issued by the WHO and FAO. The selected instrument also affects the efficiency of the analysis results.

Table 1. Method validation of DSPE combined with a UV–Vis spectrophotometer for the analysis of tetracycline residues

Samples	Recovery (%)	Linearity range (mg L^{-1})	Correlation coefficient (r^2)	LOD (mg L^{-1})	LOQ (mg L^{-1})	RSD (%) (n = 3)	Enrichment Factor (times)
(a) Milk							
Without Extraction	99.58	10-50	0.9976	2.78	9.03	2.32	-
DSPE Method in standard solution	98.86	1-5	0.9960	0.35	1.16	4.68	3.29
DSPE Method in spiked matrix samples	98.22	1-5	0.9931	0.46	1.52	2.74	3.27
(b) Egg							
Without Extraction	97.50	10-50	0.9976	1.36	4.53	1.40	-
DSPE Method in standard solution	88.20	1-5	0.9960	0.17	0.58	1.19	3.26
DSPE Method in spiked matrix samples	88.10	1-5	0.9913	0.51	1.70	1.06	3.27

CONCLUSIONS

Chi-NaTPP-based MIPs have been successfully synthesized. This material significantly improves the extraction and analysis of tetracycline residues in complex food matrices such as milk and eggs. This method not only improves sensitivity and accuracy but also contributes to the broader goal of monitoring antibiotic residues in agricultural products to safeguard public health.

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