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Application of ion-exchange cartridge clean-up in food analysis V. Simultaneous determination of sulphonamide antibacterials in animal liver and kidney using high-performance liquid chromatography with ultraviolet and mass spectrometric detection

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Abstract

A simple, rapid, and reliable method for the determination of residual sulphonamide antibacterials (SAs) (sulfadiazine, sulfamerazine, sulfadimidine, sulfamethoxypiridazine, sulfisozole, sulfamonomethoxine, sulfamethoxazole, sulfisoxazole, sulfadimethoxine, and sulfaquinoxaline) in animal liver and kidney was developed using a combination of clean-up on a Bond Elut PSA cartridge and HPLC with UV detection. The SAs were extracted with ethyl acetate and then dissolved in 5 ml of 50 v/v% ethyl acetate-*n*-hexane after being evaporated to dryness. For clean-up of the crude sample, the resuspended extract was applied to a Bond Elut PAS (primary/secondary amine cartridge), and then SAs were eluted from the cartridge using 5 ml of 20 v/v% acetonitrile–0.05 *M* ammonium formate before being analysed by HPLC. Recoveries of the SAs at the levels of 0.5 and 0.1 μg/g were 70.8–98.2%, the rerative standard deviation were less than 7.0%, and the detection limits were 0.03 μg/g. The present analysis method of SAs in animal kidney and liver using HPLC with a clean-up procedure was demonstrated to be highly applicable to the direct LC–MS–MS analysis without any modification.

Keywords: Food analysis; Clean-up methods; Sulphonamides

1. Introduction

Sulphonamide antibacterials (SAs, Fig. 1) are widely used for the treatment and prevention of animal diseases and more than ten different kinds of SAs are known to be used for domestic animals in Japan. The widespread use of SAs in factory farming without proper withdrawal periods has let residues of SAs stay in organs and muscles of slaughtered animals. Monitoring of such residues in slaughtered animals has, accordingly, become one of the most important duties for public health agencies. Since the concentration of residual drugs including SAs in liver and kidney is generally higher than that in muscles [1,2], inspection of the liver and kidney of slaughtered animals for residual SAs is considered to

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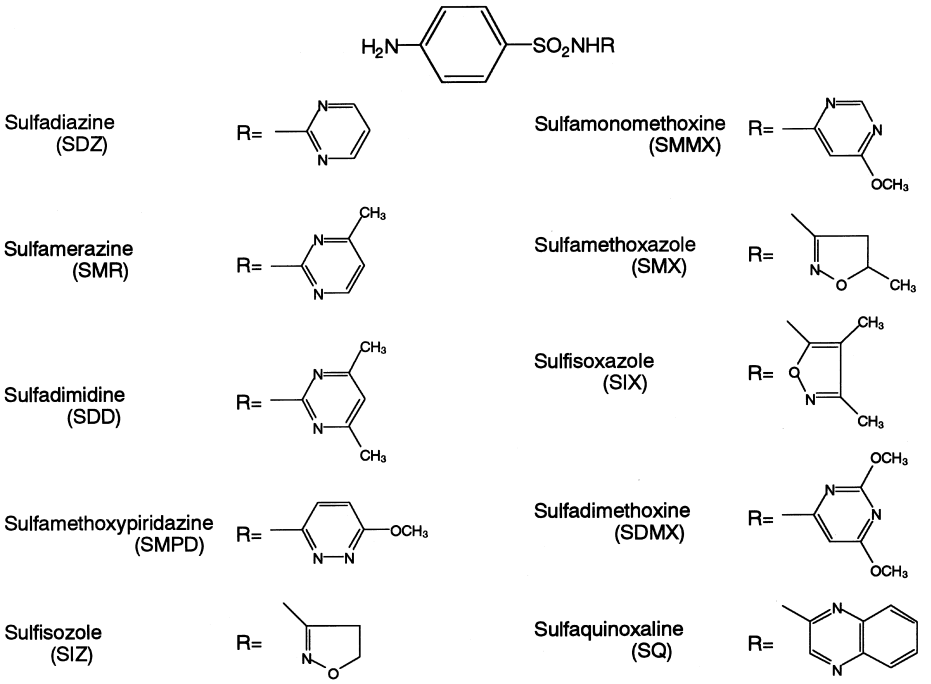

Fig. 1. Structures of sulphonamide antibacterials.

be the most effective means of monitoring these substances for maintaining the safety of foods.

In order to inspect the residues in liver and kidney of the slaughtered animals, HPLC method has been frequently used because of its reliability and sensitivity [3–11]. However, most of them require pre- [6,9,11] or post-column [3,5,7,8] derivatization using fluorescence reagents such as fluorescamine and complicated procedures [4,10], because severe matrix influences occur when SAs extracted from the liver and kidney are analysed using HPLC with UV-detection. Consequently, there is a great need for a simple and reliable analysis method using UV-detection which permits the simultaneous determination of SAs in the liver and kidney.

We have established a method for the determination of SAs in some foods using a combination of amino cartridge clean-up and HPLC with UV detection [12]. Although this method was simple and reliable, it did not yield satisfactory results for the residual analysis of SAs in animal liver and kidney. Furthermore, the sample prepared for HPLC analysis in this method could not be directly applied to LC–MS analysis because the mobile phase and sample solution contained a non-volatile acid, phosphoric acid. However, these problems can be overcome by careful consideration of the following: (1) HPLC conditions using a volatile mobile; (2) the selection of prepacked anion-exchange cartridge;
(3) the elution of SAs from the cartridge; and (4) influences of applying a solvent to the cartridge.

In this paper, a technique is described for the determination of residual SAs in animal liver and kidney using a combination of clean-up on a prepacked anion-exchange cartridge (Bond Elut PSA) and HPLC with UV and MS detection using a volatile mobile phase.

2. Experimental

2.1. Materials

Acetonitrile, methanol, formic acid, anhydrous sodium sulfate, ethyl acetate, *n*-hexane, and ammonium formate were analytical grade reagents.

Sulfamethoxazole (SMX), sulfadiazine (SDZ),

sulfamerazine (SMR), sulfisoxazole (SIX), sulfadimidine (SDD), sulfadimethoxine (SDMX), and sulfamethoxypiridazine (SMPD) were obtained from Sigma (St. Louis, MO, USA) and sulfisozole (SIZ), sulfamonomethoxine (SMMX), and sulfaquinoxaline (SQ) are from Wako Pure Chemical (Osaka, Japan).

The Bond Elut PSA (3 ml/500 mg, part No. 1210-2042) cartridge was purchased from Varian (Harbor City, CA, USA). Sep Pak Vac 3 ml QMA (3 ml/500 mg, part No. WAT020850) and Sep Pak Vac 3 ml NH_2 (3 ml/500 mg, part No. WAT020840) cartridges were obtained from Waters (Milford, MA, USA).

2.2. Preparation of standard solution

Each standard (10 mg) was accurately weighed into a 100-ml volumetric flask and diluted with acetonitrile. Subsequent dilutions were made with methanol. (0.01, 0.025, 0.05, 0.25, 0.5, 1.5, 2.5 and 5.0 μg/ml)

2.3. Clean-up procedure

A 5-*g* amount of sample and 10 *g* of anhydrous sodium sulfate were weighed into a 50-ml centrifuge tube, blended with 20 ml of ethyl acetate for 2 min using a high speed blender (Ultra-Turrax T25; IKA Werk, Staufen, Germany), centrifuged (700 *g*) for 5 min and then the supernatant was decanted. This extraction procedure with ethyl acetate was repeated once more and the combined extracts were evaporated to dryness under reduced pressure at 35°C. The residue was dissolved in 5 ml of 50% ethyl acetate-*n*-hexane and the solution was applied to a Bond Elut PSA prewashed with 5 ml of *n*-hexane. The cartridge was washed with 3 ml of *n*-hexane and air-dried by aspiration for 3 min. SAs were eluted from the cartridge with 5 ml of 20% acetonitrile–0.05 *M* ammonium formate and 20 μl of the eluate were injected into the HPLC and liquid chromatography tandem mass spectrometric (LC–MS–MS) systems for the determination and the identification of SAs, respectively.

2.4. High performance liquid chromatography

A high-performance liquid chromatograph equipped with a constant flow-pump (LC-10AD; Shimadzu, Kyoto, Japan) was used with a UV-detector (Shimadzu SPD-10A) operated at 272 nm. The separation was performed on an L-column ODS (5 μm, 250×4.6 mm, I.D.) (Chemical Inspection and Testing Institute, Tokyo, Japan) with methanol–acetonitrile–0.05 *M* formic acid (10:15:75) as a mobile phase at a flow-rate of 1.0 ml/min at 30°C.

2.5. Tandem mass spectrometry

The mass spectrometer used was a Quattro II (Micromass, Altrincham, UK) with an electrospray ion source and the instrument was operated in the positive mode with a daughter ion scan. The capillary voltage was 3.0 kV, cone voltage was 30–35 kV, the ion source temperature was 130°C, and the desolvation temperature was 350°C. A collision induced dissociation was performed using the collision energy of 18 eV with argon as the collision gas.

3. Results and discussion

3.1. Establishment of HPLC system

In consideration of the application of the present HPLC system to the LC–MS–MS analysis, we tried to separate ten different kinds of SAs shown in Fig. 1 on a C18 HPLC column using a volatile mobile phase consisting of acetonitrile, methanol, and aqueous formic acid solution. After optimization of the mobile phase parameters, concentration of formic acid, pH of the aqueous solution, and ratio of the aqueous solution and the organic solvents, satisfactory separations of these SAs were obtained using methanol–acetonitrile–0.05 *M* formic acid (10:15:75) on the L-column ODS (5 μm, 4.6×250 mm, I.D.). The flow-rate was 1.0 ml/min, the column temperature was 30°C, and the monitoring wavelength was adjusted to 272 nm, which is a maximum absorption wavelength for all of these SAs. Under these conditions, the ten different kinds of SAs were successfully separated in 35 min, as shown in Fig. 2. The calibration graphs were linear between 0.5 and 100 ng.

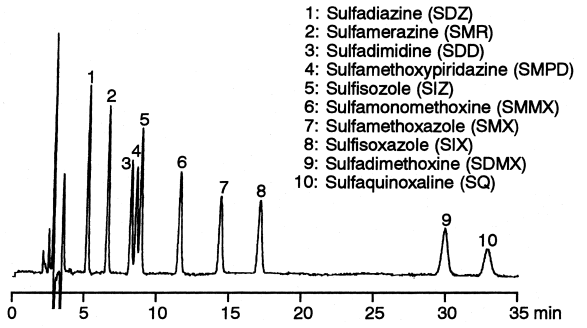

Fig. 2. HPLC separation of sulfa drugs, Conditions: See Experimental.

3.2. Establishment of clean-up system

3.2.1. Selection of prepacked cartridge

As mentioned above, recoveries of the SAs were remarkably decreased when the SAs in animal liver were analysed according to a previous amino cartridge clean-up technique [12]. In order to determine the influence of the amount of liver sample analysed on the recoveries, a decreasing amount of liver was used for the analysis of SAs recoveries at the concentrations of 0.5 μg/g. The recoveries improved with decreasing amount of the liver weight, indicating that impurities from the liver undoubtedly interfere with the retention of the SAs on the amino cartridge. These results indicate that more satisfactory results can be obtained when an anion-exchange cartridge with stronger retention power is used for the analysis of SAs in liver and kidney.

In order to select a cartridge with this property, a quaternary amine cartridge (Sep Pak QMA) and a primary/secondary amine cartridge (Bond Elut PSA) were studied to examine their power of SAs retention for the improvement of the SAs analysis. In our previous study [12], we found that ethyl acetate is most suitable for the extraction of SAs from foods including animal muscles, fish muscles, and eggs, and that direct application of the extract to the cartridge without concentration is convenient. On the bases of the findings of our previous study and a preliminary experiment of the present study, it was concluded that a cartridge to be used in the present study should possess both abilities to retain SAs with organic solvents such as ethyl acetate and then to release the SAs with a suitable eluent. The ethyl acetate solution of SAs (2.5 μg/ml), which passed through either types of the cartridges, was collected and evaporated to dryness. The SAs collected from the cartridges were determined by HPLC to calculate the amounts of SAs retained in the cartridge. These experiments revealed that the SAs were perfectly retained on both cartridges. Since both cartridges gave satisfactory retention of the SAs, they were successively compared with respect to the elution behavior of the SAs. After application of the SAs to the cartridges in the same manner as described above, the SAs were eluted from the cartridges with 10 ml of 20% acetonitrile–0.05 *M* ammonium formate, and then determined by HPLC. More than 98% of all of the SAs were recovered from the primary/secondary amine cartridge; whereas less than 80% for sulfisozole and sulfisoxazole were recovered from the quaternary amine cartridge. We, therefore, concluded that the primary/secondary amine cartridge is the most suitable for our objective.

3.2.2. Elution of SAs from the cartridge

As analytical practice, it is desirable to use a mobile-phase for sample preparation and standard solutions in order to inject sample or standard solutions into an HPLC system [13,14], it was first tried to elute the SAs from the cartridge with the mobile phase. However, satisfactory recoveries of the SAs from the cartridge could not be obtained when the mobile phase (methanol–acetonitrile–0.05 *M* formic acid, 10:15:75) was used as the eluent. We, therefore, tried to elute the SAs using an eluent having basically the same ratio of organic and aqueous composition as the mobile phase, including acetonitrile and 0.05 *M* aqueous ammonium formate solution.

Using this eluent, the influence of acetonitrile concentration was first investigated on the recoveries of the SAs. After applying the SAs (2.5 μg each) to the cartridge in the same manner as described above, the SAs were eluted with 10 ml of the eluent and determined by HPLC. It was found that all of the SAs were perfectly recovered with the acetonitrile concentrations between 15 and 25%. Based on this observation, we selected an acetonitrile concentration of 20% for the further analyses.

Because it is generally accepted that pH of the eluent is one of the most important factors for the

elution of SAs from cartridge, the effect of pH on recoveries of the SAs was investigated using 10 ml of 20% acetonitrile–0.05 *M* aqueous ammonium formate solution. All of the SAs recovered satisfactory with pH level of 3.5; whereas the SAs scarcely recovered with pH level less than 3.0. Because 0.05 *M* ammonium formate has a pH of 3.5, we decided to use a 20% acetonitrile–0.05 *M* aqueous ammonium formate solution without adjusting the pH of the solution. Next, we investigated the volume of eluent to completely elute the SAs from the cartridge using the solution. In order to elucidate an optimal volume of the solution to elute the SAs, the applied SAs (2.5 μg each) were eluted from the cartridge as 1 ml fractions for the determination of SAs in each fraction. These experiments revealed that use of the eluent more than 5 ml completely eluted the SAs; whereas 3 ml of the eluent did only between 70 and 80% of the SAs. We, therefore, used 5 ml of the 20% acetonitrile–0.05 *M* aqueous ammonium formate solution as the eluent for the primary/secondary amine cartridge.

3.2.3. Application of SAs to the cartridge

In order to examine an applicability of direct application of the extract from liver and kidney without concentration, we first applied a swine liver extract (40 ml of ethyl acetate) spiked with the SAs (2.5 μg each) to the cartridge. Recoveries of the SAs were turned out to be less than 50%, the result of which suggested that the impurities from the liver interfere with the retention of the SAs on the cartridge and that a large volume of sample cannot be applied to the cartridge to maintain good recoveries of the SAs from the cartridge. In our previous report for the analysis of pyridonecarboxylic acid antibacterials [15], we found that an anion-exchange cartridge has a stronger retention power in a mixture of *n*-hexane and ethyl acetate than that in only ethyl acetate. Accordingly, we tried to substitute a mixture of ethyl acetate and *n*-hexane for the ethyl acetate extract. After the SAs (2.5 μg each) were added to the ethyl acetate extract of the swine liver, the extract was evaporated to dryness, and then was dissolved in 10 ml of selected ratios of the mixtures of ethyl acetate and *n*-hexane before applying to the cartridge. It was found that the best recoveries(>98%) was obtained when 50% ethyl acetate-*n*-hexane was used as the applying solvent. These results indicate that the SAs could not be retained on the cartridge when applying a solvent containing more than 50% of ethyl acetate, and that the SAs in the residue cannot be dissolved in a solvent containing ethyl acetate less than 50%.

Next, influence of the volume of the applying solvent was examined using selected volumes of 50% ethyl acetate-*n*-hexane in the same manner as described above. Recoveries of over 98% were obtained when the volumes between 1 and 10 ml of 50% ethyl acetate-*n*-hexane were used. Use of the solvent more than 10 ml yielded lower recoveries (less than 75%). Judging from the results of preliminary experiments mentioned above, we had chosen a procedure: (1) to evaporate the extract to dryness, and (2) to dissolve the residue in 5 ml of 50% ethyl acetate-*n*-hexane, before applying the extracted sample to the cartridge.

3.3. Application of the various samples

Based on our experimental results, we concluded that the most suitable conditions for the analysis of residual SAs in animal liver and kidney is as follows: SAs were extracted with ethyl acetate, the extract was evaporated to dryness, the resulting residue was dissolved in 5 ml of 50% ethyl acetate-*n*-hexane, and the solution was applied to a Bond Elut PAS (anion-exchange cartridge). The SAs were eluted from the cartridge with 5 ml of 20% acetonitrile–0.05 *M* ammonium formate and determined by HPLC. In order to investigate the capability of the present method, recoveries of the SAs from fortified kidneys and livers of swine and bovine, and chicken liver at the levels of 0.5 and 0.1 μg/g were determined. The SAs (2.5 and 0. 5 μg each) dissolved in 50 μl of methanol were spiked to 5 g each of the sample, and recovered SAs from the sample were determined using the method presented here. As shown in Table 1, satisfactory recoveries (over 70%) and relative standard deviation (less than 7.0%) were obtained for these low concentrations of the SAs. The detection limits were 0.03 μg/g for all of the SAs (S/N ratio=3). Typical HPLC chromatograms of the SAs determination of the extracted from commercially available livers and kidneys are shown in Fig. 3. As shown by these chromatograms, satisfactory clean-up

Table 1
Recoveries of SAs from animal kidneys and livers

	Recovery, % (RSD, %)[a]									
	SDZ[b]	SMR[b]	SDD[b]	SMPD[b]	SIZ[b]	SMMX[b]	SMX[b]	SIX[b]	SDMX[b]	SQ[b]
Fortified at 0.5 μg/g										
Swine kidney	93.6	94.0	95.0	93.4	92.4	96.2	94.2	88.4	91.4	87.8
	(3.5)	(3.4)	(4.0)	(3.6)	(3.3)	(3.2)	(3.4)	(3.6)	(3.3)	(4.1)
Swine liver	94.2	91.0	93.0	92.4	91.0	93.2	91.4	87.2	89.4	89.0
	(2.5)	(4.6)	(3.0)	(3.7)	(3.2)	(3.0)	(4.7)	(4.4)	(3.8)	(4.8)
Bovine kidney	97.2	97.8	98.2	96.4	95.2	97.8	96.4	91.8	90.6	90.8
	(1.3)	(1.1)	(1.3)	(1.2)	(1.2)	(1.1)	(0.9)	(1.9)	(2.2)	(2.7)
Bovine liver	94.2	92.4	93.6	88.4	90.8	93.2	93.8	87.8	92.6	95.8
	(3.2)	(2.8)	(2.3)	(3.4)	(2.4)	(3.0)	(2.4)	(1.7)	(3.5)	(2.3)
Chicken liver	89.6	90.8	89.2	81.8	81.8	92.8	90.6	81.0	89.4	86.8
	(4.4)	(3.3)	(4.8)	(4.5)	(4.0)	(4.2)	(3.4)	(4.8)	(2.2)	(2.8)
Fortified at 0.1 μg/g										
Swine kidney	74.8	73.0	86.6	87.2	84.8	79.4	75.0	86.4	80.8	81.2
	(4.6)	(4.8)	(5.5)	(5.2)	(5.2)	(6.3)	(4.9)	(4.4)	(4.7)	(5.7)
Swine liver	75.4	74.0	82.6	80.0	75.6	72.0	80.6	79.8	78.0	78.8
	(5.1)	(4.6)	(4.9)	(6.8)	(4.8)	(6.5)	(6.0)	(5.6)	(6.6)	(7.0)
Bovine kidney	84.0	83.6	83.8	87.6	90.2	90.2	86.8	79.8	86.6	79.2
	(5.1)	(4.0)	(3.9)	(5.9)	(3.1)	(3.8)	(3.1)	(3.7)	(4.9)	(3.7)
Bovine liver	85.8	86.2	84.8	86.8	86.6	87.6	84.2	82.2	84.6	79.2
	(4.2)	(5.1)	(4.7)	(5.7)	(3.1)	(5.1)	(5.1)	(5.6)	(5.8)	(5.5)
Chicken liver	75.8	78.8	78.4	74.4	74.8	79.0	81.4	70.8	81.6	85.2
	(6.1)	(2.8)	(4.7)	(5.7)	(5.5)	(4.7)	(6.4)	(6.9)	(5.1)	(6.2)

[a] $n=5$, intra-assay variation.
[b] SDZ: sulfadiazine, SMR: sulfamerazine, SDD: sulfadimidine, SMPD: sulfamethoxypiridazine, SIZ: sulfisozole, SMMX: sulfamonomethoxine, SMX: sulfamethoxazole, SIX: sulfisoxazole, SDMX; sulfadimethoxine and SQ: sulfaquinoxaline.

of the extracts was achieved by our clean-up method mentioned above, without having interfering peaks in the chromatograms.

3.4. Application to the LC–MS–MS analysis

In order to investigate the applicability of the present method to LC–MS–MS analysis, we analysed a swine kidney sample and a bovine kidney sample obtained from a slaughterhouse, both samples of which were previously found to be positive for SAs by microbiological assay. Using our assay system presented here, we found that the swine kidney contained 0.48 μg/g of sulfadimidine (SDD) and that the bovine kidney contained 1.07 μg/g of sulfamonomethoxine (SMMX) as shown in Figs. 4(A) and 5(A), respectively. In addition, we were able to confirm residual SDD and SMMX in the swine and the bovine kidney samples by the tandem mass spectra of respective SAs taken at the top of corresponding peaks in the chromatograms, as shown in Figs. 4(B) and 5(B). The $[M+H]^+$ and product ions corresponding to the structures of both SAs were clearly observed at m/z 279, 186, 156, and 92 for SDD and m/z 281, 188, 156, and 92 [16,17], respectively, confirming the identity of SDD and SMMX. These results clearly demonstrated the applicability of the present method to LC–MS–MS analysis.

4. Conclusions

A method for the determination of residual SAs in animal liver and kidney was established using a combination of clean-up on a Bond Elut PSA cartridge and HPLC with UV. The present method is not only simple, rapid, and reliable, but also permits

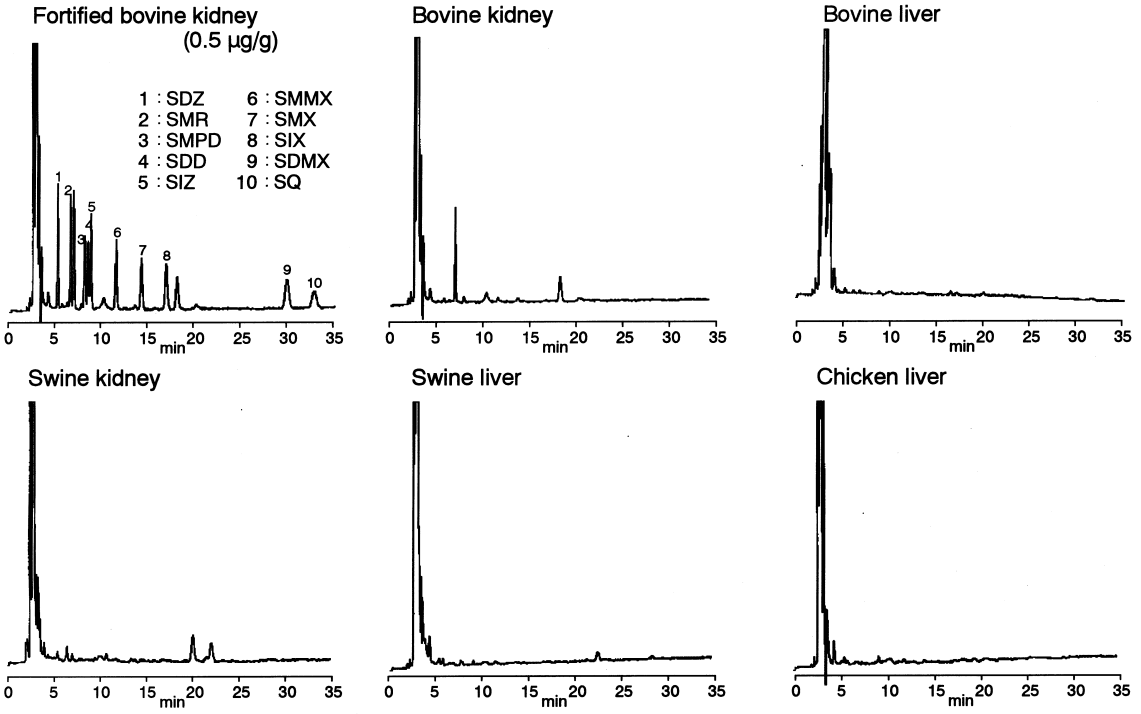

Fig. 3. Typical HPLC chromatograms of various animal kidneys and livers, Conditions: See Experimental.

the simultaneous determination of ten different kinds of SAs with good recoveries (over 70%), relative standard deviation (less than 7.0%), and detection limits (0.03 μg/g). Furthermore, it is applicable to a direct LC–MS–MS analysis. Accordingly, wide spread use of the method presented here is strongly

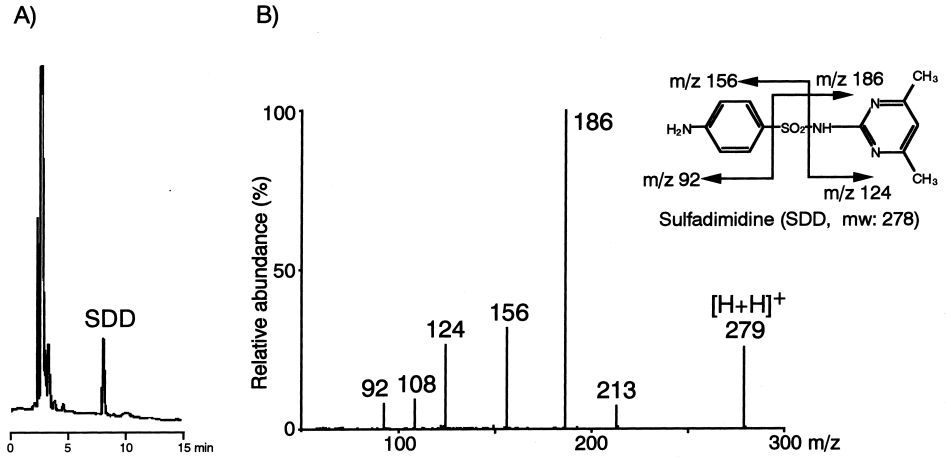

Fig. 4. ESI LC–MS–MS analysis of residual SDD at concentration of 0.48 μg/g in swine kidney: (A) Monitored at UV 272 nm; (B) ESI MS–MS spectrum of the residual SDD, Conditions: See Experimental.

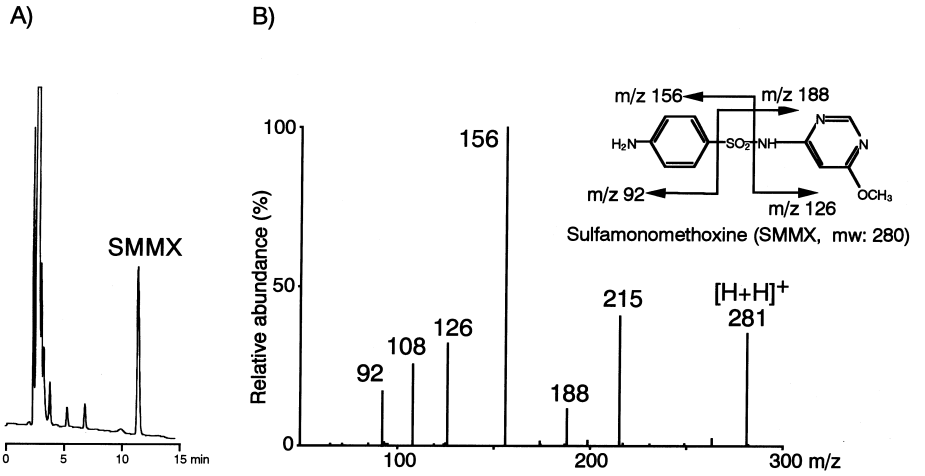

Fig. 5. ESI LC–MS–MS analysis of residual SMMX at concentration of 1.07 μg/g in bovine kidney: (A) Monitored at UV 272 nm; (B) ESI MS–MS spectrum of the residual SMMX, Conditions: See Experimental.

recommend for the routine determination of residual SAs in animal liver and kidney.

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