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Synthesis and Evaluation of Molecularly Imprinted Polymeric Microspheres for Chloramphenicol by Aqueous Suspension Polymerization as a High Performance Liquid Chromatography Stationary Phase

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Molecularly imprinted microsphere for chloramphenicol (CAP) with high adsorption capacity and excellent selectivity is prepared by aqueous suspension polymerization, in which chloramphenicol is used as template molecule and ethyl acetate as porogen. The CAP-imprinted microspheres are used as high performance liquid chromatography (HPLC) stationary phase and packed into stainless steel column (150 mm × 4.6 mm i.d.) for selective separation of chloramphenicol. HPLC analysis suggests that chloramphenicol can be distinguished from not only its structural analogs but also other broad-spectrum antibiotic such as erythromycin and tetracycline. In addition, the binding experiments of CAP-imprinted microspheres are carried out in ethanol/water (1:4, V:V), the results indicate that the maximum apparent static binding capacity of molecularly imprinted microspheres is up to 66.64 mg g^{-1} according to scatchard model.

Key Words : Molecularly imprinted polymer, Microsphere, Chloramphenicol, Stationary phase

Introduction

Chloramphenicol is a broad-spectrum antibiotic exerting activity against a variety of bacteria through protein inhibition. However, it is proved that CAP has serious side-effects on humans in the form of bone marrow depression, and fatal aplastic anemia.[1] For these health concerns, CAP has been strictly banned for use in food-producing animals in many countries including China, United States and the European Union,[2,3] but the use of CAP is still inviting in some fields like animal husbandry and aquatic farming due to its activity, availability and the low cost. Therefore, it is important to develop a sensitive and reproducible method to control and monitor CAP residues in food of animal origin. Some methods have emerged such as enzyme-linked immunosorbent assay,[4,5] gas charomatography-mass spectrometry (GC-MS),[6,7] and liquid chromatography-tandem mass spectrometry (LC-MS/MS).[8,9] Unfortunately, these methods have some drawbacks, for instance, they are complex, high-cost or easily disturbed by structural analogs of CAP and other materials. The HPLC method in combination with a solid-phase extraction (SPE) process has also been developed for the determination of CAP in foods.[10] Nevertheless, most of these SPE materials have poor selectivity to CAP, and there may be still many interfering substances in these samples after being treated by SPE materials. It may cause the complexity of chromatographic peaks and inaccurate data in the sequential HPLC analysis.

In recent years, molecularly imprinted polymers (MIPs) have been demonstrated possess unique and predetermined selectivity towards target molecule because of their many cavities complementary against template molecules in shape, size and chemical functionality.[11,12] MIPs have a variety of applications including chromatography, solid-phase extraction (SPE), enzymatic catalysis, and sensor technology.[13-15] Up to now, there have been some reports about chloramphenicol molecularly imprinted polymers (CAP-MIPs) obtained by bulk polymerization.[16-18] These CAP-MIPs are solid blocks and required grinding and sieving for use, they are very time-consuming. Additionally, spherical CAP-MIPs using CAP as template molecule without grinding are also reported.[19,20] But these reported spherical and block CAP-MIPs are mainly employed as SPE mediums. Moreover, it is found that in most of these references chloroform is employed as porogen and solvent.[17,21,22] Actually, porogen and solvent are very important to the morphology of pores and the imprinted performance of MIPs. However, the solubility of the template CAP in chloroform is very low, which may lead to the less complementary cavities and adsorption capacity of MIPs for CAP. Furthermore, chloroform may be fatal if swallowed, inhaled or absorbed through skin because it causes irritation to skin, eyes and respiratory tract and may cause cancer, and also may affect central nervous system, cardiovascular system, liver and kidneys. Therefore, its use is not desirable.

In this paper, the preparation and evaluation of CAP-MIP microspheres used as HPLC station phase is first reported. The CAP-MIPs are packed into a stainless steel column by slurry method. The resulted CAP-MIPs column is used for the analysis of CAP and its structural analogs like thiamphenicol and florfenicol by HPLC. The results indicate that CAP can be distinguished not only from thiamphenicol and

florfenicol but also erythromycin and tetracycline, and the obtained CAP-MIPs have good rigidity and recognition properties and high adsorption capacity for CAP.

Experimental

Materials. Chloramphenicol and erythomycin are supplied by J&K Chemical Ltd. Thiamphenicol, florfenicol and tetracycline are obtained from Sigma-Aldirich Chemie (Steinheim, Germany), their structures were shown in Figure 1. Functional monomer methacrylic acid (MAA) is purchased from Xilong chemical factory (Guangdong, China). Cross-linking monomer ethylene dimethacrylate (EDMA) is obtained from Yunkai chemical Co., Ltd. (Shandong, China). Free radical initiator α,α'-azoisobutyronitrile (AIBN) is supplied by Fluka (Buchs, Switzerland). Acetonitrile and methanol are obtained from Dima technology Inc. (Ontario, Canada) and trifluoroacetic acid (TFA) is supplied by Sinopharm chemical reagent Co., Ltd. Ethyl acetate (EA), chloroform (CHL) and ethanol are supplied by Beijing chemical factory (Beijing, China). Polyvinyl alcohol (PVA) is purchased from Kuraray Co., Ltd. (Japan). The water is demineralized and purified by Seralpur PRO 90C, Seral (Ransbach-Baumbach, Germany).

Preparation of CAP Imprinted and Non-imprinted Polymeric Microspheres. CAP imprinted microspheres are prepared by using suspension polymerization method as follows: CAP (2 mmol, 0.646 g), MAA (4 mmol, 0.344 g), EDMA (40 mmol, 7.92 g), 12 mL of EA/CHL (12:0, 10:2, 6:6, 0:12, V:V) and AIBN (0.1 g) are mixed together as oil phase and then purged with a stream of nitrogen for 10 min.

Then added it into 1.5% *wt*. PVA/water (150 mL) in a glass vessel and stirred at 400 rpm. Polymerization is performed at 60 °C for 24 h under stirring and N_2 atmosphere, which resulted in the creation of spherical microparticles. Those microspheres are sieved with boults and microparticles with diameter between 20 μm and 50 μm are collected. The collected microspheres are washed by hot water to remove PVA. Following that spherical MIPs are washed by ethyl acetate and methanol until no CAP is found in the rinses. Non-imprinted polymers (NIPs) for control experiments are prepared following exactly the procedure described above but excluding the template CAP from the formulation.

Morphological Characterization of Microspheres. Scanning electron microscopy (SEM, JSM-6700, JEOL) is utilized to determine the shape and morphology of microspheres. For the SEM observation, the MIPs are dried at 60 °C and then attached to silver papers and sputter coated with a gold layer. The specific surface areas (SUA) are determined by Mercury Porosimeter.

Binding Experiments and Scatchard Model Analysis. Weighing 100 mg MIPs into a 10 mL screw-cap vial and an amount of 6 mL ethanol/water (1:4, V:V) of CAP (50 μg mL^{-1}) are added into the vial. Then the vial is sealed, stirred at 30 rpm under room temperature. The concentration of CAP in ethanol/water solution at different time intervals is monitored by a UV-vis spectrophotometer at 276 nm. NIPs are processed in the same manner. Scatchard model analysis is performed in the condition of various concentration of CAP, other conditions are the same as binding experiments except for special explanation.

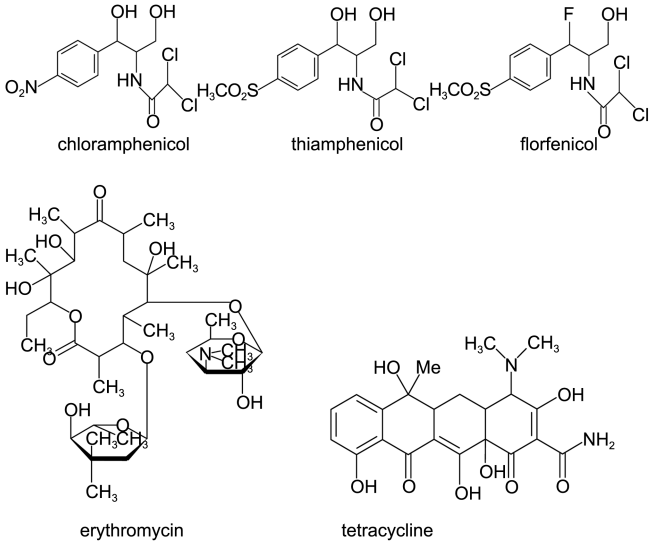

Figure 1. Chemical structures of chloramphenicol, thiamphenicol, florfenicol, erythromycin and tetracycline.

Chromatography Analysis. The microspheres (20-50 μm) are packed into a stainless steel column (4.6 mm id × 150 mm) by slurry method using a pneumatic pump at 30 MPa and using ethanol as slurry solvent. HPLC is performed using a Shimadzu-LC-20AT pump equipped with SIL-20A injector, a Shimadzu SPD-M20A US detector and a CTO-2AScolumn oven. Column oven temperature is controlled at 25 °C. All mobile phases in this paper consisting of acetonitrile and water, which have a ratio between 5:95 and 100:0 (V:V). The flow rate is constant at 1.0 mL min^{-1}. All samples are dissolved in methanol/water (5:95, V:V). Standard solutions of thiamphenicol, florfenicol, chloramphenicol, tetracycline and erythromycin are 200 μg mL^{-1}. Taking 1 mL standard solution of thiamphenicol, florfenicol and chloramphenicol respectively and mixing them together, the mixing solution is used for HPLC analysis. And the mixing solution of florfenicol, chloramphenicol and tetracycline is processed in the same way. Control sample is methanol/water (5:95, V:V) solution without CAP and other antibiotics. The wavelengths for detection of chloramphenicol, thiamphenicol, florfenicol, tetracycline and erythromycin are at 276, 226, 224, 355 and 210 nm, respectively.

Results and Discussion

Preparation and Characterization of CAP-MIP Microspheres. In this study, MIPs and NIPs are produced by aqueous suspension polymerization, in which water with PVA is used as continuous phase, and solution of chloroform/ethyl acetate containing MAA, CAP and AIBN is employed as oil phase. And the mixture of ethyl acetate and chloroform is

Table 1. Porous properties and adsorption capacity of the MIPs using different porogens

Porogen	EA	EA/CHL	EA/CHL	CHL
Dosage (mL)	12	10/2	6/6	12
Aperture (nm)	4.03	4.19	5.10	7.05
SUA (m^2/g)	323.44	312.54	270.50	206.15
Qmax2 (mg/g^{-1})	66.64	62.32	58.83	53.91

also porogenic solvent. Microspheres produced with diameter between 20 μm and 50 μm are collected, and analyzed by micromeritics ASAP 2020 and scanned by SEM. As shown in Table 1, it is found that in the same condition, aperture of micropores improved and specific surface area of MIPs decreased with the increase of chloroform. It can be ascribed to the fact that the chlorinated porogens cause severe swelling of the polymer product [23] and chloroform is easier to volatilize than ethyl acetate, thus it is inclined to form bigger micropores than ethyl acetate when used as porogen, which can also be further demonstrated from the SEMs of micropores for microspheres obtained in different ratio of chloroform to ethyl acetate (Figure 2).

In addition, the morphology of MIPs and NIPs obtained using ethyl acetate as porogen are shown in Figure 3. The results indicate that microspheres have good sphericity and surface of microspheres is smooth, which means that the obtained molecularly imprinted microspheres for CAP will be better than bulk imprinted polymers when used as chromatography media, because bulk imprinted polymers is irregular and usually lead to asymmetric and tailing peak and bad separation performance.[24]

Binding of CAP to MIPs and NIPs in Ethanol/Water (1:4, V:V). To study the recognition ability of CAP-MIPs, binding experiments are performed in ethanol/water (1:4, V:V). Figure 4 shows the binding amounts of CAP to the MIPs and the NIPs. It can be seen that the amounts of CAP binding to the MIPs is much higher than that of the NIPs. To

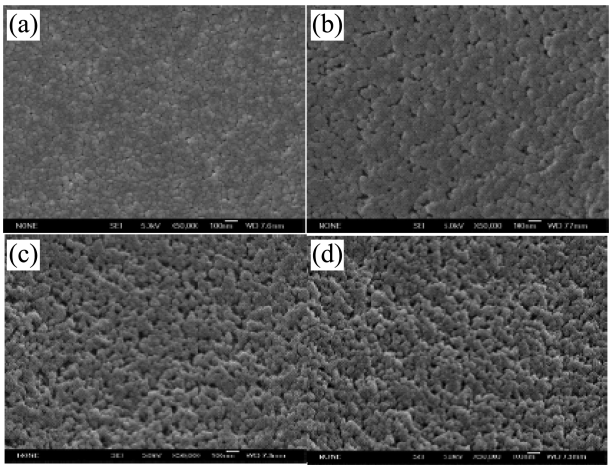

Figure 2. Scanning electron micrographs of MIPs obtained in different ratio of chloroform to ethyl acetate. (a) 12 mL ethyl acetate; (b) 10 mL ethyl acetate + 2 mL chloroform; (c) 6 mL ethyl acetate + 6 mL chloroform; (d) 12 mL chloroform.

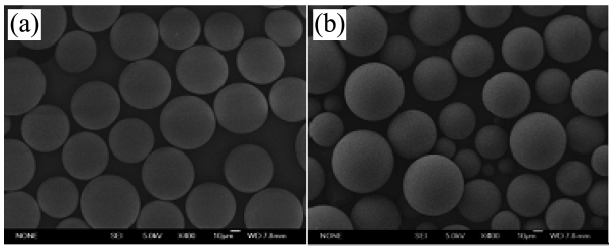

Figure 3. Scanning electron micrographs of MIPs and NIPs by aqueous suspension polymerization. (a) SEM of MIPs; (b) SEM of NIPs.

the MIPs, the value of binding amounts seem to be linearly increased with the time in the first 25 minutes, and then the adsorption velocities of CAP are slowly increased, eventually the saturated binding are observed in 100-240 min. However, there is no great difference among the amounts of CAP binding to the NIPs with the time.

The amounts of CAP binding to the MIPs and the NIPs in various CAP concentrations (0.1-0.45 mmol L^{-1}) are also studied at room temperature. According to the relation curve between the binding amounts of CAP to the MIPs and adsorption time (Figure 4), the adsorption time (2 h) is chosen as in the following experiments. Figure 5 indicates that the amounts of CAP binding to the MIPs and the NIPs improve with the increase of the concentration of CAP. Moreover, the former is always much larger than the latter, which suggests that the MIPs obtained by our method have a good imprinted performance. The reason is that there are apparent differences in tridimensional structure between the MIPs and the NIPs. In the MIPs, there are amounts of sites and cavities which are complementary to the template in size and shape, and they are contributive to the high effective selectivity of the template. To NIPs, however, there are no sites and cavities complementary to the template and so its adsorption for CAP is weak.

Scatchard Model Analysis. Scatchard[25] model is often used to evaluate the recognition characteristic of MIPs and Scatchard equation is follows: $Q/C_{CAP} = (Q_{max}-Q)/K_d$. In this equation, Q (mg g^{-1}) is binding capacity, C_{CAP} (0.0323 mg

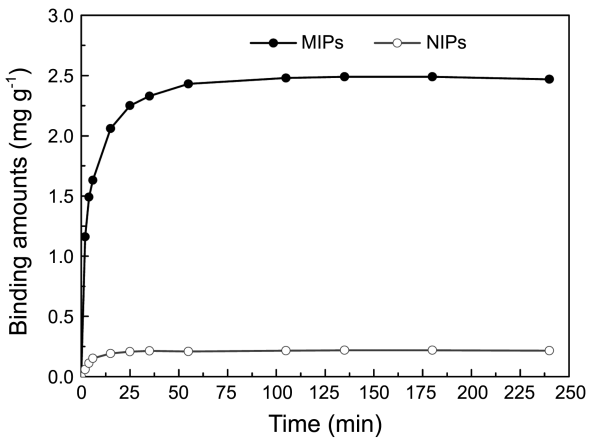

Figure 4. Time course of binding to the MIPs and NIPs in ethanol/water (1:4, V:V).

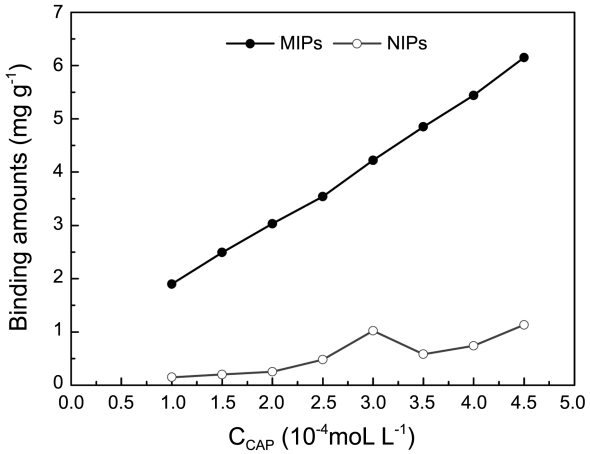

Figure 5. The logarithmic bonding isotherm of MIPs and NIPs for chloramphenicol.

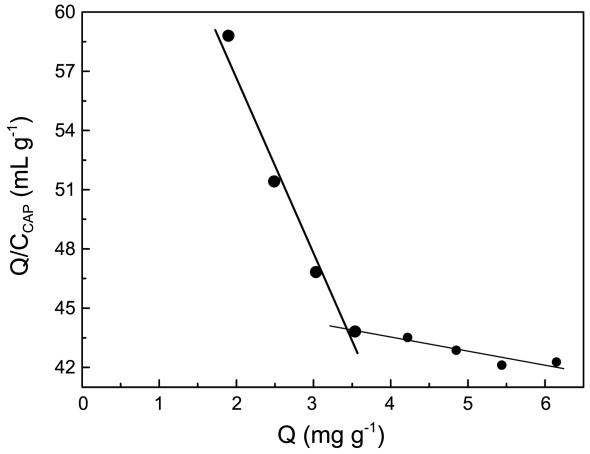

Figure 6. Scatchard plot of MIPs in ethanol/water (1:4, V:V).

mL^{-1}) is the concentration of CAP, Q_{max} is maximum apparent binding capacity, and K_d (mg mL^{-1}) is disassociation constant at binding site. The relations between Q/C_{CAP} and Q are shown in Figure 6. It can be seen that Scatchard plot was not linear indicating that binding sites in MIPs are heterogeneous in respect to the affinity for CAP. In Scatchard curve, there are two distinct straight lines. It suggests that two kinds of binding sites including high affinity and low affinity are formed in MIPs. From the slop of Scatchard curve, we can obtain disassociation constants K_{d1}=0.11 mg mL^{-1} and K_{d2}=1.44 mg mL^{-1}. According to the intercepts of two lines, it can be obtained that Q_{max2} of binding site with high affinity is 66.64 mg g^{-1} and Q_{max1} of binding site with low affinity is 8.27 mg g^{-1}.

According to the same method described above, maximum binding capacities (Q_{max2}) of MIPs prepared in the porogen solutions consisted of different ratio of chloroform/ethyl acetate are also obtained. The results are shown in the Table 1, which exhibit that the adsorption capacity of MIPs for CAP is the highest when ethyl acetate is used as porogen. Maybe there are two aspects of reasons: one is that the solubility of CAP in ethyl acetate is the biggest, which can

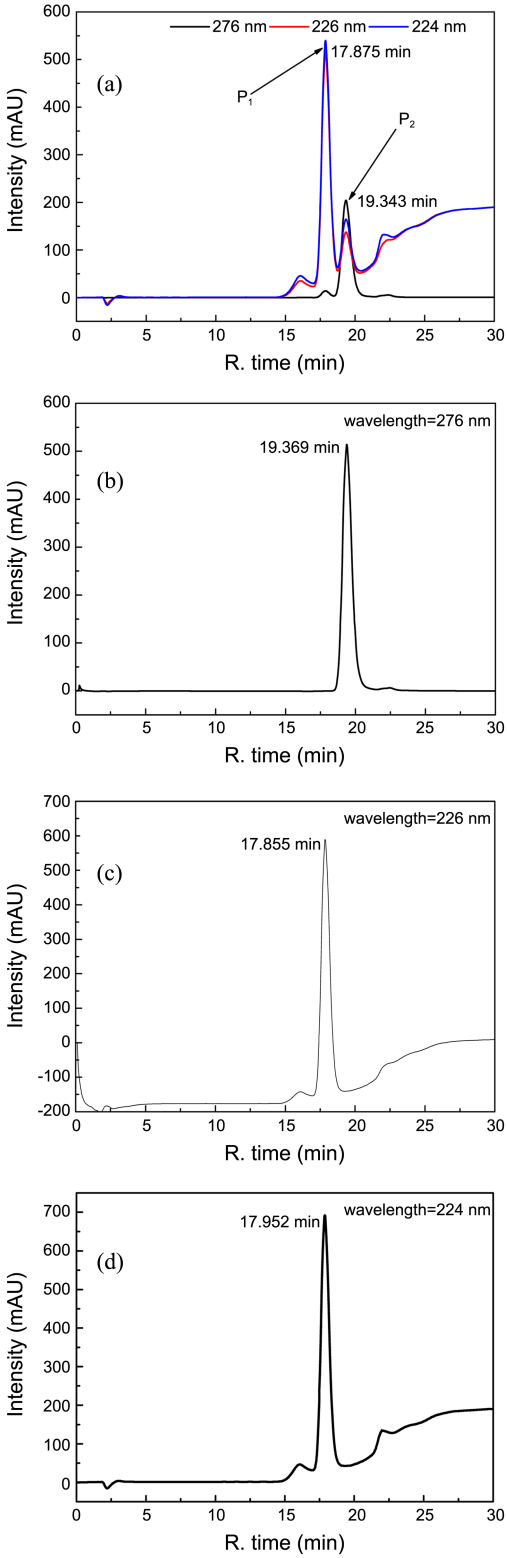

Figure 7. Chromatograms for separation of chloramphenicol, thiamphenicol and florfenicol on the MIPs column (a was the mixture of chloramphenicol, thiamphenicol and florfenicol, b was chloramphenicol standard solution, c was thiamphenicol standard solution, and d was florfenicol standard solution), HPLC conditions: mobile phase = mixture of water + 0.1% TFA (solvent A) and methanol + 0.1% TFA (solvent B); gradient = 5-100% B in 30 min; flow rate = 1 mL min^{-1}; injection volume = 50 μL; column dimension = 150 mm × 4.6 mm i.d.

create the most effective imprinted cavities and sites. The other reason is that CAP is a small molecule (< 1 nm) and aperture of micropores is bigger than 4 nm, it may be thought that the more specific surface area, the more adsorption capacity. Additionally, the toxicity of ethyl acetate is much smaller and the solubility of CAP in it is bigger than chloroform. Therefore, ethyl acetate is used as porogen in the following experiments.

Separation of Chloramphenicol and its Structural Analogs by CAP-MIPs Column. Chloramphenicol, thiamphenicol and florfenicol are broad-spectrum antibiotic with similar structures (Figure 1) leading to difficult separation. In this paper, their mixture is loaded on CAP-MIPs column and eluted by acetnitrile/water solution (containing 0.1% TFA). The results are shown in Figure 7, which suggest that whatever the detection wavelength (276 nm, 226 nm, 224 nm) is used, several peaks can be seen. Comparing with the retention times of standard solutions for CAP, thiamphenicol and florfenicol in the same HPLC conditions, it can be figured out that P_1 in Figure 7(a) is corresponding to thiamphenicol and florfenicol, and P_2 corresponding to CAP (19.343 min). When the detection wavelength is 226 nm or 224 nm, the baseline is not always horizontal or steady due to the interference caused by the mobile phase. However, it is clearly that CAP-MIPs column can distinguish CAP from its structure analogs thiamphenicol and florfenicol in such HPLC conditions.

In addition, the mixture of chloramphenicol, thiamphenicol and florfenicol is analyzed on NIPs column. The results are shown in Figure 8, which indicate that chloramphenicol, thiamphenicol and florfenicol are not separated at all. It also further demonstrate that CAP-MIPs obtained have good imprinted performance.

Separation of CAP, Erythromycin and Tetracycline by HPLC Using CAP-MIPs Column. In order to further investigate the separation performance of CAP-MIPs column, the CAP-MIPs column prepared in our lab is also used to separate chloramphenicol and other broad-spectrum antibiotic such as erythromycin and tetracycline, the chemical structure of erythromycin and tetracycline are shown in Figure 9. When their mixture is analyzed on HPLC, only one or two peaks appeared in the chromatogram if the detection wavelengths are 355 nm or 276 nm (Figure 9(a)). On the

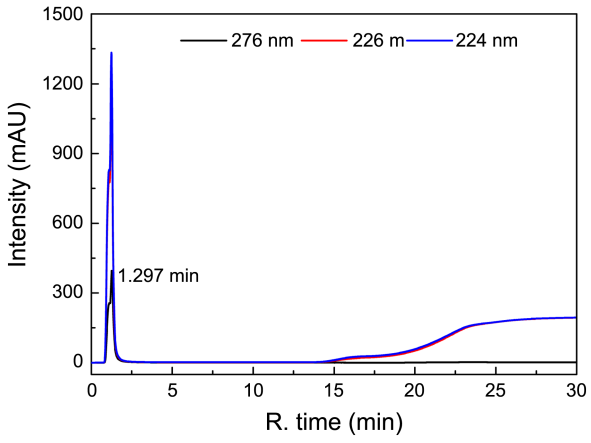

Figure 8. Chromatograms for separation of effect of chloramphenicol, florfenicol and thiamphenicol on the NIPs column. HPLC conditions: mobile phase = mixture of water + 0.1% TFA (solvent A) and methanol + 0.1% TFA (solvent B); gradient = 5-100% B in 30 min; flow rate = 1 mL min^{-1}; injection volume = 50 μL; column dimension = 150 mm × 4.6 mm i.d.

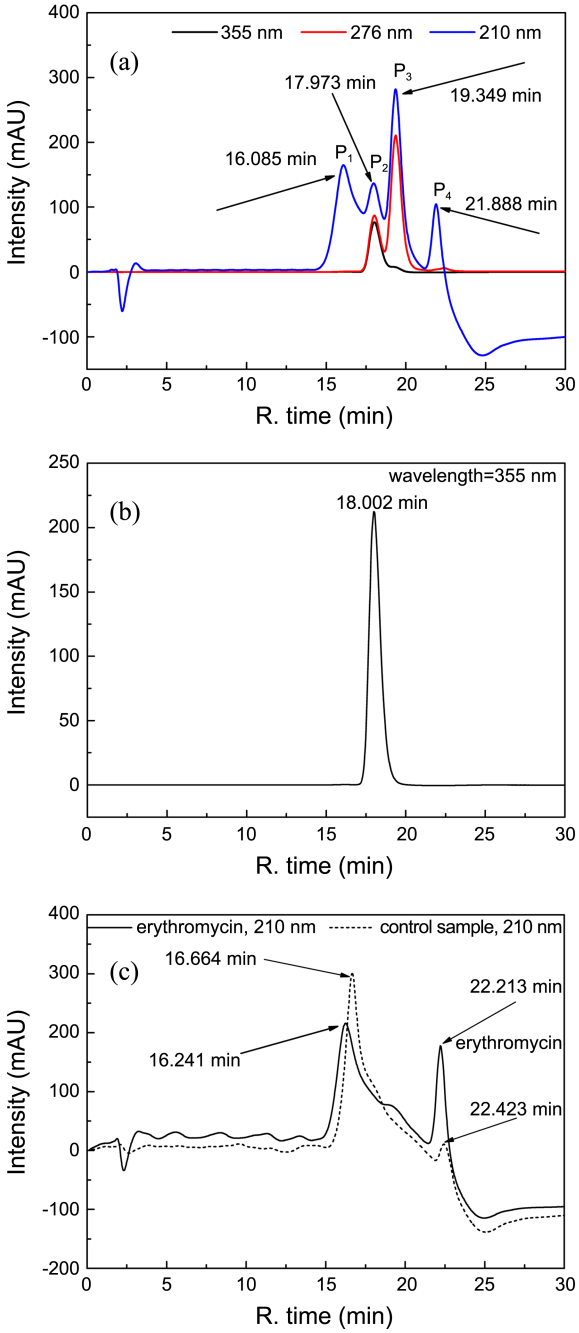

Figure 9. Chromatograms for detection of the mixture consisting of chloramphenicol, tetracycline, and erythromycin, on the MIPs column, b and c were tetracycline, and erythromycin standard solution respectively. HPLC conditions: mobile phase = mixture of water + 0.1% TFA (solvent A) and acetonitrile + 0.1% TFA (solvent B); gradient = 5-100% B in 30 min; flow rate = 1 mL min^{-1}; injection volume = 50 μL; column dimension = 150 mm × 4.6 mm i.d.

other hand, more than three peaks appeared when the detection wavelength is 210 nm. It is found that the retention time of standard solutions for chloramphenicol, tetracycline and erythromycin are 19.369 min, 18.002 min and 22.213 min, respectively (Figure 7(b), Figure 9(b) and Figure 9(c)). Comparing with retention times of P_2, P_3, P_4 in Figure 9(a), it can be concluded that P_2, P_3, P_4 are corresponding to tetracycline, chloramphenicol and erythromycin, respectively. In addition, we can know that P_1 is an interference peak caused by mobile phase by comparing Figure 9(c) with Figure 9(a). Therefore, the above results indicate that these three substances are effectively separated by the CAP-MIPs column. Furthermore, it is also demonstrated that resulted CAP-MIPs for CAP have good selectivity and imprinted performance.

Conclusions

The molecularly imprinted polymer microspheres by aqueous suspension polymerization using CAP as template molecule and ethyl acetate as porogen are obtained. The resulted MIPs have good selectivity and high adsorption capacity against CAP. The CAP-MIPs with spherical shape are utilized as chromatographic separation medium and packed into 4.6 mm id × 150 mm stainless steel column. On the CAP-MIPs column, CAP can be separated from not only its structural analogs but also erythromycin and tetracycline in such a condition that mobile phase is mixture of water + 0.1% TFA (solvent A) and acetonitrile + 0.1% TFA (solvent B), the gradient is 5-100% B in 30 min and flow rate is 1 mL min^{-1}. It is promising that the prepared CAP-MIPs can be used as HPLC stationary phase to detect and separate CAP from structural analogs.

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