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Determination of trace macrolide antibiotics in milk with online solid-phase extraction with an ionic-liquid-based monolithic column

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ABSTRACT: An ionic liquid (IL)-based monolithic poly(ionic liquid glycidylmethacrylate-*co*-ethylene glycol dimethacrylate) column was prepared via *in situ* free-radical polymerization with 1-vinyl-3-butylimidazolium chlorine as one of the comonomers. The obtained monolithic column was used as the sorbent of solid-phase extraction (SPE) and coupled with high-performance liquid chromatography for the simultaneous determination of the macrolide antibiotics roxithromycin (ROX) and acetylspiramycin (ACE) in processed pure milk. The monolithic column was characterized by Fourier transform infrared spectrometry, scanning electron microscopy, nitrogen absorption–desorption, mercury intrusion porosimetry, and thermogravimetric analysis. The results reveal that the monolithic column exhibited a high selectivity and good permeability to the macrolide antibiotics in milk. The optimized method offered excellent linearity with a linear regression coefficient greater than 0.998. The precisions for interday and intraday were both less than 7.7%. The accuracies expressed by the recoveries for ROX and ACE were in the ranges 92.5–103.8 and 93.0–107.6%, respectively. Compared to the previous methods, this method had a low limit of detection and a good accuracy. As a result, the polymer IL based monolithic column could feasibly be used as a high-selectivity online SPE sorbent for determining trace macrolide antibiotics in milk. © 2016 Wiley Periodicals, Inc. J. Appl. Polym. Sci. **2016**, *133*, 43943.

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INTRODUCTION

Roxithromycin (ROX) and acetylspiramycin (ACE) both macrolide antibiotics. They are lipophilic molecules with a 12- to 16-member central ring, and several amino and/or neutral sugars are bound.[1] Among which, ACE contains four similar components (mono-ACE II/III and di-ACE II/III).[2] They are widely used for the treatment of infections in humans and animals. In addition, macrolide antibiotics are usually used as an addition reagent and added directly to food, mainly milk, to prolong its freshness.[3] Milk is the normal drink in human life. In China, most people drink processed pure milk. Recently, some research investigations have pointed out that various types of pharmaceuticals (including antibiotics) could exert adverse effects on the human body, even at low concentrations.[4] However, various types of antibiotics are being constantly detected in milk at relatively low concentrations;[5] they can have toxic effects on consumers. It is an essential task for developing a multiresidue analytical method to detect and quantify antibiotics in milk.[5,6]

Milk is a complex matrix sample containing lots of proteins and fat, which may interfere with the analysis. Therefore, as a crucial step for the whole chromatographic analysis of biological matrices, the sample pretreatment for protein, including fat removal and analyte enrichment, is required.[3,7–9] There are many methods for preparing pharmaceuticals at different concentrations; these include protein precipitation, supercritical fluid extraction, liquid–liquid extraction, solid-phase microextraction, molecularly imprinted extraction, and solid-phase extraction (SPE). Among them, SPE is one of the most practical technologies for sample preparation. Offline SPE has been used to remove the interference from biomatrix substances, but it is time consuming and prone to error.[10] Although the online SPE method is more simple, rapid, and accurate than offline SPE,[11] traditional reversed-phased sorbents (C18, C8, and C2) always have poor specificity.[12] Therefore, the development of a proper sorbent for the simultaneous enrichment and analysis of macrolide antibiotics in milk is necessary.

Additional Supporting Information may be found in the online version of this article

Over the past 20 years, monolithic materials for separation science and their wide bioanalytical applications have been reviewed extensively.[13] These monoliths have been prepared from homogeneous polymerization mixtures containing monomers (functional and crosslinking monomers), radical initiator, and porogenic solvents.[14] The advantages of monolithic columns are a low column backpressure, high permeability, and simple preparation. Compared to conventional particle-packed columns, porous monoliths have a high porosity.[15] The large pores of organic monoliths allow large biomacromolecules to go through easily; then, the biomolecules can be removed in the sample pretreatment process.[16] However, there are some disadvantages that limit the wider application of polymer monoliths in separation; these include their nonuniform structure and shrinking or swelling in the organic solvent. To improve the characteristics of polymer monoliths, ionic liquid (IL) has been used in the polymerization.

An IL is a solution of organic salts; it consists of organic cations paired with organic or inorganic anions. The melting points of these compounds is often less than or equal to 100 °C.[17,18] Because of their properties of nonvolatility, nonflammability, high ion density, and high ionic conductivity, new materials have been successfully applied as stationary phases in gas chromatography,[19] liquid chromatography,[20] capillary electrochromatography,[21,22] and SPE.[23–25] Recently, ILs have been used in the preparation of polymeric materials, and IL-based monolith columns have shown good results when they have been applied in SPE because of their high selectivity and wide operating pH range.[23] The imidazolyl group is normally used as the organic cation in IL compositions because the carbocation is easily obtained from the imidazolyl ring. ILs have good compatibility with most solvents, and this enhances the miscibility of the prepolymerization solution and then improves the structural uniformity of the monolith. Furthermore, the imidazolyl cation of the IL extends the reactions between the monolith and the target analytes, such as π–π interactions and ionic exchange actions. This enhances the adsorption capacity of the IL-based sorbent.

In this study, an IL was used to improve the uniform structure of the monolith, and the IL-based monolithic column was prepared by *in situ* free-radical polymerization. The obtained monolith material was used as an SPE sorbent to enrich macrolide antibiotics from milk, and this was followed by analysis with high-performance liquid chromatography (HPLC).

EXPERIMENTAL

Chemicals and Reagents

Glycidylmethacrylate, 1-chlorobutane, and 1-vinylimidazole were purchased from Shanghai Aladdin Co. (Shanghai, China). Ethylene dimethacrylate and 2,2′-azobisisobutyronitrile were obtained from Fushun Anxin Chemical Co, Ltd. (Liaoning, China). 1-Dodecanol and cyclohexanol were supplied by Tianjin Guangfu Fine Chemical Research Institute (Tianjin, China). Acetocaustin and lead acetate were supplied by Tianjin University Chemical Plant (Tianjin, China). HPLC-grade acetonitrile (ACN), Na_2HPO_4, NaH_2PO_4, $NH_4H_2PO_4$, and triethylamine were supplied by Kermel Chemical Reagent Co, Ltd. (Tianjin, China). ROX and ACE were obtained from Maya Reagent (Zhejiang, China). Ultrapure water was used for all of the experiments. All liquids and solutions were filtered through a 0.45-μm membrane before the HPLC procedure. The processed pure milk sample was the product of Inner Mongolia Yili Industrial Group Co., Ltd. (Inner Mongolia, China). The marked components were as follow: fat = 6%, protein = 5%, carbohydrate = 2%, sodium = 3%, and calcium = 13%. (The processed pure milk was obtained by the heating of the raw milk at a certain temperature.)

Apparatus and Analytical Conditions

The pore type was determined by the nitrogen adsorption–desorption measurement on a TriStar II 3020 instrument (Micromeritics). Macropore size distribution was determined on an AutoPore IV 9500 instrument (Micromeritics). Morphological images of the monoliths were obtained with a scanning electron microscopy (SEM) instrument (Hitachi S-3400, Hitachi High Technologies, Japan). Molecular weight data for the IL was obtained by an MSD Trap XCT instrument with an electrospray ionization source (Agilent Technologies). The Fourier transform infrared (FTIR) spectra were assayed on an FTIR-8400S IR apparatus (Shimadzu, Japan). Thermogravimetric analysis was assayed by Setsys 16/18 instrument (Setaram, Caluire, France). The stainless steel columns (*i.d.* = 50 × 4.6 mm) were purchased from Beijing Xinyu Instrument, Ltd. Co. (Beijing, China). The HPLC setup was composed of the following parts: a double P3000A pump, a 7725i manual-injection valve equipped with a 20-μL sample injection loop, a variable-wavelength UV-3000 detector, and LC-3000 acquisition and processing software; this was all obtained from CXTH Co. (Beijing, China). The polymer monolithic column was used as a precolumn, and a C18 Cosmosil packed column (*i.d.* = 150 × 4.6 mm) was used as the analytical column. Water was used as the mobile phase for enrichment, and an ACN/Na_2HPO_4 aqueous solution (0.02 mol/L, 60/40 v/v) as the mobile phase for separation and analysis. The detection wavelength was 215 nm. The flow rate was 1.0 mL/min. The system was operated at ambient temperature.

Sample Preparation

Stock solutions of 1.0 mg/mL of ROX and ACE were prepared, respectively, and further diluted with an ACN/Na_2HPO_4 aqueous solution (0.02 mol/L, 60/40 v/v) to prepare working solutions with ROX and ACE concentrations of 5.0, 2.0, 1.0, 0.5, 0.25, 0.1, and 0.05 μg/mL, respectively. The standard solutions were prepared at concentrations of 500.0, 200.0, 100.0, 50.0, 20.0, 10.0, and 5.0 ng/mL, respectively, by the addition of desired amount of working solutions to milk. Quality control samples (5, 20, and 500 ng/mL) were also prepared in the same way. All of the solutions were stored in the refrigerator at 4 °C before use.

Pretreatment of Milk

Before analysis, 5.0 mL of bovine milk was dissolved with 25.0 mL of 0.1% trichloroacetic acid and 1.0 mL of 2% lead acetate. Afterward, the tube with the mixed solution was homogenized by ultrasound for 20 min and finally centrifuged twice at 8000 rpm for 10 min at 4 °C to separate the fat layer.

Preparation of the Monolithic Columns

Synthesis of 1-Vinyl-3-Butylimidazolium Chloride. 1-Vinyl-3-butylimidazolium chloride was synthesized by the same method

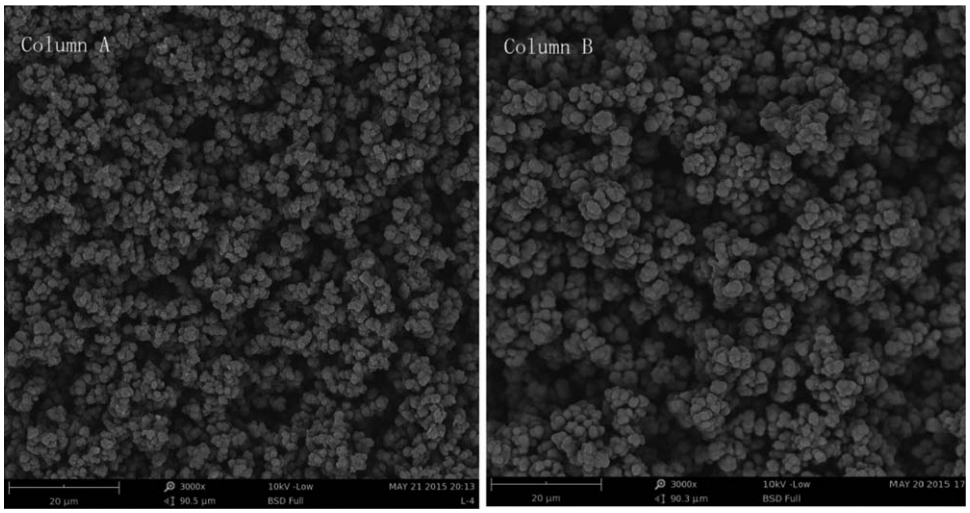

Figure 1. SEM images of columns A and B.

used in our previous study.[26] The synthesis and mass spectrometry spectrum of the 1-vinyl-3-butylimidazolium chloride IL are illustrated in Figures S1 and S2 in the Supporting Information, respectively.

Preparation of the IL-Based Monolithic Column. The monolithic column (column A) was prepared via *in situ* polymerization with a solution composed of 2,2′-azobisisobutyronitrile (1% w/w of monomer and crosslinker), IL (0.1 mL), glycidylmethacrylate (0.25 mL), ethylene dimethacrylate (0.4 mL), and a binary porogenic solvent of 1-dodecanol (0.4 mL) and cyclohexanol (0.8 mL). After being homogenized by ultrasound for 20 min and treated with nitrogen for 10 min, the homogeneous solution was transferred into a stainless steel column (*i.d.* = 50 × 4.6 mm). The column was submerged in a water bath at 60 °C for 10 h after the ends were sealed. After that, the monolithic column was connected to a HPLC pump and washed online with methanol to remove porogens and other soluble compounds. Compared to column A, column B was prepared following the conditions of column A except for the addition of IL. The schematic preparation of the column A is illustrated in Figure S3 in the Supporting Information.

Online SPE–HPLC Procedure

The synthesized monolithic column was used as an SPE column for sample preconcentration; it was placed in the sample-loop position of the injection valve. At first, 20 μL of milk standard solutions were directly injected into the SPE column when the six-port injector valve was on the load position. Then, a washing mobile phase (5.0 mL of water, 1.0 mL/min) was used to remove the fat and protein selectively, but the analytes, ROX and ACE, were trapped on the SPE column. Then, the six-port valve was switched to the inject position to couple the SPE column to the analytical column. ROX and ACE were transferred from the SPE column to the analytical column according the afflux of the mobile phase.

RESULTS AND DISCUSSION

Structures and Morphological Characteristics of the Monolithic Column

FTIR Spectrum of the Monolith. Columns A and B were characterized by FTIR spectroscopy with the results shown in Figure S4 in the Supporting Information. In the spectrum of column A, the absorbances at 3015, 1470, and 912 cm^{-1} were due to $\nu_{C=H}$, $\nu_{C=N,C=C}$, and β_{C-H} obtained from the imidazolyl group. However, these peaks did not appear on the spectrum of column B; this meant that the imidazolyl group was successfully bonded to column A.

SEM Images of the Monolith. Figure 1 shows the morphologies of the polymer-based monolithic materials characterized by SEM. Figure 1, obtained from column A, demonstrated that the monolith possessed a uniform porous structure, which offered a large number of channels formed by the accumulation of spherical particles. Compared with column A, column B showed bigger and fewer pores. The results indicate that the IL-based monolith had more and smaller pores than the non-IL one. This was because the IL had good compatibility with the prepolymerization solution; this created more accumulated pores and smaller through pores than the non-IL-based one.

Thermal Stability of the Materials. To confirm that the pretreatment of the pore size distribution assay would not destroy the pore structure of the monolith, before the assay, column A was tested by thermogravimetric analysis with the results shown in Figure S5 in the Supporting Information. Thermogravimetric analysis showed that aside from the loss of residual solvent, column A did not undergo any significant thermal degradation until the temperature reached 294.10 °C; this indicated that the polymer monolithic column exhibited good thermal stability and confirmed that the following pore type and the pore size distribution assays were credible.

Pore Type of the Monolith. The pore types of columns A and B were first examined by nitrogen adsorption–desorption measurements. The pretreatment for the measurement was as follows: the

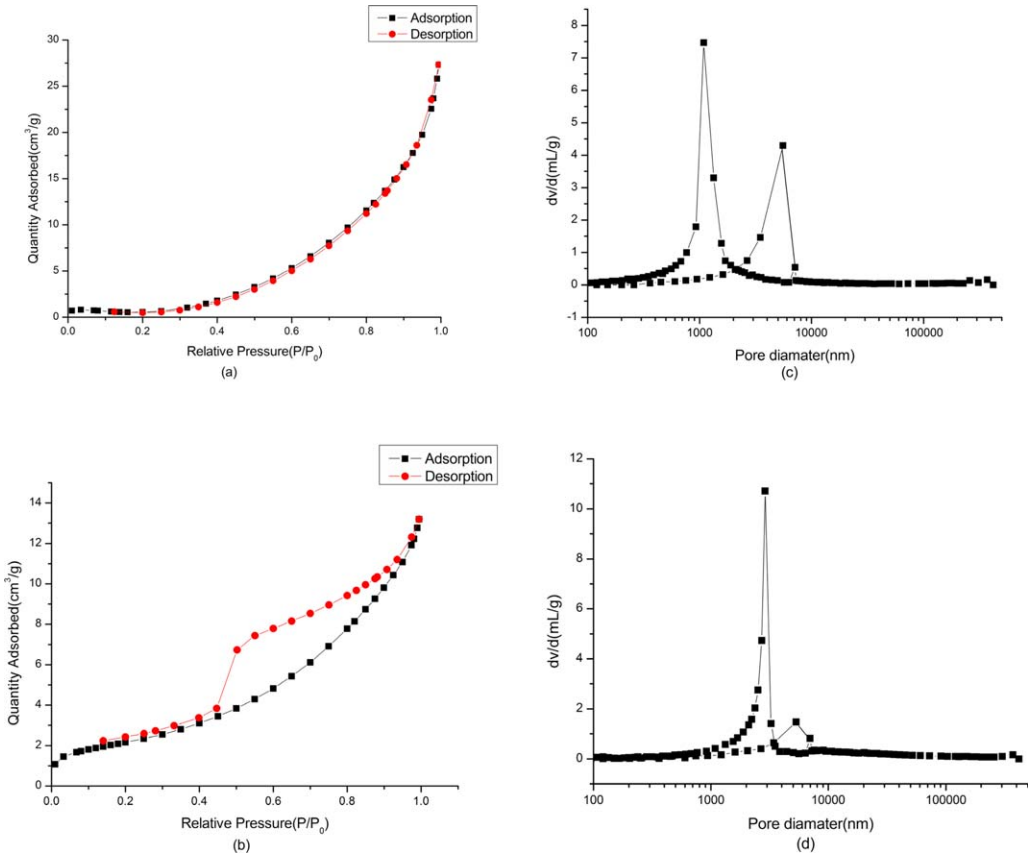

Figure 2. Pore size distributions of the monoliths: (a) nitrogen adsorption–desorption isotherms of column A, (b) nitrogen adsorption–desorption isotherms of column B, (c) macropore size distribution curve of column A by mercury porosimetry, and (d) macropore size distribution curve of column B by mercury porosimetry. P, adsorption partial pressure; P_0, adsorbent saturated vapor pressure; $d_{v/d}$, volume of mercury injection. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

polymer monolith was pushed out from the stainless steel tube and then set in a vacuum oven at 60 °C for 24 h. The dried monolith was weighed after it was blown with nitrogen at 120 °C for 4 h. At last, the pretreated monolith was assayed by the nitrogen absorption–desorption instrument. Figure 2(a,b) shows the nitrogen absorption–desorption isotherms of columns A and B, and the total pore areas of the monoliths were 1.89 and 7.93 m^2/g, respectively. However, the two nitrogen absorption–desorption isotherms were both adjusted to typical isotherms of type III; this showed that there were weak interactions between N_2 and the polymer monolith. So, there were a few micropores and mesopores in the structure. The two isotherms also indicated that macropores were the main roles of the porous structures.

Pore Size Distributions of the Monoliths. Therefore, mercury intrusion porosimetry was used to investigate the macropore distribution of columns A and B; the results are shown in Figure 2(c,d). Figure 2(c) shows that column A, the IL-based monolithic column, typically contained two types of macropores, accumulated pores and through pores, and the modal pore diameters were 1.09 and 5.53 μm, respectively. Furthermore, the total intrusion volume, porosity, and pore area were 1.70 mL/g, 65.94%, and 16.82 m^2/g, respectively. Figure 2(d) was obtained from column B, which also contained two types of macropores, as the modal pore diameters were 2.89 and 5.31 μm. The total intrusion volume, porosity, and pore area of column B were 1.65 mL/g, 41.63%, and 3.05 m^2/g, respectively. Furthermore, compared to column A, with a volume of the through pore of 4.50 mL/g, column B had a through-pore volume of 1.75 mL/g; this gave it a high mass transfer resistance in the online procedure. All of the results mentioned previously show that the pore structure of column A was optimal.

Investigation of the Pretreatment Ability of the Monolith

Blank milk was directly injected into an empty column and an IL-based monolithic column, respectively, and water was used to elute the samples with the chromatogram, as shown in Figure S6(a,b) in the Supporting Information. The peaks of the two chromatograms had similar peak areas; this indicated that the biological matrix compounds could be largely removed from the milk. Meanwhile, the enrichment ability of the IL-based monolithic column was also investigated by the injection of 3.0 μL of ROX and ACE solutions (0.1 mg/mL), respectively. Figure S7(a–c) in the Supporting Information shows that when water was used as the mobile phase, antibiotic drugs were enriched on the monolithic column [Figure S7(a)]. When the ACN/Na_2HPO_4 aqueous solution (0.02 mol/L, 60/40 v/v) was used as the mobile phase, ROX and ACE were eluted from the monolithic column [Figure S7(b,c)], respectively. So, the IL-based monolithic column could be used as an SPE column to remove matrix compounds and extract ROX and ACE from milk.

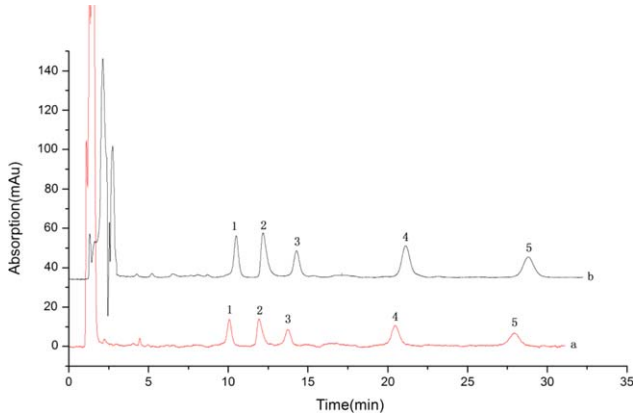

Figure 3. Chromatograms of matrix effect evaluation: (a) chromatogram obtained from the column with C18 only and (b) chromatogram obtained from the SPE column-*co*-C18 column. The conditions were as follows: monolithic column A, *i.d.* = 50 × 4.6 mm; analysis column, C18 Cosmosil packed column (*i.d.* = 150 × 4.6 mm), flow rate = 1.0 mL/min, concentration = 200 ng/mL, room temperature, detector wavelength = 215 nm, elution solution = 60/40 v/v ACN/Na_2HPO_4 aqueous solution (0.02 mol/L), and amount of injection = 20 μL. The analytes were (1) mono-ACE II, (2) ROX, (3) mono-ACE III, (4) di-ACE II, and (5) di-ACE III. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Optimization of the Online SPE–HPLC Conditions

A feasible mobile phase played an important role in removing the interference from the milk and retaining analytes on the SPE column.

ROX and ACE were stable in neutral solutions, and because of the tolerance of the analytical column (C18 column), the pH of the mobile phase for separation and analysis was adjusted to 7.0 for further study.

Various solutions, including ACN/triethylamine aqueous solution (0.1%, 60/40 v/v), ACN/$NH_4H_2PO_4$ aqueous solution (0.02 mol/L, 60/40 v/v), ACN/NaH_2PO_4 aqueous solution (0.02 mol/L, 60/40 v/v), and ACN/Na_2HPO_4 aqueous solution (0.02 mol/L, 60/40 v/v), were investigated, respectively, with the results shown in Figure S8(a) in the Supporting Information. Among them, the ACN/Na_2HPO_4 aqueous solution (0.02 mol/L, 60/40 v/v) provided better recovery for ROX and ACE than the other solutions, and it was used as the mobile phase for separation and analysis.

To confirm the optimal concentration of the buffer in the ACN/Na_2HPO_4 mobile phase, different concentrations, including 0.005, 0.01, 0.02, and 0.05 mol/L, were evaluated. The results, shown in Figure S8(b) in the Supporting Information, indicate that the recovery of ROX and ACE increased with increasing concentration from 0.005 to 0.02 mol/L and then remained almost constant at concentrations of 0.02–0.05 mol/L. To ensure good recovery and because of the protection of the HPLC system, a concentration of 0.02 mol/L was adopted.

Matrix Effects

To investigate the effect of the matrix on the analysis, the standard sample with a concentration of 200 ng/mL was injected to the HPLC system and analyzed by the column with C18 only and SPE column-*co*-C18 column, respectively, with the results shown in Figure 3(a,b). Figure 3(a) shows that the chromatogram obtained from the column with C18 only presented a large peak of the milk matrix at the dead time; this acutely reduced the duration of life of the C18 column. However, the chromatogram shown in Figure 3(b) obtained from the SPE column-*co*-C18 column had no large peak. Furthermore, the five peaks in Figure 3(b) had larger peak areas than those in Figure 3(a). These results indicate that most fat and protein in the milk were cleaned up in the SPE step, and the analytes were more efficiently separated from the interfering matrix than in the column with C18 only.

Method Validation

Selectivity. The selectivity of the method was assessed by a comparison of the chromatograms obtained from the blank milk (without ROX and ACE) and the standard milk sample (with ROX and ACE) under the same chromatographic conditions. As shown in Figure 4, at the retention times of ROX and ACE, we detected no peak of interfering endogenous compounds; this indicated that in this study, this was a high-selectivity method for ROX and ACE in milk.

Linearity. The linearity of this method was assessed by the correlation coefficient obtained from the calibration equation according to the calibration curve, which was produced by the injection of a series of standard solutions. The correlation coefficients, which were all higher than 0.998, confirmed the linear relationship in the drug concentration range of 5–500 ng/mL. Furthermore, the limit of detection (LOD) and limit of quantification (LOQ) for ROX and ACE in milk were all less than 0.8 and 2.7 ng/mL, respectively. Table I lists the calibration equations, correlation coefficient, LOD, LOQ, and confidence interval values in detail.

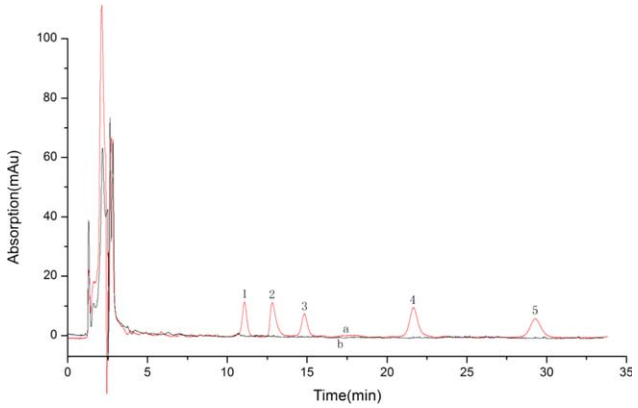

Figure 4. Chromatograms of the spiked milk sample and real milk sample: (a) chromatograms for the separation of ROX and ACE in the milk sample and (b) no peak found in the real milk sample. The conditions were as follows: monolithic column A, *i.d.* = 50 × 4.6 mm; analysis column, C18 Cosmosil packed column (*i.d.* = 150 × 4.6 mm); flow rate = 1.0 mL/min; concentration = 100 ng/mL; room temperature; detector wavelength = 215 nm; elution solution = 60/40 v/v ACN/Na_2HPO_4 aqueous solution (0.02 mol/L); and amount of injection = 20 μL. The analytes were (1) mono-ACE II, (2) ROX, (3) mono-ACE III, (4) di-ACE II, and (5) di-ACE III. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Table I. Calibration Curves, LODs, LOQs, and Confidence Intervals of ROX and ACE from Milk Samples

Analyte (sample)	Calibration equation	Correlation coefficient	LOD (ng/mL)	Confidence interval (LOD)	LOQ (ng/mL)	Confidence interval (LOQ)
ROX	$y = 6.19 \times 10^5 x - 2.96 \times 10^4$	$r^2 = 0.9987$	0.5	0.3837-0.6203	1.7	1.554-1.842
Mono-ACE II	$y = 4.95 \times 10^5 x - 3.81 \times 10^3$	$r^2 = 0.9995$	0.8	0.6336-0.9784	2.7	2.551-2.961
Mono-ACE III	$y = 3.52 \times 10^5 x - 8.92 \times 10^3$	$r^2 = 0.9998$	0.6	0.4321-0.7576	2.0	1.834-2.217
Di-ACE II	$y = 6.17 \times 10^5 x - 5.87 \times 10^3$	$r^2 = 0.9989$	0.6	0.4719-0.7225	2.0	1.813-2.268
Di-ACE III	$y = 5.09 \times 10^5 x - 3.64 \times 10^3$	$r^2 = 0.9997$	0.5	0.3774-0.6125	1.7	1.514-1.879

Table II. Precision, Accuracies, and Confidence Intervals of ROX and ACE from Milk Samples at Three Different Concentrations

Analyte (sample)	Concentration (ng/mL)	Precision relative standard deviation (%)				Accuracy (recovery, %)	Confidence interval
		Intraday	Confidence interval	Interday	Confidence interval		
ROX	500	2.76	2.484-2.904	3.67	3.446-3.886	103.8	99.47-106.0
	20	3.42	3.255-3.609	5.04	4.738-5.322	98.2	95.95-101.8
	5	6.70	6.455-6.910	7.76	7.478-7.933	92.5	89.25-95.4
Mono-ACE II	500	2.23	2.012-2.467	3.03	2.824-3.279	102.1	99.28-105.7
	20	4.65	4.476-4.819	6.56	6.357-6.755	94.5	91.62-97.3
	5	4.27	4.028-4.435	5.87	5.672-6.110	102.5	99.26-105.4
Mono-ACE III	500	2.43	2.246-2.684	4.31	4.109-4.522	100.8	96.45-103.0
	20	2.92	2.775-3.173	3.42	3.243-3.667	105.7	102.13-108.2
	5	5.08	4.825-5.224	5.13	4.907-5.335	107.6	104.52-110.1
Di-ACEII	500	3.57	3.317-3.768	2.89	2.627-3.049	102.8	99.53-105.3
	20	3.01	2.810-3.254	4.57	4.338-4.772	96.0	93.27-99.2
	5	6.52	6.321-6.785	5.28	5.086-5.430	105.3	102.81-108.3
Di-ACE III	500	2.30	2.190-2.579	3.90	3.761-4.118	103.8	100.20-106.7
	20	5.75	5.543-5.974	5.68	5.463-5.789	93.0	90.74-96.8
	5	4.22	4.015-4.448	6.65	6.432-6.843	104.5	101.29-107.4

Precision and Accuracy. The analytes with low, middle, and high concentrations were all prepared in duplicate and injected five times, respectively. The precision was evaluated by the calculation of the relative standard deviation values of the measured concentrations on the same day (intraday) and different days (interday). The relative standard deviation values were calculated by eq. 1. The accuracy, expressed by the recovery, was the value of the measured concentration divided by the target concentration and multiplied 100%. Table II lists the values of precision (<7.70%), recovery (92.5–107.6%), and confidence interval in detail; these values confirmed the feasibility of the method:

$$\text{Relative standard deviation}\,(\%) = \frac{\text{Standard deviation}}{\overline{x}} \times 100\% \quad (1)$$

The standard deviation is expressed as follows:

Table III. Comparison of This Method with Other Methods for the Determination of ROX and ACE Residues

Technique	Sample	Target analytes	Linearity range	LOD	Recovery	Determination	Reference
CE	Human urine, tablets	ACE, etc.	0.5-100 μmol/L	7.1×10^{-3} μmol/L	85.3-100.2%	ECL	1
MSPD	Sheep milk	ROX, etc.	24.0-96.5 μg/kg	8.0 μg/kg	84.7-95.0%	LC-DAD-UV	3
SPE	Wastewater	ROX, etc.	20-2000 ng/L	1.1 ng/L	78.0%	LC-MS/MS	15
MMIPS	Pork, fish, shrimp	ROX, etc.	0.05-10 μg/g	0.015-0.2 μg/g	82.5-113.1%	HPLC-UV	27
Online SPE	Milk	ACE, ROX	5-500 ng/mL	0.5-0.8 ng/mL	92.5-107.6%	HPLC-UV	This study

CE, capillary electrophoresis; DAD, diode array detector; ECL, electrogenerated chemiluminescence; LC, liquid chromatography; MMIPS, magnetic molecularly imprinted polymers; MS/MS, tandem mass spectrometry; MSPD, matrix solid-phase dispersion; UV, ultraviolet.

$$\text{Standard deviation} = \sqrt{\frac{\sum_{i=1}^{n} (x_i - \bar{x})^2}{n-1}} \quad (2)$$

where $\bar{x}$ is the average of the measured value, x_i is the measured value at different times, and n ($n \geq 5$) is the number of the measured values.

Repeatability. To investigate the repeatability of the method, five IL-based monolithic columns were prepared under the same conditions as described in the Preparation of the IL-Based Monolithic Column section, and were then used as the SPE columns in the online SPE–HPLC procedure. The relative standard deviation values for the retention times and peak areas were less than 5.15% ($n = 5$) and 6.68% ($n = 5$), respectively; this showed the good repeatability of the method.

Application in the Real Milk Sample

The proposed online SPE–HPLC procedure was successfully applied in the analysis of macrolide antibiotics in the real milk sample. A spiked milk sample (100 ng/mL) and real milk sample were analyzed by online SPE–HPLC. Figure 4 shows that ROX and ACE were detected in the spiked sample [Figure 4(a)], and no peak was found in the real milk sample [Figure 4(b)]. The results indicate that the concentrations of ROX and ACE in the real milk sample were lower than the LOD values of 0.5 and 0.8 ng/mL, respectively. Furthermore, this method is promising for the determination of trace macrolide antibiotics in milk.

Comparison with Other Methods. To evaluate the performance of the proposed method, Table III lists the LOD values and recoveries of this method and other methods. The results show that the method exhibited remarkable advantages: ROX and ACE could be analyzed simultaneously with this method, the LOD values (except in comparison with the liquid chromatography tandem/mass spectrometry method) and recoveries with the method improved, and the preparation of the monolithic sorbent for SPE and the online procedure were simple.

CONCLUSIONS

An IL-based monolithic column was prepared and used as the SPE sorbent. Under the experimental conditions tested, the SPE sorbent exhibited good performance in terms of cleanup efficiency and achieved a high recovery for specific analytes. Generally, the method was effective for the analysis of the trace macrolide antibiotics in biological samples, particularly for the sample pretreatment of milk.

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REFERENCES

1. Liu, Y. M.; Shi, Y. M.; Liu, Z. L.; Tian, W. *Electrophoresis* **2010**, *31*, 364.
2. Wang, M. J.; Zheng, C.; Hu, C. Q.; Jin, S. H.; Hoogmartens, J.; Schepdael, A. V. J. *Chin. Pharm. Sci.* **2011**, *20*, 572.
3. García-Mayor, M. A.; Gallego-Picó, A.; Garcinuño, R. M.; Fernándze-Hernando, P.; Durand-Alegría, J. S. *Food. Chem.* **2012**, *134*, 553.
4. Gros, M.; Rodríguze-Mozaz, S.; Barceló, D. *J. Chromatogr. A* **2013**, *1292*, 173.
5. Llorca, M.; Gros, M.; Rodríguez-Mozaz, S.; Barceló, D. *J. Chromatogr. A* **2014**, *1369*, 43.
6. Zhou, J. L.; Maskaoui, K.; Lufadeju, A. *Anal. Chim. Acta* **2012**, *731*, 32.
7. Heidari, H.; Razmi, H.; Jouyban, A. *J. Sep. Sci.* **2014**, *37*, 1467.
8. Handa, T.; Singh, S.; Singh, I. P. *J. Pharm. Biomed.* **2014**, *89*, 6.
9. Luo, Y.; Xu, L.; Rysz, M.; Wang, Y. Q.; Zhang, H.; Alvarez, P. J. *J. Environ. Sci. Technol.* **2011**, *45*, 1827.
10. Khalikova, M. A.; Šatínský, D.; Šmidrkalová, T.; Solich, P. *Talanta* **2014**, *130*, 433.
11. Shang, D. W.; Wang, X. P.; Zhao, X. T.; Huang, F. M.; Tian, G. Z.; Lu, W.; Zhou, T. Y. J. *Chromatogr. B* **2011**, *879*, 3459.
12. Yan, H. Y.; Sun, N.; Han, Y. H.; Yang, C.; Wang, M. Y.; Wu, R. J. *J. Chromatogr. A* **2013**, *1307*, 21.
13. Connolly, D.; Currivan, S.; Currivan, S.; Paull, B. *Proteomics* **2012**, *12*, 2904.
14. Arrua, R. D.; Talebi, M.; Causon, T. J.; Hilder, E. F. *Anal. Chim. Acta* **2012**, *738*, 1.
15. Rahayu, A.; Lim, L. W.; Takeuchi, T. *Talanta* **2015**, *134*, 232.
16. Jandera, P. *J. Chromatogr. A* **2013**, *1313*, 37.
17. Ho, T. D.; Canestraro, A. J.; Anderson, J. L. *Anal. Chim. Acta* **2011**, *695*, 18.
18. Lara, F. J.; Olmo-Iruela, M.; García-Campaña, A. M. *J. Chromatogr. A* **2013**, *1310*, 91.
19. Poole, C. L.; Poole, S. K. *J. Sep. Sci.* **2011**, *34*, 888.
20. Yang, L.; Yu, H.; Zhou, S. *Chromatographia* **2010**, *72*, 307.
21. Wang, Y.; Deng, Q. L.; Fang, G. Z.; Pan, M. F.; Wang, Y.; Yu, S. *Anal. Chim. Acta* **2012**, *712*, 1.
22. Han, H. F.; Wang, Q.; Liu, X.; Jiang, S. X. *J. Chromatogr. A* **2012**, *1246*, 9.
23. Guo, L.; Deng, Q. L.; Fang, G. Z.; Gao, W.; Wang, S. *J. Chromatogr. A* **2011**, *1218*, 6271.
24. Vidal, L.; Riekkola, M.-L.; Canals, A. *Anal. Chim. Acta* **2012**, *715*, 19.
25. Vidal, L.; Parshintsev, J.; Hartonen, K.; Canals, A.; Riekkola, M. L. *J. Chromatogr. A* **2012**, *1226*, 2.
26. Qin, J. X.; Bai, L. G.; Wang, J. F.; Ma, Y. M.; Liu, H. Y. *Anal. Methods* **2015**, *7*, 218.
27. Zhou, Y. S.; Zhou, T. T.; Jin, H.; Jing, T.; Song, B.; Zhou, Y. K.; Mei, S. R.; Lee, Y. *Talanta* **2015**, *137*, 1.