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Matrix solid-phase dispersion extraction and high-performance liquid chromatographic determination of residual sulfonamides in chicken

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Abstract

Simultaneous determination of the six sulfonamides (SAs) sulfadiazine, sulfadimidine, sulfamonomethoxine, sulfamethoxazole, sulfadimethoxine and sulfaquinoxaline in chicken using matrix solid-phase dispersion (MSPD) with neutral aluminium oxide as an MSPD sorbent and high-performance liquid chromatography (HPLC) is presented. In the present MSPD, six SAs could be isolated by only one step, elution with a 70% (v/v) aqueous ethanol solution, without the sorbent conditioning and the sorbent–tissue matrix washing. For the HPLC determination, a LiChrospher 100 RP-8 and a mixture of 1% acetic acid solution (pH 3.0, in water)–acetonitrile–*N*,*N*-dimethylformamide (78:22:5, v/v/v) as the mobile phase with a photodiode array detector were used. Average recoveries were greater than 87.6% with relative standard deviations between 0.5 and 8.6%. The total time and amount of solvent required for the analysis of one sample were <1.5 h and <12 ml, respectively.

Keywords: Matrix solid-phase dispersion; Extraction methods; Food analysis; Sulfonamides

1. Introduction

Sulfonamides (SAs) are regularly used by veterinarians in chickens for therapeutic, prophylactic, or growth-promoting purposes. Use of SAs in chickens may result in SA residues being present in the marketed tissues if the adequate withdrawal times for the chickens have not been observed or if these drugs have been improperly administered. To ensure the safety of food to the consumers, the European Union (EU) has established maximum residue limits (MRLs) for SAs in foods of animal origin at 0.1 ppm [1].

An analytical method for routine monitoring of SA residues in chickens must be precise, simple, economical on cost and time to permit monitoring of large number of samples, and capable of detecting the residues below MRLs. For the determination of SAs in animal tissues by high-performance liquid chromatography (HPLC), more effective extraction and deproteinization is required. At present, discharging the waste of toxic organic solvents is a severe problem on a world scale. The extraction/clean-up procedure should avoid the use of toxic solvents and reagents [2–4].

To improve the problem of classical extraction and

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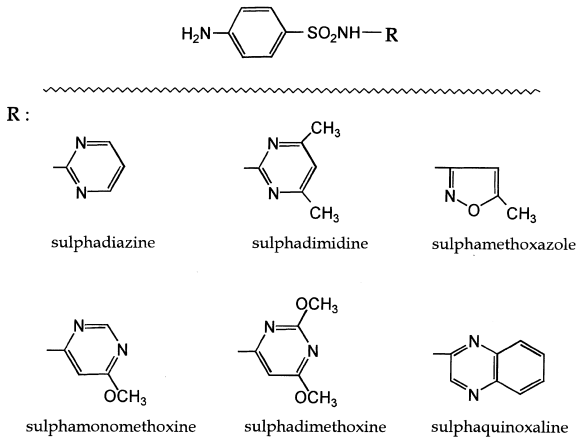

Fig. 1. Structures of sulfonamides. R: Substituent.

deproteinization techniques which involve numerous analytical steps and extensive use of organic solvents, some researchers have applied matrix solid-phase dispersion (MSPD) using a non-polar sorbent, C_{18} (C_{18}-MSPD). This technique has also been used for the extraction of SAs in animal tissues [5–10], however the recoveries were sometimes low and variable.

The aim of this study is to develop a simplified procedure for simultaneous HPLC determination of six SAs (Fig. 1) in chicken muscle tissues, which uses less toxic solvents. This paper presents: (1) using the polar sorbents and the above non-polar sorbent (C_{18}) as the MSPD sorbent, an optimum MSPD is determined; (2) the simultaneous determination of the SAs by the isocratic HPLC system.

2. Experimental

2.1. Materials and reagents

Chicken muscle tissues, which were purchased from local food markets, served as samples and were deep-frozen until analysis. Acetonitrile, distilled water, ethanol, *N*,*N*-dimethylformamide (DMF) (HPLC grade), hexane (residual pesticide grade), and acetic acid (analytical chemical grade) were obtained from Wako (Osaka, Japan). Six SA standards [sulfadiazine (SDA), sulfadimidine (SDD), sulfamonomethoxine (SMM), sulfamethoxazole (SMX), sulfadimethoxine (SDM), and sulfaquinoxaline (SQ)] were obtained from Wako or Sigma (St. Louis, MO, USA). Respective stock standard solutions of SAs, were prepared by accurately weighing SDA, SDD, SMM, SMX, SDM, and SQ (10 mg) and dissolving in ethanol (100 ml). Working mixed standard solutions of these six SAs were prepared by diluting the stock solutions with ethanol. These solutions can be kept at 4°C for up to 1 month.

Six polar sorbents for the normal-phase MSPD were used: three types of aluminium oxide 90 (activity I, 70–230 mesh), alumina active acidic (alumina A), active basic (alumina B), active neutral (alumina N) (Merck, Darmstadt, Germany); alumina active neutral super I (activity super I, 70–200 mesh) (Alumina N-S) (ICN Biomedicals, Eschwege, Germany); silica gel 60, (70–230 mesh) (silica) (Merck); Florisil PR, (60–100 mesh) (Florisil) (Wako) were used. Silica and Florisil were pre-heated at 200°C for 3 h and 140°C for 12 h, respectively, and cooled in a desiccator and stored in a sealed bottle until analysis. A non-polar sorbent for the reversed-phase, Wakosil 40 C_{18} (Wako) (C_{18}) was pre-washed twice with hexane and dried at 45°C.

2.2. Apparatus

The following apparatus were used in the sample preparation: vacuum manifold for solid-phase extraction (SPE), VacMaster 10 (International Sorbent Technology, Mid Glamorgan, UK); homogenizer, Model NS-50 (Microtec, Chiba, Japan); rotary evaporator, Model EYELA N-N (Tokyo Rikakiki, Tokyo, Japan); 0.45 μm disposable syringe filter unit, Millex-LH low protein binding hydrophilic LCR (PTFE) membrane (Millipore, Bedford, MA, USA).

Five silica-based reversed-phase columns (5 μm, 250×4.6 mm I.D.) with their guard columns (5×4.6 mm I.D.) (LiChrospher 100 RP-18 and LiChrospher 100 RP-8) (Mightysil RP-18 GP and Mightysil RP-18 GP *Aqua*) were obtained from Merck or Kanto (Tokyo, Japan).

Analyses of standard and extracted SAs were conducted using a Jasco HPLC system (Model PU-980 pump and DG-980-50 degasser) (Jasco, Tokyo, Japan) equipped with an SPD-M10A_{vp} diode array detector (Shimadzu, Kyoto, Japan) interfaced with a

Fujitsu FMV-5133D7 personal computer (Fujitsu, Tokyo, Japan).

The separation was performed on a LiChrospher 100 RP-8 with a guard column using a mixture of 1% acetic acid solution (pH 3.0, in water)–acetonitrile–DMF (78:22:5, v/v/v) as the mobile phase at a flow-rate of 1.0 ml/min at an ambient temperature.

2.3. Procedure

Chicken muscle tissues samples were cut into pieces and blended. An accurately weighed 0.5 g amount of the sample was placed in a porcelain mortar (external diameter 90 mm). A 2-g amount of alumina N-S was added to the mortar and gently ground with the sample using a pestle to obtain a homogeneous material. The mixture was transferred to a 15-ml syringe barrel pre-plugged with a filter disc, and the barrel was then placed on a vacuum manifold. Flow was controlled at 0.3 ml/min. Six SAs were eluted with 10 ml of a 70% (v/v) aqueous ethanol solution. The eluate was evaporated to dryness, and the residue was dissolved in 1 ml of the HPLC mobile phase. The resulting solution was filtered through a 0.45 μm disposable syringe filter unit. A 10 μl volume of the filtrate was injected into the HPLC system.

2.4. Recovery test

The recoveries of six SAs from chicken muscle samples fortified at 0.05, 0.1, 0.2, 0.5, and 1.0 ppm were determined. These fortification concentrations were prepared by adding 50 μl of five mixed standard solutions of SAs (0.5, 1, 2, 5, and 10 μg/ml, respectively) to a separated 0.5 g portion of the sample.

3. Results and discussion

3.1. HPLC operating conditions

Nevado et al. [11] previously reported that SAs could be determined by HPLC using a C_{18} column and the low-pH environment of the mobile phase. In preliminary experiments, when similar HPLC conditions were used, SMM and SMX, or SDM and SQ could not be separated. We therefore tested a C_8 column and a mixture of acid solution and acetonitrile or ethanol as the mobile phase, which were compared with respect to the separation of the six SAs, and of SDA from the interfering peaks. It was difficult to separate SMM and SMX with the mobile phase of ethanol–acid solution, and to separate SDA from the interference originating in the resulting extract. The best chromatogram with complete separation of all target compounds and interfering peaks with clear/short retention time was obtained on the C_8 column with an isocratic mobile phase of 1% acetic acid solution (pH 3.0, in water)–acetonitrile–DMF (78:22:5, v/v/v). The peaks of SMM and SMX, and of SDM and SQ were separated by increasing the concentrations of DMF in the mobile phase.

Using a photodiode array detector, absorption spectra of SDA, SDD, SMM, SMX, SDM, and SQ standards in the mobile phase were measured for the selection of the HPLC monitoring wavelength. The measurement was conducted at 267 nm which gave an average maximum absorbance for all of the SAs. The minimum detectable amounts (signal-to-noise ratio$>$5) were 0.09 ng for SDA, 0.12 ng for SDD, 0.17 ng for SMM, 0.20 ng for SMX, 0.33 ng for SDM, and 0.36 ng for SQ. The target compounds were successfully detected within 16 min when the flow-rate was 1.0 ml/min.

3.2. Sample preparation

Previous C_{18}-MSPD methods require methylene chloride, acetonitrile, or methanol, as the eluting solvent. According to the Swiss Toxicity Classification [12], the above solvents are handled as toxic solvents (i.e., poison class=very strong toxin). In contrast, the influence of ethanol on the environment and humans is negligible (poison class=not subject to toxicity). On the other hand, some studies showed that a commercial normal-phase (pre-packed alumina A or N) cartridge or the column chromatography using alumina B as the packing material was suitable for clean-up of residual SAs in animal products [13–16]. Therefore, six types of polar sorbents were examined for the normal-phase MSPD in the present study. Ethanol was used as an eluting solvent, and the sorbents were deactivated by adding water to the

eluent. The material mixture of a 2 g of sorbent and a 0.5 g of fortified (=0.5 ppm for each drug) chicken muscle sample were used.

Table 1 presents the effect of ethanol concentration in the eluting solvent on the recoveries of SAs from the MSPD examined here. The elution volume was standardized at 10 ml. Of each MSPD, the eluents, ranging 100–70% (v/v) ethanol concentrations in water, were applied to the syringe barrel consecutively starting with 100% ethanol. The target compounds in 10 ml of each eluted fraction were determined by HPLC. The better eluents that recovered all compounds from the six polar sorbents were: a 70% (v/v) ethanol solution for alumina N-S, alumina N, and alumina B; a 90% ethanol solution for alumina A and Florisil; 100% ethanol for silica. The alumina N-S and silica sorbents gave good recoveries (>80%) of all the target compounds simultaneously under the conditions. The alumina N-S showed better precision (RSD) for each SA than the silica (Table 1). The silica needed to make it activity I by pre-heating at 200°C for 3 h, but the alumina N-S could be used directly without pre-heating. The alumina N-S was therefore used as a MSPD sorbent (Table 1).

In the case of C_{18}-MSPD with 100% ethanol as the eluent, low recoveries for SMM, SMX, SDM, and SQ were obtained (Table 1). Since the best solvent that facilitates retention due to non-polar interaction is water, the recoveries of all SAs from C_{18}-MSPD are decreased by increasing concentrations of water in the eluent. No further study was performed.

C_{18}-MSPD techniques for the extraction of SAs in animal tissues has required sorbent conditioning with hexane (washing solvent) followed by methylene chloride (the elution solvent) and C_{18}–tissue matrix washing with hexane to remove lipid materials. With the proposed MSPD method, all six SAs could be isolated by only one step [the elution procedure with a 70% (v/v) aqueous ethanol solution] without sorbent conditioning and sorbent–tissue matrix washing to remove the interfering compounds. Moreover, no use of toxic solvents was achieved. The elution from the muscle tissue dispersed evenly onto the alumina N-S sorbent with a 70% (v/v) aqueous ethanol solution isolates six SAs and, under this condition, interfering compounds of the sample origin remain on the sorbent. This is borne out by the fact that the HPLC trace of blank chicken muscle extract (Fig. 2A) is free from interfering compounds.

Fig. 2 shows representative HPLC chromatograms of blank and fortified (0.1 ppm) chicken muscle samples obtained under the established method. The resulting extracts were free from interference. Target compounds could be successfully separated within 16 min. These findings demonstrate that the extraction and the HPLC condition worked well. The present method made it unnecessary to use a gradient HPLC system to improve the separation and did not require ''pre-column washing'' after analysis.

3.3. Recoveries, calibration and identification

Table 2 summarizes the average recoveries from known negative chicken muscle samples at five different fortification levels (0.05, 0.1, 0.2, 0.5, and 1 ppm), correlation coefficients of calibration graphs, and inter-/intra-assay variabilities of the SAs isolated.

Average recoveries were greater than 87.6% with RSDs between 0.5 and 8.6% ($n=5$). These results are much better than those for previous methods [5,6]. The calibration graphs were generated by plotting peak heights obtained from fortified samples at levels ranged 0.05–1 ppm and passed through the origin (slopes: 6.36 for SDA, 6.20 for SDD, 4.13 for SMM, 4.64 for SMX, 2.08 for SDM, and 3.10 for SQ). The correlation coefficients for the six SAs were statistically highly significant ($P<0.01$). Inter- and intra-assay variabilities ranged from 4.0 to 5.5% and 1.0–3.3%, respectively, indicating that the proposed method has high precision.

The limit of detection (LOD) and limit of quantitation (LOQ) for the target compounds were calculated in accordance with the CCMAS 1993 (Codex Committee for Methods Analyses and Sampling). Based on the peak heights in HPLC chromatograms obtained from blank and fortified samples, LOD was defined as the average background of samples (=fluctuations of the baseline) plus three times the SD and LOQ was defined as the average background of samples plus 10 times the SD. In a practical analysis for the residue monitoring, the LOQs for the six compounds ranged from 0.006 to 0.04 ppm. These LOQs were well below the MRL (=0.1 ppm).

Table 1
Effect of the ethanol concentration in the eluent and on the recoveries of sulfonamides from MSPD systems

Sorbent	Drug	Recovery (%)				
		Ethanol (%) in the eluent (ethanol–water, v/v)				
		100	90	80	70	Better eluent[a]
Alumina N-S	SDA	10	28	55	ND[b]	92 (5)
	SDD	60	41	5	5	112 (7)
	SMM	40	16	50	12	117 (6)
	SMX	20	33	38	ND	90 (7)
	SDM	21	29	35	ND	85 (10)
	SQ	34	53	5	ND	90 (2)
Alumina N	SDA	11	18	45	15	89 (29)
	SDD	52	48	4	4	103 (2)
	SMM	24	14	33	26	96 (8)
	SMX	14	22	40	ND	77 (8)
	SDM	14	23	62	ND	99 (30)
	SQ	32	55	10	ND	93 (15)
Alumina A	SDA	75	12	ND	ND	87 (9)
	SDD	91	ND	ND	ND	91 (5)
	SMM	70	16	ND	ND	86 (11)
	SMX	59	ND	ND	ND	59 (39)
	SDM	36	ND	ND	ND	36 (74)
	SQ	62	ND	ND	ND	62 (32)
Alumina B	SDA	13	13	57	9	89 (7)
	SDD	42	42	7	4	92 (6)
	SMM	22	16	49	ND	87 (4)
	SMX	13	13	37	ND	62 (7)
	SDM	9	13	8	ND	29 (22)
	SQ	25	28	9	ND	62 (5)
Silica	SDA	94	ND	ND	ND	94 (8)
	SDD	89	ND	ND	ND	89 (10)
	SMM	91	ND	ND	ND	91 (9)
	SMX	94	ND	ND	ND	94 (9)
	SDM	89	ND	ND	ND	89 (10)
	SQ	96	ND	ND	ND	96 (4)
Florisil	SDA	66	42	ND	ND	108 (5)
	SDD	95	6	ND	ND	101 (5)
	SMM	62	10	ND	ND	69 (13)
	SMX	84	17	ND	ND	101 (1)
	SDM	72	8	ND	ND	77 (13)
	SQ	91	13	ND	ND	100 (2)
C_{18}	SDA	85	NT[c]	NT	NT	85 (5)
	SDD	80	NT	NT	NT	80 (1)
	SMM	50	NT	NT	NT	50 (19)
	SMX	44	NT	NT	NT	44 (15)
	SDM	32	NT	NT	NT	32 (19)
	SQ	38	NT	NT	NT	38 (26)

Data are averages ($n=3$). Values in parentheses are relative standard deviations. A 0.5 g amount of fortified chicken muscle sample (0.5 ppm for each drug) was applied to the MSPD system. The volume of eluent was uniform, 10 ml.

[a] A 70% (v/v) ethanol solution for alumina N-S, alumina N, and alumina B; a 90% ethanol solution for alumina A and Florisil; 100% ethanol for silica.

[b] Not detected.

[c] Not tried.

Table 2
Recoveries of sulfonamides from chicken muscle tissues

Fortification level (ppm)	Recovery (%) (mean±SD, n=5)					
	SDA	SDD	SMM	SMX	SDM	SQ
0.05	97.7±2.2	96.6±4.0	93.5±4.3	104.1±6.9	95.1±4.8	97.0±3.9
0.1	108.6±6.0	105.7±4.3	108.1±7.5	91.6±2.9	108.7±6.1	117.5±8.5
0.2	90.5±6.8	100.2±4.9	95.4±2.4	93.1±5.5	88.0±6.9	87.6±6.7
0.5	99.7±3.6	99.2±3.0	93.0±3.0	92.2±3.0	98.0±6.9	101.6±2.6
1.0	94.7±7.1	103.7±4.2	93.6±8.1	100.8±5.8	96.9±0.4	102.0±3.0
Correlation coefficient	0.999	0.999	0.999	0.999	0.996	0.999
Inter-assay variability (%±SD)	5.3±2.3	4.0±0.6	5.2±2.5	5.0±1.6	5.5±2.7	4.8±2.4
Intra-assay variability (%, n=5)	2.5	1.0	2.8	3.3	1.5	2.1
LOD[a] (ppm)	0.003	0.005	0.006	0.009	0.016	0.012
LOQ[b] (ppm)	0.006	0.010	0.012	0.022	0.038	0.025

[a] LOD=Limit of detection.
[b] LOQ=Limit of quantitation.

A photodiode array for HPLC analysis gives spectral information and is an easy way to confirm peak identity. HPLC system equipped with the diode array proved to be able to ensure identification of the compounds. The six SAs examined here could be identified in the chicken muscle sample with their retention times and absorption spectra. The spectra of SAs obtained from sample are practically identical with those of the standard. The present sample preparation allowed a reliable confirmation.

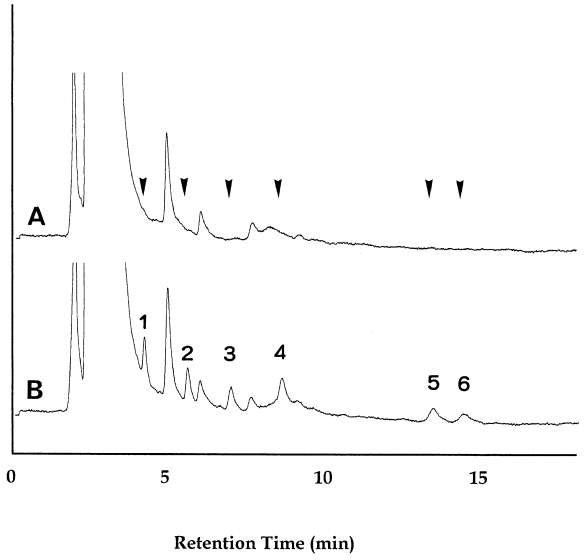

Fig. 2. Representative HPLC chromatograms obtained from chicken muscle samples (photodiode array detector set at 267 nm): (A) blank chicken muscle tissue, (B) fortified (0.1 ppm for each drug) chicken muscle tissue. Peaks: 1=sulfadiazine (SDA, t_R=4.3 min); 2=sulfadimidine (SDD, t_R=5.6 min); 3=sulfamonomethoxine (SMM, t_R=7.0 min); 4=sulfamethoxazole (SMX, t_R=8.7 min); 5=sulfadimethoxine (SDM, t_R=13.6 min); 6=sulfaquinoxaline (SQ, t_R=14.5 min).

The proposed MSPD–HPLC method for the simultaneous determination of SDA, SDD, SMM, SMX, SDM, and SQ in chicken muscle tissues offers shorter analysis time (total <1.5 h per sample), low organic solvent consumption (total<12 ml per sample), high precision (RSD<9% in the recovery test). These findings demonstrate that this method is useful for the routine residue monitoring of these compounds in chicken muscle tissues.

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