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Separation Science and Technology
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713708471

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Online publication date: 12 February 2010

To cite this Article Khorrami, Afshin Rajabi and Edrisi, Mahtab(2010) 'Synthesis and Evaluation of a Molecularly Imprinted Polymer for Solid Phase Extraction of Ethopabate from Chicken Tissue', Separation Science and Technology, 45: 3, 404 — 412
To link to this Article: DOI: 10.1080/01496390903409567
URL: http://dx.doi.org/10.1080/01496390903409567

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Separation Science and Technology, 45: 404–412, 2010
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ISSN: 0149-6395 print/1520-5754 online
DOI: 10.1080/01496390903409567

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Synthesis and Evaluation of a Molecularly Imprinted Polymer for Solid Phase Extraction of Ethopabate from Chicken Tissue

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In this paper the development and evaluation of a molecularly imprinted polymer (MIP) for ethopabate is described. Ethopabate (ETP), 4-acetamido-2-ethoxybenzoic acid methyl ester, is one of the antibiotics which is used as coccidiostat in poultry feeds. In the present study, two widely used functional monomers, methacrylic acid (MAA) and 4-vinylpyridine (4-VP) were compared theoretically and experimentally as the candidates for MIP preparation. Hyperchem software was employed to estimate binding energies between ETP and functional monomers and batch rebinding experiments were performed to study the binding characteristics of the polymers. The results showed that MAA is a better functional monomer to prepare MIP. UV/Vis and NMR spectroscopy were used as two common tools to study the interactions between ETP and MAA in the pre-polymerization mixture. Liquid chromatography experiments showed that the prepared MIP has recognition capability toward ETP in comparison with other structurally related compounds. The ETP-imprinted polymer was further applied for selective solid phase extraction (SPE) of ETP from a chicken tissue sample. The extraction yield of ETP was found to be quantitative ($87 \pm 3\%$) and the LOD and LOQ based on 3 and 10 times of the noise of HPLC profile were 0.05 and 0.32 ng ml^{-1}, respectively. It was confirmed that the binding ability of the prepared MIP for ETP was essentially sufficient in the presence of other compounds coexisting in tissue sample. Therefore, as a selective and efficient solid phase material, ETP-imprinted polymer has a high potential application in the analysis of residues of this antibiotic in chicken tissue samples.

Keywords chicken tissue; ethopabate; molecularly imprinted polymer; solid phase extraction

INTRODUCTION

An antibiotic is a chemotherapeutic agent that inhibits the growth of microorganisms such as bacteria, fungi, or protozoa. The term originally referred to any agent with biological activity against a living organism, however, antibiotics now refer to substances with anti-bacterial, anti-fungal, or anti-parasitical activity. Poultry industries commonly use a wide selection of antibiotics as anticoccidial drugs and as growth promoters. For decades, ethopabate (ETP), 4-acetamido-2-ethoxybenzoic acid methyl ester, has been one of the popular antibiotics, which were frequently used as coccidiostat in poultry feeds (1). At the present time, the use of this antibiotic in poultry production has been banned in the EU and USA in accordance with the directives and regulations of the European Union numbered 98/19/EC, 45/1999/EC and 2205/2001/EC (2). However, because of the lack of control and legislation regarding antibiotic usage or residue in food, ETP is not phased out in many developing countries. Consequently, the development of reliable analytical approaches for the determination of ETP residue in low concentration levels in chicken tissue samples seems worthwhile.

Chromatographic methods such as gas chromatography (GC) (3) and high performance liquid chromatography (HPLC) with UV detection (4–6) or by fluorimetry (7,8) have been applied to the determination of this compound after sample clean-up by (multiple) solvent extraction(s), conventional column chromatography and solid phase extraction (SPE). SPE has received much attention in recent years for the sample preparation and analysis of trace concentration in samples. However, typical SPE sorbents lack selectivity and this constitutes a problem when a selective extraction from a complex matrix has to be performed.

To enhance the molecular selectivity in SPE, immunoassay sorbents and molecularly imprinted polymers (MIPs) have been developed. Recent developments have demonstrated that MIPs are well alternatives to antibody based sorbent (9). Molecular imprinting is a well-established strategy of polymer synthesis to prepare MIPs with high selectivity towards a particular molecule, which is known as a template. Generally, the imprinting process involves pre-arrangement of the functional monomers around a template molecule, then polymerization in the presence of a cross-linking monomer and finally removing the template in order to leave a cavity specific for template molecule. SPE using MIPs (MISPE) are extensively used in

Received 2 February 2009; accepted 30 September 2009.
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extraction of various biologically active compounds such as hormones (10,11), antibiotics (12–15) and drugs (16–18).

Because of a very complex matrix of tissue samples, only a few works have been carried out to MISPE from these types of samples (19–21). In the present work, we study the evaluation and characterization of a MIP in a non-covalent approach, able to bind ETP selectively for its applications in the selective clean-up and quantification of this compound in chicken tissue sample. In order to choose the suitable functional monomer, Hyperchem software was used to predict the possible interactions and binding energies between ETP and functional monomers. Methacrylic acid (MAA) and 4-vinylpridine (4-VP) as the most widely used functional monomers were chosen for this purpose. Binding characteristics of the two MIPs including dissociation constants, K_d, and number of binding sites, Q_{max}, were evaluated and compared using Scatchard analysis in equilibrium batch rebinding experiments. In order to confirm the non-covalent interactions of ETP-functional monomer in the pre-polymerization mixture, the UV/Vis and ^{1}H NMR spectroscopy were employed. The selectivity and recognition ability of the prepared MIP toward ETP and other compounds structurally related to it were evaluated chromatographically on a column packed with the ETP-imprinted polymer. Finally, the resultant MIP was used for development of a MISPE protocol for pre-concentration of ETP antibiotic from chicken tissue sample.

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EXPERIMENTAL

Reagent and Standards

ETP was purchased from Iran veterinary drugs Co. (Tehran, Iran). All analytical grade solvents, functional monomers (MAA and 4-VP), ethylene glycol dimethacrylate (EGDMA), methyl-4-aminobenzoate (MBZ), and N-phenylacetamide (NPA) were purchased from Merck (Darmstadt, Germany). Acetonitrile (ACN) and methanol were LC-grade and purchased from (Darmstadt, Germany). Methyl-4-acetamidobenzoate (MAB) was synthesized from methyl-4-aminobenzoate. In order to remove the stabilizer, MAA, 4-VP and EGDMA were distilled under reduced pressure immediately before use. 2,2′-Azobisisobutyronitrile (AIBN) as the initiator of polymerization was obtained from Acros Organics (Geel, Belgium) and recrystallized from methanol before use.

Apparatus

A chromatographic system (PerkinElmer, USA) consisting of binary pump series 200, Rheodyne injector with a 20 µl loop, and UV detector series 200 was used. The TotalChrom software was used to acquire and process spectral and chromatographic data from the detector. All separations were performed using Spherisorb ODS (5 µm, 4.6 mm i.d. × 22 cm length) from PerkinElmer under isocratic condition. The column temperature was adjusted at 25°C by a Knauer column thermostat (Berlin, Germany). For ETP determination, the detector was set at 270 nm for peak area measurements and the mobile phase was composed of 0.05 M phosphate buffer at pH = 3/ACN (20/80, v/v) at a flow rate of 1.1 ml min^{-1}. All UV/Vis measurements and spectra were obtained with a UVIKON 922 (Kontron) spectrophotometer. ^{1}H NMR spectra were recorded on a Brucker 300 MHZ (Karlserohe, Germany) instrument at 25°C.

Synthesis of Polymers

Two MIPs were prepared according to the conventional bulk polymerization using MAA and 4-VP as their functional monomer. 0.237 g (1 mmol) ETP and 0.347 g (4 mmol) MAA or 0.438 g (4 mmol) 4-VP were first dissolved in 9 ml ACN in a 20 ml thick-walled glass tube and incubated for 4 h to ensure of hydrogen bond formation between ETP and functional monomers. Then, 4.044 g (20 mmol) EGDM and 0.05 g AIBN were added to the mixtures. After oxygen-free nitrogen gas bubbling into the solution for 5 min, the tubes were sealed under vacuum, and the mixtures kept in an oil bath at 60°C for 24 h. The resulting bulk rigid polymers were crushed and ground manually with a mortar and pestle and sieved, under water through 50 and 25 µm sieves. Particles between sieves were collected for further experiments. Then the template molecule was completely extracted in a Soxhlet apparatus by repeated extractions with ACN, water, methanol/trifluoroacetic acid (90/10, v/v) and methanol, until the template was not detected by HPLC in effluents after its evaporation and reconstitution of the residue in 200 µl mobile phase. A blank polymer with MAA as functional monomer that did not contain any template was prepared simultaneously using the same protocol.

Synthesis of Methyl-4-Acetamidobenzoate (MAB)

The synthesis of MAB was performed as follows. 0.15 g methyl-4-aminobenzoate (1 mmol) dissolved in 2 ml THF was placed in a test tube and 0.07 ml acetyl chloride (1 mmol) was added. The mixture was stirred over 1 h at room temperature. The MAB was precipitated out as a white precipitate, filtered under reduced pressure and washed with 3 ml THF. The final product was dried under vacuum at 60°C over night and had the following ^{1}H NMR and melting point data: ^{1}H NMR ($CDCl_3$) δ (ppm), 2.07 (s, 3H), 3.88 (s, 3H), 7.38–7.88 (m, 4H), 10.49 (s, 1H); m.p.:135°C.

Molecular Modeling Studies

Simulating the interactions between ETP and monomers (MAA and 4-VP) was performed using Hyperchem 7.0 (Hyperchem Inc. Gainesville, FL) according to the procedure described by Farrington et al. (22). After drawing molecular

structures of ETP, MAA, and 4-VP, their conformations were evaluated and refined using molecular mechanic MM+ and the semi-empirical PM3 method, respectively. Ab initio (3-21 G) quantum mechanic basis set was used to refine the conformation of the lowest energy. For analyzing the possible interactions between ETP and monomers and for calculating the binding energies, the amber MM method was used. The force filed was set up with constant dielectric and the scale factors of Van der Waals and electrostatic were selected at 0.5. The binding energies between ETP and monomers were calculated using Eq. (1):

$$\Delta E = [E_{complex} - E_{ETP} - E_{monomer}] \quad (1)$$

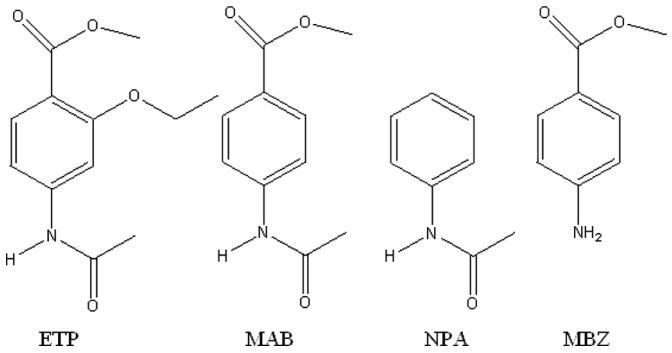

FIG. 1. Chemical structures of ethopabate (ETP), methyl-4-acetamido benzoate (MAB), N-phenylacetamide (NPA) and methyl-4-aminobenzoate (MBZ).

Spectroscopic Analysis

In order to approve the interaction between ETP and MAA in the pre-polymerization mixture, UV/Vis and ^{1}H NMR spectroscopy were used. For UV/Vis studies, several solutions with different mole ratios of MAA to ETP in ACN were prepared by adding different amounts of MAA to 0.2 mM ETP solutions. The resulting concentrations of MAA in the solutions were from 0 to 1.0 mM. In order to ensure complete interaction between ETP and MAA, the solutions were incubated at 25°C for 4 h and their spectra were recorded at 230–320 nm using corresponding MAA solutions as blank. For ^{1}H NMR spectroscopy, ETP and MAA individually were dissolved in deuterated ACN to obtain their ^{1}H NMR spectra. Then, they were mixed in a molar ratio like the pre-polymerization mixture and the spectrum of the mixture was recorded after 4 h incubation time.

Binding Experiment

Twenty milligrams of the washed and dried MIP particles (20–50 μm) were added to 5 ml conical flasks containing 2 ml of ACN with different concentration of ETP (1×10^{-3}–5×10^{-1} mM). The flasks were incubated at 25°C for 12 h with stirring. Then, the mixtures were transferred into centrifuge tubes and centrifuged at 6000 rpm for 20 min. The concentrations of free ETP in supernatant solutions were determined using UV/Vis spectrophotometry by measuring the absorbance at 267 nm. The amount of ETP bounded to the MIPs was calculated by subtracting the concentration of free ETP from the initial concentration. The number of binding sites in the polymers, Q_{max}, and the sequilibrium dissociation constants, K_d, for two MIPs were calculated using Scatchard analysis.

Liquid Chromatography Experiment

The retention of ETP and other similar compounds whose chemical structures are shown in Fig. 1 on the imprinted and the blank columns were studied by liquid chromatography. The experiments were performed with a PerkinElmer series 200 HPLC pump and detector system. MIP particles between 25 and 50 μm sieves after removing their fine particles by sedimentation in acetone were slurry packed into a 120 mm × 6 mm HPLC stainless steel column. The UV detector wavelength was set at 297 nm and the analysis performed at room temperature. The mobile phase was ACN at a flow rate of 0.3 ml min^{-1}. Before the evaluation of the polymer, the packed column was washed with a mixture of methanol/acetic acid (95/5, v/v) for about 10 h at a flow rate of 0.1 ml min^{-1} to remove the template and the un-reacted monomers and establish a constant baseline. 20 μl of acetone 0.1% (v/v) in ACN was used as void marker. 20 μl (0.05 mg ml^{-1}) of ETP and other similar structures in ACN were injected and the retention time for each one was obtained.

MISPE Studies

It has been proved that MIPs offer their highest selectivity and rebinding ability when samples dissolved in the solvent used for the MIPs preparation (23,24). On the other hand, ETP is fairly soluble in ACN and has been successfully employed as a good extractant of ETP from chicken muscle samples (17). Therefore it was decided to use ACN as the extracting and loading solvent for further MISPE experiments. MIP column was prepared by packing 200 mg of the polymer into a 4 ml empty SPE cartridge. The polymer in the cartridge was secured by polyethylene frits at the top and the bottom. The cartridge was conditioned sequentially with 3 ml of methanol/acetic acid (90/10, v/v), 3 ml of methanol, and 5 ml of ACN at 1 ml min^{-1}. Extraction experiments entailed loading the cartridge with ACN solution containing ETP at 0.5 ml min^{-1}. After loading, the column was washed with 1 ml of ACN at 1 ml min^{-1}. Finally, the elution was performed by passing 3 ml of methanol/acetic acid (90/10, v/v) at 0.5 ml min^{-1}. All the fractions from the sample loading, washing, and elution steps were collected and then evaporated to dryness at 40°C under a stream of N_2 gas.

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The residues were reconstituted by dissolution in 200 µl ACN. Then, 20 µl of each sample was injected onto the analytical column of HPLC. The recoveries were calculated using the constructed calibration plot.

Extraction of ETP from Chicken Muscle Tissue

Chicken muscle samples used for this study were collected from local market and were minced with a meat grinder and stored frozen at −20°C until assay. One gram of tissue sample was put in a test tube and 1 ml of ACN added to it. The mixture was spiked with the prescribed volume of the working standard solution and mixed well. Then the tube was centrifuged for 15 min at 12,000 rpm and the clear supernatant was passed through a 0.45 µm Nylon filter. Finally, 0.5 ml portion of the filtered supernatant was loaded into the conditioned MISPE cartridge as described previously. A blank sample was prepared with the same method in the absence of ETP for comparison.

RESULTS AND DISCUSSION

Molecular Modeling

Hyperchem has been frequently used as a conventional tool to predict the structure of species in the prepolymerization mixtures (25,26). By means of this software the validity of different interactions between functional groups of ETP and monomers was investigated. Optimized structures of ETP, MAA and 4-VP are shown in Fig. 2 and calculated partial charges of their atoms are listed in Table 1. These data suggest the most probable interaction sites between different functional groups of molecules. Along with these data, H_{29} is H-bond donor of ETP and O_{16}, O_8, O_9, and O_{11} are its H-bond acceptors. Because of spatial considerations, the probability of interactions using O_9 and O_{11} is not significant and very weak interactions are expected by them. It can be seen in the MAA molecule that the H_{12} is the H-bond donor and O_5 is the H-bond acceptor. Also, N_1 in 4-VP is its single interacting site which acts as H-bond acceptor. The binding energies, ΔE, of the most possible interactions between ETP, MAA and 4-VP which are shown in Fig. 3, were calculated and the results are listed in Table 2. The strength of these complexes which can be estimated from their ΔE values determines the ability of the synthesized polymer to selective binding of the template. As can be seen, high binding energies of complexes between ETP and MAA suggest that the imprinted polymer using this monomer would be more favorable with the highest selectivity than the polymer uses 4-VP as functional monomer.

UV/Vis and ^{1}H NMR Spectroscopy

UV/Vis spectroscopy has been used frequently to study the interactions between template and functional

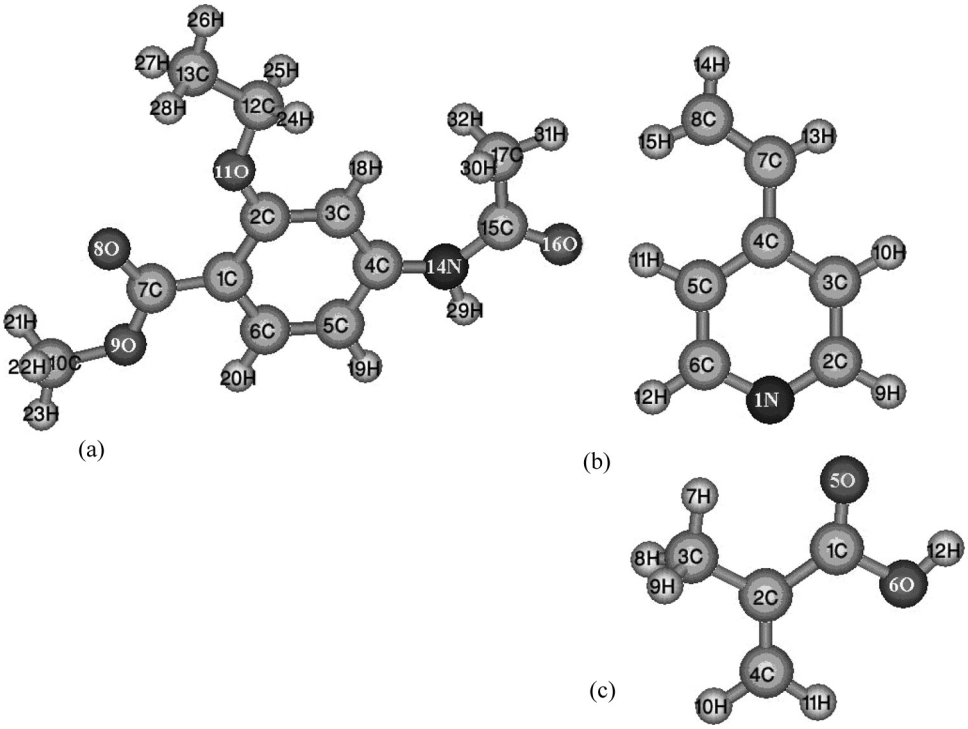

FIG. 2. Optimized conformation of ETP (a), 4-VP (b) and MAA (c) obtained by Hyperchem. Carbon = green, nitrogen = blue, oxygen = red and hydrogen = white.

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TABLE 1
The partial charges of atoms in ETP, MAA and 4-VP

Molecule	No.	Atom	Charge
ETP	1	C	−0.337
	2	C	0.536
	3	C	−0.310
	4	C	0.446
	5	C	−0.282
	6	C	−0.152
	7	C	1.022
	8	O	−0.623
	9	O	−0.728
	10	C	−0.271
	11	O	−0.736
	12	C	−0.1
	13	C	−0.599
	14	N	−1.098
	15	C	0.861
	16	O	−0.603
	17	C	−0.712
	18	H	0.272
	19	H	0.241
	20	H	0.299
	21	H	0.219
	22	H	0.219
	23	H	0.216
	24	H	0.206
	25	H	0.206
	26	H	0.238
	27	H	0.238
	28	H	0.196
	29	H	0.383
	30	H	0.241
	31	H	0.241
	32	H	0.271
MAA	1	C	0.913
	2	C	−0.218
	3	C	−0.609
	4	C	−0.350
	5	O	−0.612
	6	O	−0.729
	7	H	0.258
	8	H	0.224
	9	H	0.224
	10	H	0.228
	11	H	0.266
	12	H	0.415
4-VP	1	N	−0.672
	2	C	0.130
	3	C	−0.323
	4	C	−0.032
	5	C	−0.317
	6	C	0.133
	7	C	−0.202
	8	C	−0.403
	9	H	0.256
	10	H	0.246
	11	H	0.244
	12	H	0.256
	13	H	0.237
	14	H	0.227
	15	H	0.219

monomers in pre-polymerization mixtures (16). As shown in Fig. 4, the maximum absorption of ETP is increased by increasing the mole ratio of MAA to ETP which confirms some interaction between them. As can be seen, when the mole ratio of MAA to ETP exceeds four, the spectral changes are less pronounced. Thus, it was concluded that the optimum mole ratio of MAA:ETP was 4:1. Consequently, this mole ratio was selected as the best value for further synthesis of MIP. Because the formation of a hydrogen bond leads to a change in the chemical shift, ^{1}H NMR spectroscopy has been generally used to clarify the nature of non-covalent interactions between the template and the functional monomers in the pre-polymerization mixture (27). Comparing individual ^{1}H NMR spectra of ETP and MAA and their mixture spectrum in deuterated ACN revealed that there are two apparent chemical shift changes in the mixture spectrum. The first was a downfield shift from $\delta = 8.52$ to $\delta = 8.60$ corresponding to the interaction between H_{29} of ETP and O_5 of MAA. The second was another downfield shift from $\delta = 9.86$ to $\delta = 10.23$ corresponding to the possible interactions between H_{12} of MAA with O_{16} and O_8 of ETP. By considering the extent of the chemical shift changes in the mixture spectrum, it is obvious that the strongest hydrogen bond occurs between ETP as the proton acceptor and MAA as the proton donor.

Scatchard Analysis

The Scatchard analysis was performed to predict the binding parameters of the MIPs prepared using MAA and 4-VP as functional monomers (28,29). The equilibrium dissociation constant, K_d, and the apparent maximum number of binding sites, Q_{max}, were calculated using the following equation (30):

$$\frac{Q}{[ETP]} = \frac{Q_{max} - Q}{K_d} \quad (2)$$

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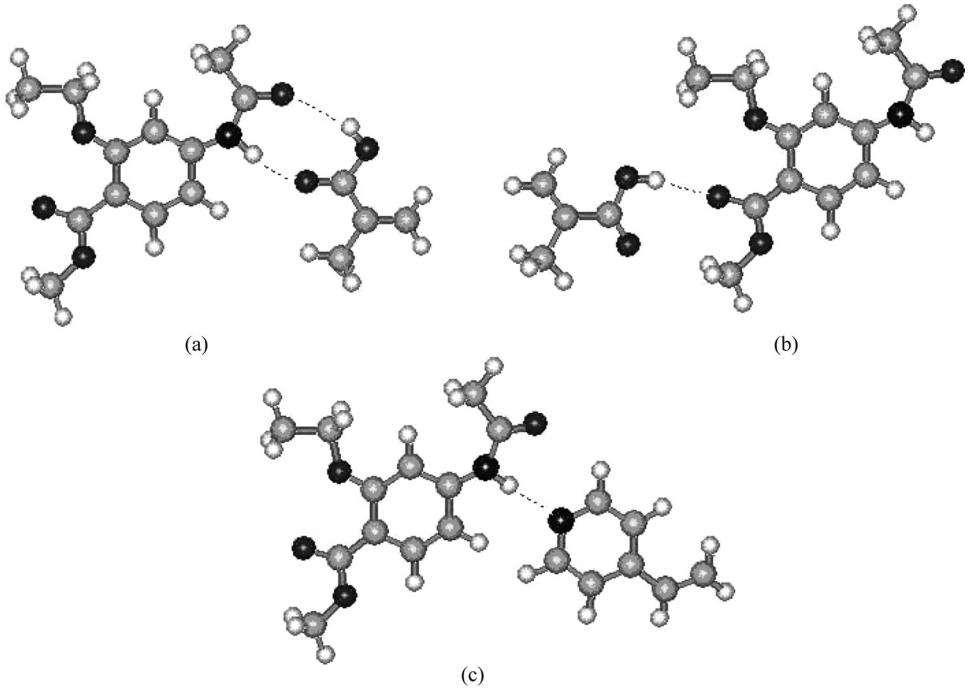

FIG. 3. The most possible interactions between ETP and functional monomers are represented.

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Where K_d ($\mu mol l^{-1}$) is defined as the equilibrium dissociation constant of the binding sites, Q and Q_{max} (μmol) are the amount of ETP bonded to the polymer and the apparent maximum number of binding sites, respectively. $[ETP]$ ($\mu mol l^{-1}$) is the equilibrium free concentration of ETP. Therefore, a graph of $Q/[ETP]$ versus Q yields a straight line, with a slope of $-1/K_d$ and an intercept of Q_{max}/K_d.

TABLE 2
The binding energies between ETP and functional monomers

Species	Energy ($kJ\,mol^{-1}$)	ΔE (binding energy) ($kJ\,mol^{-1}$)
ETP	−158.912	–
MAA	−141.96	–
4-VP	−16.77	–
Complex between ETP and MAA, ($H_{29}...O_5$ and $H_{12}...O_{16}$)	−348.200	−47.328
Complex between ETP and MAA, ($H_{12}...O_8$)	−328.548	−27.676
Complex between ETP and 4-VP, ($H_{29}...N_1$)	−193.530	−17.84

Figure 5 shows the Scatchard plots for the MIP prepared by MAA as its functional monomer. The obvious curvature in the plot is due to the heterogeneity of the underlying binding sites of the MIP. This heterogeneity

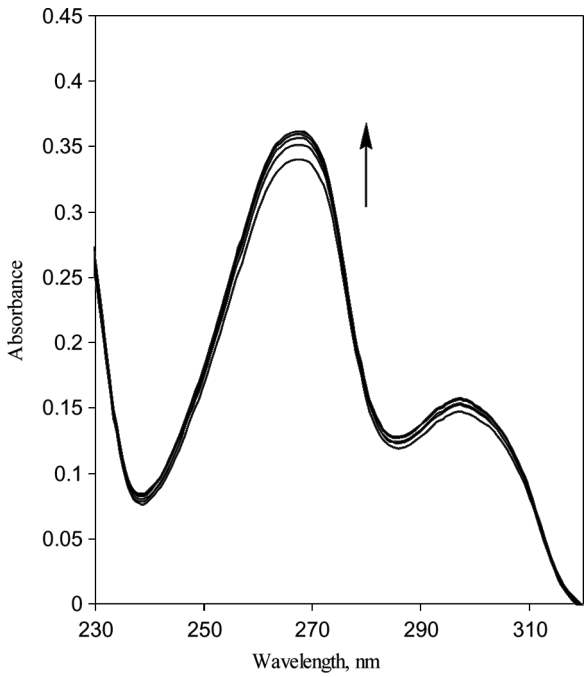

FIG. 4. The UV/Vis spectra of ETP in the presence of different concentrations of MAA in ACN.

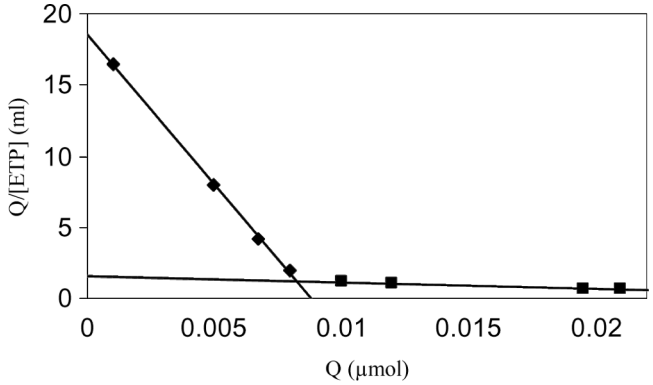

FIG. 5. The Scatchard plot for MIP using MAA as functional monomer.

can be modeled by the limiting slopes method by two linear parts with different slopes, yielding two sets of binding parameters corresponding to the high and low affinity sites. The least squares regression equations for the two parts are $Q/[ETP] = -2103.5\ Q + 18.55$ ($r = 0.9985$) and $Q/[ETP] = -44.166\ Q + 1.5577$ ($r = 0.9971$) for the high and the low affinity sites, respectively. The K_d and Q_{max} of higher affinity sites were $0.47\,\mu mol\,l^{-1}$ and $0.04\,\mu mol\,g^{-1}$ dry polymer and for lower affinity sites were $22.64\,\mu mol\,l^{-1}$ and $0.17\,\mu mol\,g^{-1}$ dry polymer, respectively. Figure 6 shows the Scatchard plot of 4-VP imprinted polymer which is a straight line with the equation $Q/[ETP] = -65.322\ Q + 0.7177$ ($r = 0.9916$). The calculated K_d and Q_{max} were $15.31\,\mu mol\,l^{-1}$ and $0.5\,\mu mol\,g^{-1}$ dry polymer, respectively. The calculated dissociation constants were in similarity to Hyperchem studies in which it predicts that in the prepolymerization mixture ETP forms more strong complexes with MAA than 4-VP.

Liquid Chromatography

To confirm the imprinting effect and selectivity of the ETP-imprinted polymer synthesized using MAA, its ability to resolve structural analogs was measured chromatographically. ETP and its analogs, methyl-4-acetamido benzoate (MAB), N-phenylacetamide (NPA), and methyl-4-aminobenzoate (MBZ), which have almost similar molecular structures, were used for this study.

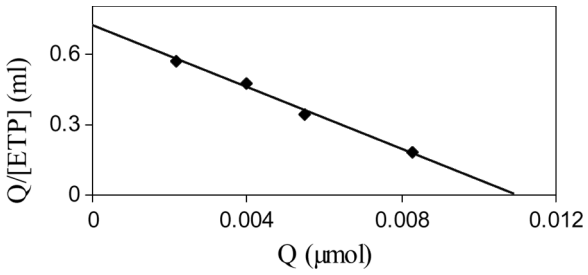

FIG. 6. The Scatchard plot for MIP using 4-VP as functional monomer.

TABLE 3
The retention times for ETP and other structurally related compounds

Compounds	Retention time (min)
ETP	11.35
MBZ	7.8
MAB	15.7
NPA	16.51

Experiments showed that all the compounds had little or no retention on the column packed with NIP and were co-eluting with the void marker. Table 3 shows that these compounds clearly showing more or less retention capabilities on the column packed with the ETP-imprinted polymer. As can be seen, NPA, MAB, and ETP have relatively high retention times on the column. This could be explained by their similarity in possessing the acetamide group on their molecules. These results were along with the Hyperchem studies which had predicted a high binding energy for interaction between ETP and MAA via this functional group. As a result, it could be concluded that the short retention time of MBZ on MIP column could be owing to the absence of this group. Unexpected stronger retention of NPA and MAB on MIP column says that although the polymer was prepared using ETP as the template molecule, but a comparatively smaller size of NPA and MAB and the presence of acetamide substitution enable them to fit better than ETP into the specific binding sites in the polymer network. Also, weak retention of MBZ in comparison with NPA on MIP column confirms that the H on the nitrogen atom has not a significant role in forming hydrogen bond which this observation was in agreement with the previously NMR studies. The chromatographic evaluation of the ETP imprinted polymer elucidated that both structural features and functional groups were the main recognition mechanisms of the imprinted polymer.

SPE Using ETP-Imprinted Polymer

The prepared ETP-imprinted polymer was used to MISPE by means of cartridges. The breakthrough volume of the sample solution was tested by dissolving 1 μg ETP in 1, 2, and 3 ml of ACN and the recommended procedure followed. The results of the recovery experiments which are summarized in Table 4 showed that ETP could be quantitatively recovered from MISPE cartridge after extraction from 1 ml sample solution, washing with 1 ml of ACN, and eluting with 3 ml of methanol/acetic acid (90/10, v/v). The capacity of the MISPE cartridge was examined by passing 2 ml portions of ACN solution containing different amounts of ETP, followed by the recommended

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TABLE 4
Percent recoveries of 1 μg ETP from different volumes of ACN[a]

	Cartridge packing			
	MIP	MIP	MIP	NIP
Sample volume (ml)	1	2	3	2
Loading effluent + 1 ml of washing ACN	2.8 ± 3	5.8 ± 2	8.1 ± 3	96 ± 3
Elution (3 ml of methanol/acetic acid (90/10, v/v))	97 ± 2	94 ± 4	90 ± 5	4.3 ± 5

[a] $\bar{x} \pm$ R.S.D. ($n = 3$).

procedure (Table 5). ETP was not detected considerably (1.5%) in the effluent after the passing solution containing 1 μg, showing that the loaded ETP was completely retained. This concentration is significantly higher than the concentration of ETP likely to be present in chicken muscle samples.

Analysis of Standard Spiked Chicken Tissue Sample

In order to examine the performance of the imprinted polymer in complicated matrix, the prepared MIP was used to MISPE of ETP from the chicken tissue sample. MISPE from tissue extracts were performed under the same conditions as extraction of the standard solutions discussed earlier. The fractions from the loading and wash steps were found to be free of the analyte. No signal was detected at the retention time of the analyte when the non-spiked blank muscle sample was analysed. In spiked sample of 1 μg, the adsorption recovery for ETP was 87 ± 3%. The limit of detection (LOD) and the limit of quantification (LOQ) based on three and ten times of the noise of HPLC profile were 0.05 and 0.32 ng ml^{-1}, respectively. The chromatograms in Fig. 7 clearly show the difference between the elution profiles obtained by RP-HPLC analysis of tissue extract before and after MISPE on the ETP-imprinted polymer.

TABLE 5
Percent recoveries of ETP at different concentrations in 2 ml of ACN

	Added value, μg		
	1	10	100
Loading effluent + 1 ml of washing ACN	1.5	4.5	25
Elution I (3 ml of methanol/acetic acid (90/10, v/v))	98	81	44
Elution II (3 ml of methanol/acetic acid (90/10, v/v))	<0.1	4	29

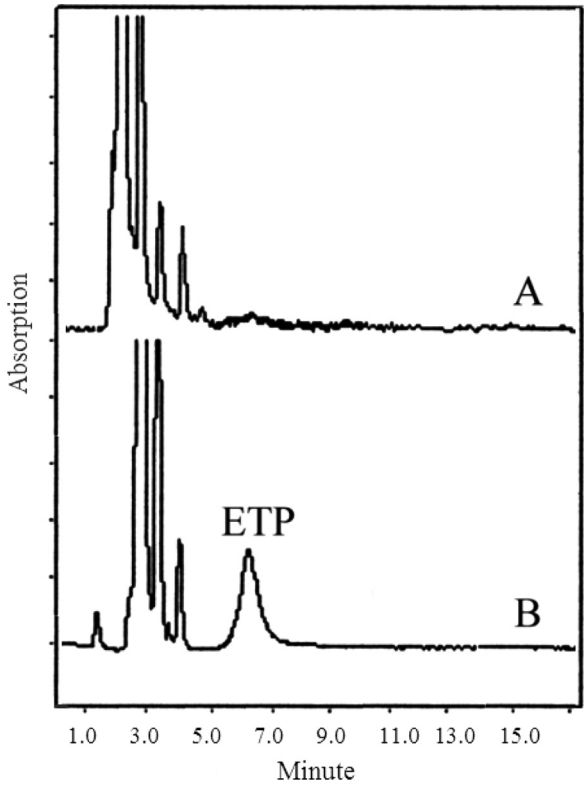

FIG. 7. Chromatograms obtained by injecting tissue extract without preceding MISPE (A), and after MISPE (B).

CONCLUSION

In this work a theoretical and experimental study has been carried out on the design, development, and evaluation of a molecularly imprinted polymer specific to antibiotic ethopabate (ETP). The molecular modeling results predicted that the binding energy between ETP and MAA is stronger than the 4-VP functional monomer. Also, rebinding studies using Scatchard analysis showed that the ETP-imprinted polymer with MAA has satisfactory characteristics in terms of number of binding sites and dissociation constant. The occurrence of non-covalent interaction between the template and MAA in the pre-polymerization mixture was studied using UV/Vis and ^{1}H NMR spectroscopy. Chromatographic studies revealed the recognition capability of the ETP-imprinted polymer and confirmed its suitability as a selective MISPE sorbent. The recommended MISPE procedure allowed the selective preconcentration of ETP residues from chicken tissue samples prior to chromatographic detection. The results suggested that this MIP exhibited acceptable adsorption ability toward ETP even in the presence of other matrix compounds in chicken tissue sample and the matrix of the tested samples caused no difficulty in the analysis.

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