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Article

Preparation and Evaluation of a Mixed-Bed Immunoaffinity Column for Selective Purification of Sixteen Sulfonamides in Pork Muscle

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This paper describes the preparation of a novel mixed-bed immunoaffinity chromatography (IAC) column by coupling four monoclonal antibodies against different sulfonamides (SAs) to Sepharose 4B. The IAC column can be used to simultaneously extract and purify 16 SAs in pork muscle. The dynamic column capacities for all SAs in mixed standard solution were between 312 and 479 ng/mL gel. After simple extraction and IAC cleanup, the sample solution can be directly injected for liquid chromatography-ultraviolet analysis. The recoveries of SAs from spiked samples at levels of 25, 50 and 100 μg/kg ranged from 83.3 to 103.1% with variation coefficient less than 8.6%. The comparison of IAC with liquid–liquid extraction and solid phase extraction indicated that IAC has better purification effect and needs less organic solution than conventional methods, thus it would be an ideal method for selective purification of SAs in pork muscle.

Introduction

Sulfonamides (SAs, Figure 1) are a class of antibacterial compounds widely used in veterinary practice because of their inexpensiveness and wide spectrum of activity. They can be used to prevent or treat bacterial and protozoan infections in animals or added to animal feed to promote growth (1). Because improper use of SAs and insufficient withdrawal time for treated animals can result in SA residue of in edible tissues, thus posing potential carcinogenic risk to consumers (2), regulation 281/96 of the EU Commission has set maximum residue limits (MRLs) of 100 μg/kg for total SAs in target tissues and milk of all food-producing species (3). The Ministry of Agriculture of the People's Republic of China also established a MRL of 100 μg/kg for the sum of SAs and a MRL of 25 μg/kg for sulfamethazine only (4).

Numerous methods for the detection of SAs in various matrixes have been reported in literature. These methods involved immunoassays (5–7), capillary electrophoresis (CE) (8, 9), liquid chromatography (LC) (10–12) and liquid chromatography–tandem mass spectrometry (LC–MS-MS) (13–15). Immunoassay is generally used as screening method because of its susceptibility to environmental factors, and suspected noncompliant results detected by immunoassay should be further validated by instrument methods. For instrument methods, these approaches usually use solid phase extraction or liquid–liquid extraction to purify the samples and concentrate the analytes of interest. They have the pitfall of requiring a lot of organic solvent and elaborate sample pretreatment. In contrast, immunoaffinity chromatography (IAC) cleanup is a good alternative method to cleanup samples (16). It is based on the specific interaction of antigen-antibody and can provide a more simple and effective mean to purify extracts and reduce the use of organic solvent. IAC has been reported to successfully extract SAs from complex samples. Märtlbauer et al. described a monoclonal antibody-based IAC for the detection of sulfadimidine (SM2) and sulfadiazine (SDZ) in milk (16), Crabbe et al. developed an IAC for purifying sulfamethoxazole (SMZ) and its major metabolites in urinary samples (17), and Li et al. prepared a generic IAC for the cleanup of multiple SAs in meat using a group-specific polyclonal antibody (18). More recently, Li et al. reported an IAC–LC–MS-MS method for simultaneous determination of fluoroquinolone and sulfonamide antibiotics in animal muscle tissues (19). However, all of these IAC columns can maximally trap eight SAs (18), which does not meet the requirement for monitoring the total amount of SAs. In the current study, we prepared a mixed-bed IAC column that can be used to purify the total of sixteen normally used SAs in pork muscle. The evaluation of the IAC column and the comparison of IAC purification with conventional methods were also demonstrated.

Experimental

Chemicals and materials

Sulfacetamide (SA), sulfisomindine (SIM2), SDZ, sulfathiazole (ST), sulfapyridine (SPD), sulfamerazine (SMR), sulfamethoxydiazine (SMD), sulfamethiazole (SMT), SM2, sulfachloropyridazine (SCP), SMZ, sulfamonomethoxine (SMM), sulfisoxazole (SIZ), sulfachloropyrazine (SCPA), sulfadimethoxine (SDM) and sulfaquinoxaline (SQX) were obtained from Sigma-Aldrich. CNBr-activated Sepharose 4B was purchased from Pharmacia Corporation (Uppsala, Sweden). High-performance liquid chromatography (HPLC) grade methanol (MeOH) was obtained from Fisher Scientific (Pittsburgh, PA). All other chemicals and solvents were of analytical grade and were obtained from Beijing Chemical Reagent Co. (Beijing, P.R.C.). Deionized water was prepared using a Milli-Q water purification system (Millipore, Bedford, MA). The monoclonal antibodies (Mab,

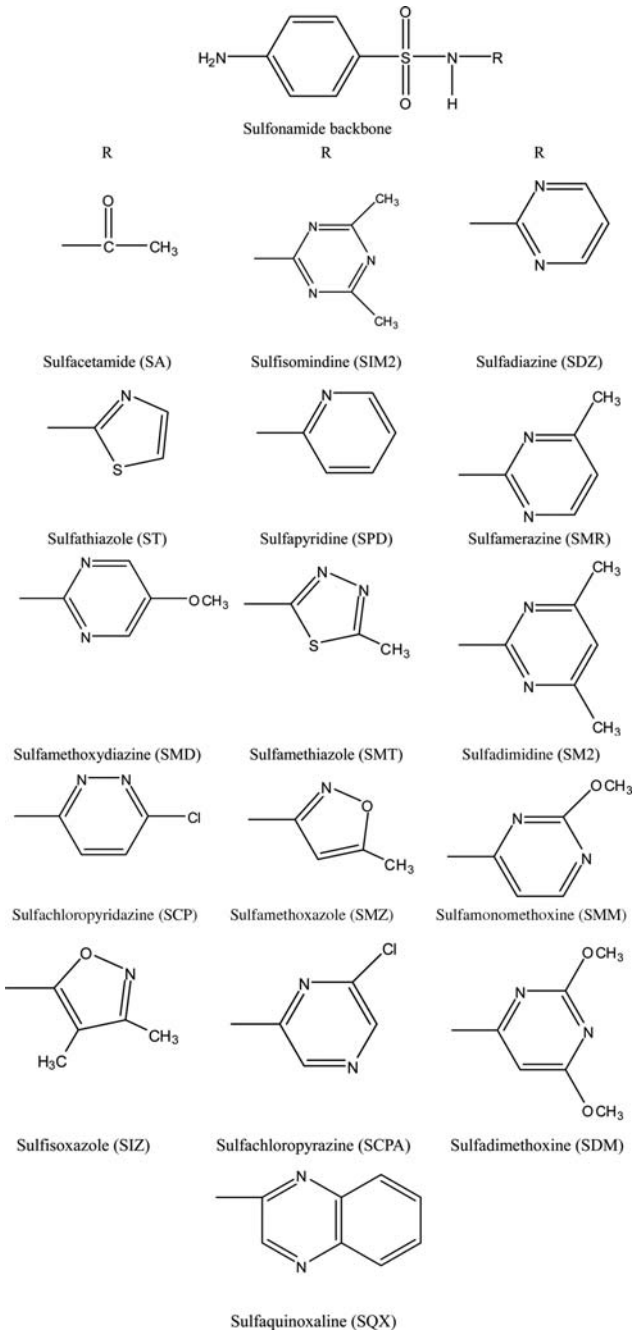

Figure 1. Chemical structures of SAs.

Cloning No.: E3D5A1, B2F7E8, C9H5D4 and C7A2F7) used in this study were provided by Clover Technology Group. By enzyme-linked immunosorbent assay, the IC_{50} values of the four Mabs were 11.2 ng/mL (Mab E3D5A1 against SIM2), 5.6 ng/mL (Mab B2F7E8 against SMM), 15.5 ng/mL (Mab C9H5D4 against SQX) and 8.9 ng/mL (Mab C7A2F7 against SMR). Individual stock standard solution of SAs (100 μg/mL) was prepared by dissolving 5.00 mg of each SA standard and diluting to a final volume of 50 mL with MeOH. The individual stock solutions were stored at 4°C in amber glass bottles and were stable for at least three months. Mixed standard solution (1 and 5 μg/mL) was prepared by mixing and diluting appropriate volumes of each standard solution to a final volume of 100 mL with MeOH.

Apparatus

The vortex mixer was from Fischer Scientific (Norcross, GA) and the centrifuge was purchased from Hettich Co. (Kirchlengern, Germany). The LC equipment was a Shimadzu LC-10ATvp system (Shimadzu, Japan). The chromatography column was a Cloversil C_{18} (4.6 × 250 mm, 5 μm).

IAC column preparation

The immunosorbent was produced as described by the manufacturer. Three grams of CNBr-activated Sepharose 4B (1 g powder gives approximately 3.5 mL final volume of gel) was added into 15 mL of 0.001 M HCl and poured to a sintered-glass funnel (40–60 μm). After being washed with 200 mL of HCl (0.001 M) and 400 mL of $NaHCO_3$ solution (0.1 M), the gel was mixed with Mab (Mab E3D5A1 6.3 mg, Mab B2F7E8 36.8 mg, Mab C9H5D4 36.8 mg and Mab C7A2F7 42 mg) dissolved in 5 mL of 0.1 M $NaHCO_3$ solution and stirred gently at 4°C for 24 h. The mixture was then washed with 50 mL phosphate buffer saline (PBS) to remove the uncombined Mabs. The eluant was collected to calculate the coupling efficiency and determine the antibody amount by Bradford protein assay method. The mixture was subsequently transferred to 10 mL of Tris-HCl buffer (0.1 M, pH 8.0) to block the unreacted sites on CNBr-activated Sepharose 4B. After the sites were blocked at 4°C for 2 h, the gel was washed with 3 cycles of 20 mL acetate buffer (0.1 M, pH 4.0) and 20 mL Tris-HCl buffer. Finally, 1 mL of gel was transferred to the glass column (10 × 0.8 mm, i.d.), and stored in PBS containing 0.01% (w/v) sodium azide at 4°C.

Column capacity determination

One milliliter of mixed standard solution (1 μg/mL) was dissolved with 20 mL of PBS. The solutions were then transferred into the IAC column (pre-conditioned with 10 mL of PBS) at a flow rate of 1 mL/min. The saturated column was washed with 10 mL of water. Finally, 2 mL of MeOH was used to elute the analytes. After filtering through a 0.2 μm PTFE filter (Jinteng Ltd, Tianjin, P.R.C.), 50 μL of the eluting solution (2 mL) was injected into the LC system. The column was regenerated by equilibrating with 10 mL of water and 10 mL of PBS, and stored in PBS (containing 0.01% sodium azide) at 4°C.

Sample preparation with IAC column

Five grams (± 0.01 g) of comminuted sample (200 g) were added into a 50 mL polypropylene centrifuge tube. Twenty milliliters of ethanol–water (80:20) were added and the sample tube was then vortexed for 2 min and shaken for 10 min. After being centrifuged at 8000 rpm for 5 min, 5 mL of the supernatant was transferred to a 50 mL volumetric flask and diluted to 50 mL with water. The mixed solution was filtrated through glass cellulose membrane and 20 mL of the filtrate was subsequently added to IAC column with flow rate of 1 mL/min. The column was then washed with 10 mL of water and eluted with

2 mL of MeOH. The collected eluting solution was evaporated to dryness at 50°C under a gentle stream of nitrogen and then reconstituted with 0.5 mL of chromatographic mobile phase, which was finally subjected to HPLC analysis.

HPLC analysis

The HPLC system consisted of a DGU-12A degasifier, a LC-10ATvp pump and a 7725 sample injector fitted with a 100-μL loop. For each analysis, 100 μL of the sample solution was injected. Chromatographic separation was achieved using a Cloversil C_{18} column (4.6 × 250 mm, 5 μm). Detection was performed by an ultraviolet (UV) detector with the wavelength of 270 nm. Data analysis was carried out on Class VP 5.03 Chemstation. The mobile phase was the mixture of MeOH (phase A) and 1.1% acetic acid in PBS (0.01 M) (phase B), and it was delivered to the HPLC column at a flow rate of 1 mL/min. The run time was 45 min for one analysis. An isocratic elution (A:B = 15:85) was used for the first 15 min of the chromatographic program. Mobile phase A was then gradually increased to 40% from 15 to 40 min. The solvent composition was then returned to the initial ratio at 40.1 min and equilibrated for another 5 min before the next injection.

Analysis of spiked samples

For control SAs, free pork muscle was obtained from piglets that had not been exposed to SAs. The tissue samples were minced and homogenized and then frozen at −20°C until analysis. For recovery study, mixed standard solution (1 and 5 μg/mL in MeOH) was added into homogenized muscle samples to produce spiked concentration of 25, 50 and 100 μg/kg. For each spiked level, six replicates were analyzed for one assay and three assays were repeated for three consecutive days.

Comparison of IAC purification with conventional methods

To compare the purification effect of IAC column with liquid–liquid extraction and solid phase extraction, 12 pork muscle samples spiked at 100 μg/kg of SAs were divided into three batches and were subjected to sample preparation by the three methods. Both of the two conventional methods have been employed as routine methods in our lab, and the procedures are briefly described in the following.

For liquid–liquid extraction (20), 1 g of samples was mixed with 2 g of anhydrous sodium sulfate in a 50-mL centrifuge tube and then extracted twice by 40 mL (20 × 2) of acetonitrile. After the extracts were combined, 10 mL of isopropanol was added and then rotary evaporated to dryness. The residue was redissolved with 1 mL of 15% acentonitrile aqueous solution and transferred to a 10-mL centrifugation tube. Two milliliters of hexane (saturated with acentonitrile) was subsequently added to degrease. After centrifugation, an aliquot of the lower solution was subjected to LC determination.

For solid phase extraction (21), 1 g of samples was mixed with 2 g of anhydrous sodium sulfate and then extracted twice by 40 mL (20 × 2) of acetonitrile. After the extracts were combined, 10 mL of hexane was added to degrease. After standing and partition, the hexane layer was discarded and 10 mL of isopropanol was added. The mixture was then rotary evaporated to dryness and re-dissovled with 1 mL of 0.2% formic acid aqueous solution and 1 mL of 5% acetic acid aqueous solution. The mixture was subsequently loaded to MCX column that was previously conditioned with 3 mL of MeOH and 3 mL of water, respectively. After being washed with 2 mL of 2% formic acid aqueous solution and 2 mL of MeOH, the column was eluted with 4 mL of 6% ammonia–MeOH (v/v). The collected elute was evaporated to dryness at 40°C under a gentle stream of nitrogen and then reconstituted in 1 mL of 15% acetonitrile aqueous solution. After being filtered, the solution was finally injected into the LC system for analysis.

For IAC cleanup, the samples were pretreated and determined according to the method described previously.

Results and Discussion

Preparation of immunoaffinity column

In previous reports, several groups have prepared IAC columns against SAs, but in these studies, only one antibody was coupled with Sepharose 4B; thus, the prepared IAC column can only trap a maximum of eight SAs due to the limitation of antibody specificity (16, 17, 19). Because the MRLs for total SAs were set by EU (3) and China (4), an IAC column that can adsorb more SAs is preferred. To achieve this goal, two strategies were generally employed: to prepare more generic antibody and to mix multiple antibodies using one IAC column. Although a generic antibody against 15 SAs has been reported

Table I
Capacity (ng/mL gel) of Individual SAs for IAC Columns Coupled with Different Mabs

	Column capacity (ng/mL gel)			
Analytes	E3D5A1	B2F7E8	C9H5D4	C7A2F7
SA	0	0	0	2188
SIM2	1535	0	0	0
SDZ	0	2276	0	2286
ST	0	2038	0	2306
SPD	0	2215	0	0
SMR	1489	2329	0	2367
SMD	0	2304	0	0
SMTZ	0	0	607	2269
SM2	1554	625	0	0
SCP	0	388	246	2320
SMZ	0	2157	0	2284
SMM	0	2401	1069	0
SIZ	0	0	0	2311
SCPA	0	2288	1043	2304
SDM	243	0	1032	0
SQX	0	0	1179	2405

Table II
IAC Column Capacity for SAs in Mixed Standard Solution

Analytes	Column capacity (ng/mL gel)	Analytes	Column capacity (ng/mL gel)
SA	312	SM2	407
SIM2	359	SCP	336
SDZ	448	SMZ	478
ST	326	SMM	324
SPD	398	SIZ	364
SMR	405	SPZ	479
SMD	357	SDM	367
SMTZ	415	SQX	453

(5), this antibody was not available in our laboratory, thus the second protocol was carried out in our study. Four Mabs (E3D5A1, B2F7E8, C9H5D4 and C7A2F7) against different SAs were used to couple with Sepharose 4B, respectively. The amount for each Mab is 3 mg/mL gel and each IAC column was poured with 1 mL gel. The adsorption specificity and capacity of the IAC columns are shown in Table 1. The results indicated that Mabs E3D5A1, B2F7E8, C9H5D4 and C7A2F7 can strongly trap three, eight, four and 10 SAs, respectively. To obtain equal column capacities for all SAs in mixed standard solution, we adjusted the antibody amount and mixed the four Mabs before coupling. The final antibody amounts for Mab E3D5A1, B2F7E8,

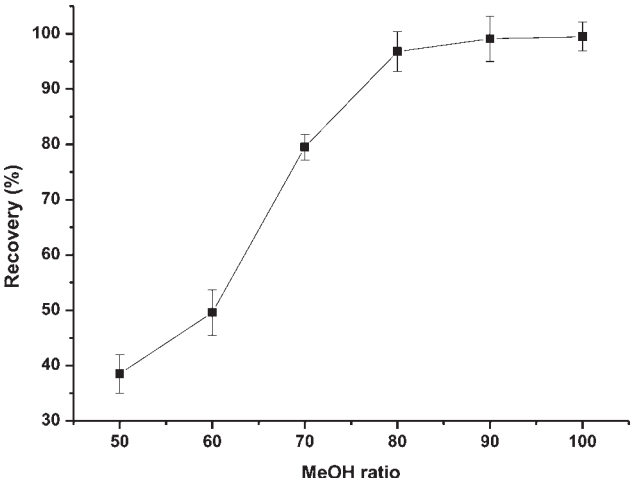

Figure 2. The elution curve of SA on an IAC column with different ratios of MeOH–water.

Table III
Recoveries and CV of SAs from Pork Muscle ($n = 18$)

SAs	Spiked level (μg/kg)					
	25		50		100	
	Recovery (%)	CV %	Recovery (%)	CV %	Recovery (%)	CV %
SA	95.1	5.1	90.2	8.6	89.1	6.1
SIM2	89.6	6.7	86.7	6.4	103.1	6.2
SDZ	83.4	4.9	95.2	6.1	96.4	6.0
ST	102.3	6.8	94.6	6.3	91.3	2.8
SPD	87.4	5.3	86.3	5.2	86.3	6.3
SMR	95.7	4.9	85.4	4.8	100.3	5.3
SMD	84.1	5.2	83.3	7.9	93.4	3.7
SMTZ	100.2	7.3	91.5	5.5	88.3	4.2
SM2	102.1	4.6	101.4	5.6	100.2	5.4
SCP	84.9	3.3	83.5	7.3	100.3	6.2
SMZ	95.2	5.8	95.6	6.8	88.4	8.0
SMM	86.3	4.8	92.4	4.8	86.7	7.6
SIZ	96.7	3.1	87.3	4.9	99.3	5.4
SPZ	99.0	4.6	88.2	6.5	97.5	6.3
SDM	83.5	6.4	91.3	4.9	86.8	8.4
SQX	97.3	8.1	83.3	4.9	85.6	5.4

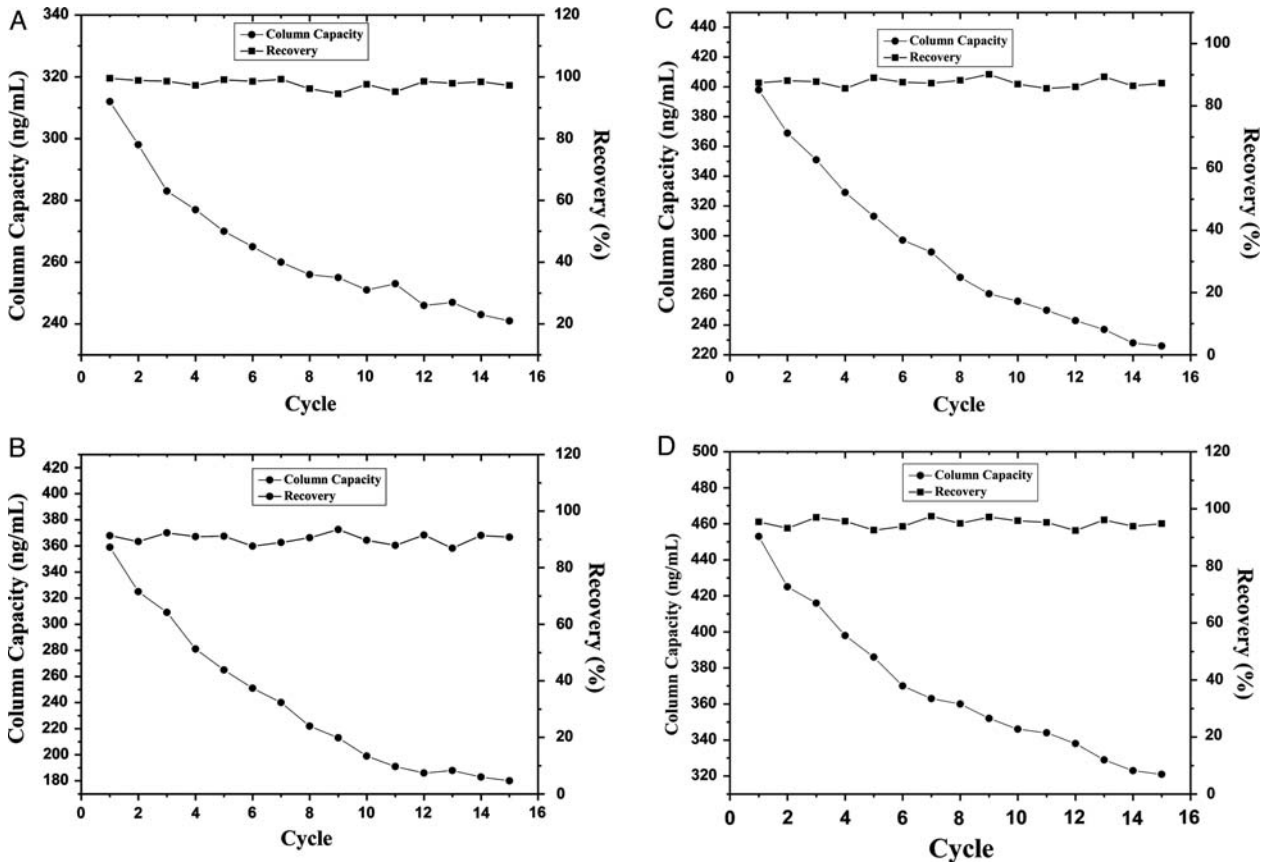

Figure 3. The IAC capacity and recovery (300 μg/kg) variation curves after 15 cycles in 45 days for (A) SA, (B) SIM2, (C) SPD, (D) SQX.

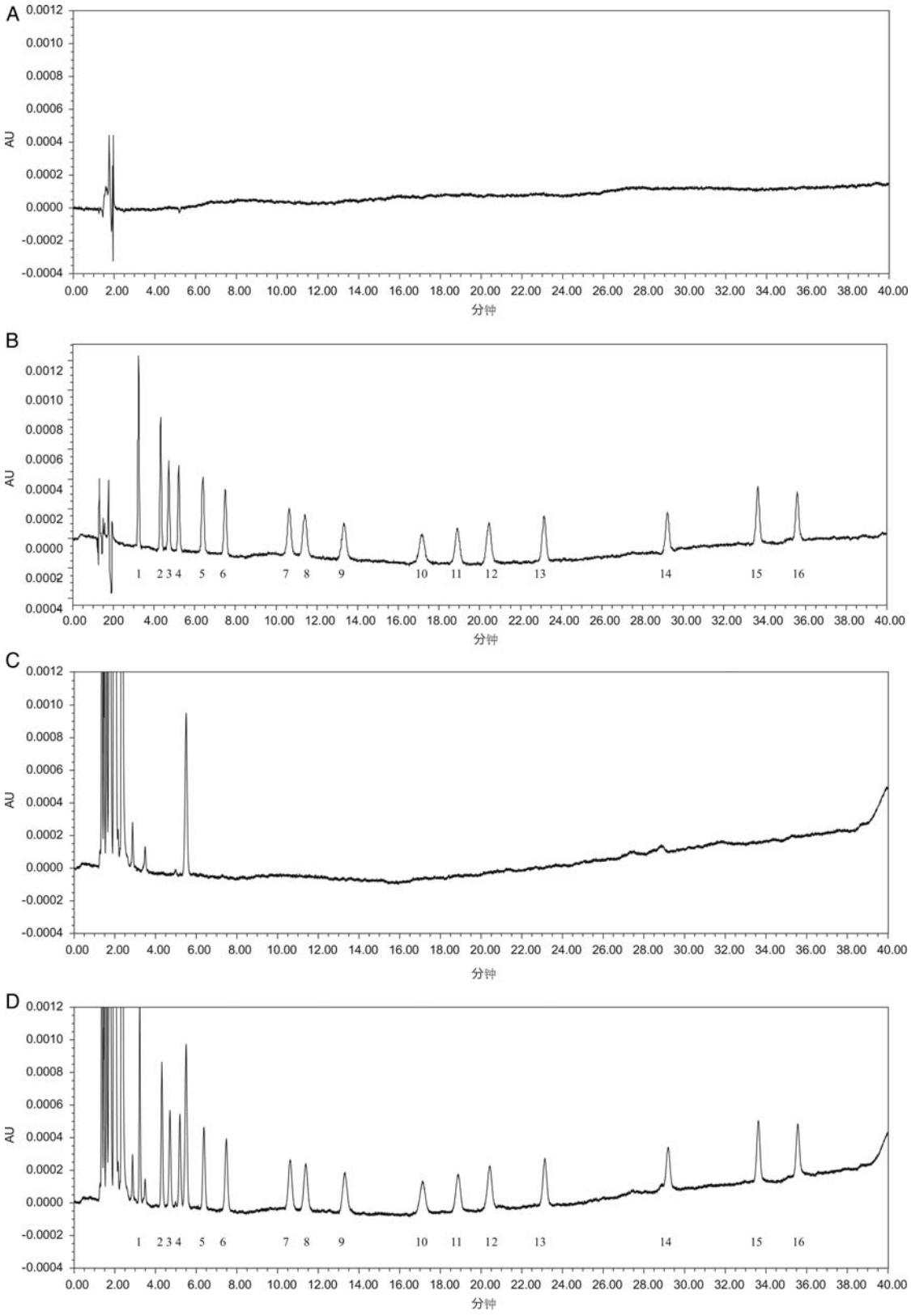

Figure 4. Typical chromatograms of control and spiked samples: (A, B) purified by IAC, (C, D) purified by liquid−liquid extraction, (E, F) purified by solid phase extraction; the spiked concentration was 100 μg/kg of SAs; the elution order of the SAs was SA (1, 3.2 min), SIM2 (2, 4.3 min), SDZ (3, 4.8 min), ST (4, 5.2 min), SPD (5, 6.3 min), SMR (6, 7.5 min), SMD (7, 10.5 min), SMTZ (8, 11.4 min), SM2 (9, 13.2 min), SCP (10, 17.1 min), SMZ (11, 18.8 min), SMM (12, 20.4 min), SIZ (13, 23.1 min), SPZ (14, 29.2 min), SDM (15, 33.6 min) and SQZ (16, 35.6 min).

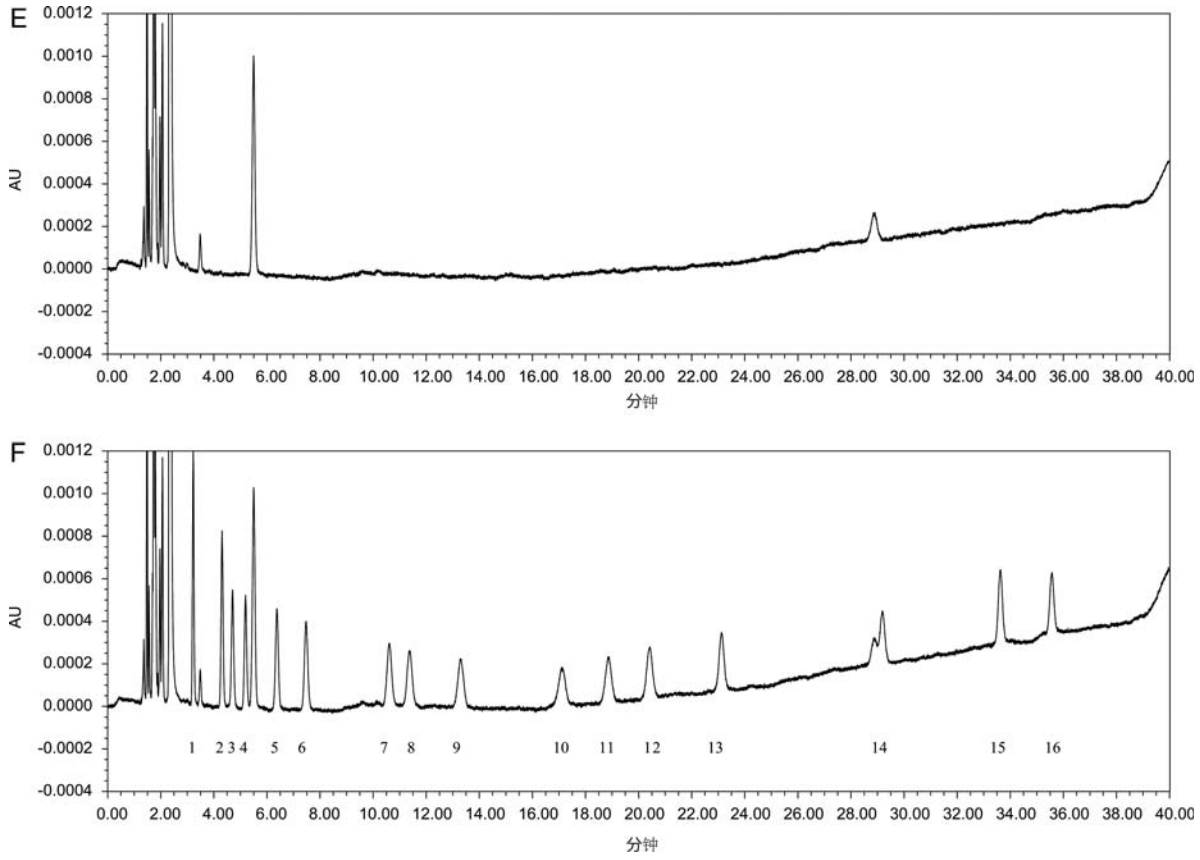

Figure 4. (Continued).

C9H5D4 and C7A2F7 were 0.6, 3.5, 3.5 and 4 mg/mL gel. The capacity of this mixed-bed IAC column for all SAs was tested to be 312–478 ng/ mL gel (Table 2**)**, which implies that the IAC column can maximally absorb SA amounts in samples with six-fold MRL level of sixteen SAs. To demonstrate that SA was specially bound to the immobilized Mab and there was no non-specific absorption, the control column (without coupled antibody) was employed to measure the capacity as described previously. When the proposed sample preparation procedures were employed, none of the SAs wwere found to adsorb on the control Sepharose 4B column.

Optimization of the IAC conditions

Optimization of loading, washing, and elution conditions is necessary to IAC cleanup because these conditions have a strong influence on the association and dissociation of antigen-antibody complex (19). To investigate whether a loading buffer containing different ratios of ethanol has an effect on the recovery, 0.1 mL of mixed standard solution (5 μg/mL) was loaded in 10 mL of PBS–ethanol (95:5), PBS–ethanol (90:10) and PBS–ethanol (80:20), respectively. After these solutions were loaded, the columns were washed by 10 mL of water followed by eluting with 2 mL of MeOH and detecting by HPLC-UV. The results indicated that the average recovery has no significant change when the ratio of ethanol was increased from 5 to 10%, and has a slight decline from 99.1 to 96.3% when the ratio was increased up to 20%. Considering the solubility of SAs in PBS–ethanol, PBS–ethanol (90:10) was selected as loading medium. The effect of flow rate (0.5, 1, 1.5 and 2 mL/min) of the loading solution on recovery was also investigated. Increase of flow rate from 1 to 2 mL/min resulted in the decrease of average recovery from 98.3 to 84.4%. However, the recoveries for SAs at flow rate of 0.5 and 1 mL/min have no significant difference, thus the flow rate at 1 mL/min was chosen for the subsequent study.

In IAC cleanup, the target analyte in samples could be selectively captured by specific antibodies immobilized on gel; at the same time, the interfering substance may also be retained because of nonspecific absorption. These interferences could be largely removed by washing procedure. Generally, washing buffer consisted of water and organic solvent such as MeOH (18). Three ratios of water–MeOH (100:0, 95:5 and 90:10) were tested for washing effect. It was found that the recovery and washing effect is similar. To save organic solvent, pure water was used as washing solution in this study.

Following the washing procedure, the trapped analyte could then be released from the IAC column by dissociating the antibody-analyte complex with eluting buffer. In this study, 2 mL of different ratios of MeOH in water (from 50 to 100%) were employed as eluting solution, respectively. The eluting curves (Figure 2) indicated that the recoveries of SA (as a representative drug) were dramatically increased from 38.5 to

96.8% when the MeOH ratio was elevated from 50 to 80%. When the MeOH ratio further rose from 80 to 100%, the recovery had no significant change. Because pure MeOH is easy to be further concentrated, it was finally selected as eluting solution.

The reusability of IAC was subsequently evaluated. For each use, more than 60 min for antibody revival is needed. The curves of column capacity and recoveries after 15 cycles of sample cleanup are shown in Figure 3. Although the column capacity gradually decreased as the cycles increased, the recoveries of SA, SIM2, SPD and SQX (as representative drugs) at spiked concentrations of 300 μg/kg in pork muscle were observed without any loss after 15 cycles in 45 days.

Method validation

The standard calibration curve was constructed by plotting the peak area versus concentration and used to determine the concentration of SAs in all subsequent analysis. It showed that the calibration curves for all SAs were linear in the range of 20–2000 ng/mL with satisfactory correlation coefficients (r^2) of more than 0.99. The limits of quantification (LOQ) for SAs in pork muscle, which were defined as signal-to-noise ratio of 10:1, respectively, were determined to be 25 μg/kg. The average recovery of 18 replicate blank tissues fortified at LOQ level was more than 83.4% with coefficient of variation (CV) of less than 8.1% (Table 3). The accuracy and precision of the method was evaluated at four levels according to recovery and coefficient of variability. When the blank pork muscle samples were spiked at concentrations of 25, 50 and 100 μg/kg, the recoveries of SAs from fortified samples ranged from 83.3 to 102.1% with intra-day and inter-day CVs of less than 8.6% (Table 3). The LOQ of this method was below or equal to current SA MRLs established by EU and China, and the accuracy and precision also met the requirements for quantitative analysis; therefore, the developed method can be used to monitor SA residue in pork muscle.

Typical chromatograms of blank and spiked samples purified by IAC, liquid–liquid extraction and solid phase extraction are presented in Figure 4. For IAC cleanup, the lack of interference to SAs peak in the chromatogram suggests a high specificity of the IAC column and a good resolution of the chromatographic method. In contrast, sample preparation by liquid–liquid extraction and solid phase extraction brings obvious interferences to SAs peaks in the chromatogram. Also, as described previously, IAC purification is simpler and requires less organic solution than both liquid–liquid extraction and solid phase extraction. Thus, the IAC procedure can be a good alternative to conventional methods for sample preparation.

Conclusions

In this study, we prepared a mixed-bed IAC column for the purification of SAs from pork muscle, which was then followed by LC separation and UV detection. The IAC extraction can give satisfactory recovery and leads to low detection limit in LC analysis. Furthermore, the IAC column can obtain better purification effects while requiring fewer purification steps and consuming less organic solution than liquid–liquid extraction and solid phase extraction. Therefore, the developed IAC column would be an ideal approach for the cleanup of SAs in pork muscle.

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