Determination of Sulfonamide Residues in Eggs by Liquid Chromatography

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A method was developed for determining residual sulfonamide antibacterials such as sulfamethazine (SMZ), sulfamonomethoxine (SMM), sulfadimethoxine (SDM), and sulfaquinoxaline (SQ) in eggs using liquid chromatography with a photodiode array detector. The spiked and blank samples were cleaned up by using an Ultrafree®-MC/PL centrifugal ultrafiltration unit. A Mightysil® RP-4 GP column and a mobile phase of 28% (v/v) ethanol–H2O with a photodiode array detector were used for the determination. Average recoveries from eggs spiked with each drug at 0.1, 0.2, 0.4, and 1.0 ppm were ≥80.9%, with relative standard deviations between 1.3 and 4.7%. The limits of quantitation were 0.060 ppm for SMZ, 0.045 for SMM, 0.044 for SDM, and 0.093 for SQ. The analysis of one sample required <30 min and <5 mL ethanol as solvent.

Sulfonamides (SAs) are regularly used by veterinarians for therapeutic, prophylactic, or growth-promoting purposes in chickens. In Japan, sulfamethazine (SMZ), sulfamonomethoxine (SMM), sulfadimethoxine (SDM), and sulfaquinoxaline (SQ) are mainly used for prevention or treatment of poultry leucocytozoonosis and coccidiosis, and are generally coadministered in feed. Use of SA-supplemented feed in laying hens may result in SA residues in marketed eggs if adequate withdrawal times for laying hens have not been observed or if these drugs have been improperly administered.

Eggs are a very important food because they are highly nutritious, inexpensive, and readily available. However, evidence has implicated SMZ as a possible carcinogen (1), which has magnified risk concerns because of its possible presence as a residue in eggs. An analytical method for routine monitoring of SA residues in eggs must be precise, simple, economical in both cost and time to permit monitoring of large number of samples, while reducing or eliminating toxic wastes for disposal in the environment. Because discharging the waste of toxic organic solvents is a severe problem, the use of toxic solvents and reagents should be avoided (2–4).

The difficulties in drug analyses in eggs are caused by formation of an emulsion that hinders recovery of residues, or by interfering co-extracts, which require many cleanup steps. For determination of target compounds in eggs using liquid chromatography (LC), more effective extraction and deproteinization are required. To improve the problem of classical extraction and deproteinization techniques which involve numerous analytical steps and extensive use of organic solvents, some researchers have recently applied the matrix solid-phase dispersion (MSPD) first developed in 1989 (5). This technique has also been used to extract SMZ, SMM, SDM, or SQ from animal tissues (6–10) and SDM from catfish tissues (11), but the recoveries were sometimes low and variable. Pensabene et al. (12) described a technique for extraction of SAs from eggs using supercritical fluid extraction (SFE) with supercritical CO2. However, the sample preparation followed by SFE was complicated and time-consuming. Previous methods (5–12) have required toxic solvents, e.g., methylene chloride, acetonitrile, and methanol, for the extraction or the LC mobile phase, which may be harmful to the environment.

This study describes a simplified sample preparation procedure for determining residual SMZ, SMM, SDM, and SQ in laying hen’s eggs, which uses fewer toxic solvents. Determination is performed by LC equipped with a photodiode array detector, which measures retention time and absorption spectrum simultaneously and allows instant identification of residues.

Experimental

Apparatus

(a) LC system.—JASCO Model PU-980 pump and DG-980-50 degasser (Jasco Corp., Tokyo, Japan) equipped with SPD-M10Avp diode array detector (Shimadzu, Kyoto, Japan) interfaced with a Fujitsu FMV-5133D7 personal computer (Fujitsu, Tokyo, Japan). Operating conditions: analytical column, Mightysil® RP-4 GP (end-capped; 5 μm), column (250 × 4.6 mm id; Kanto Chem. Co., Inc., Tokyo, Japan) with a guard column (5 × 4.6 mm id; Kanto Chem.;) mobile phase, 28% (v/v) ethanol–H2O; flow rate, 0.8 mL/min; column temperature, ambient; injection volume, 20 μL.

(b) Homogenizer.—Model PH-91-1 (processing quantities: 5–30 mL; Mitsui Denki-Seiki, SMT Co., Chiba, Japan).

(c) Microcentrifuge.—Biofuge® fresco (Kendo Lab. Products, Hanau, Germany).

(d) Centrifugal ultrafilter units.—Three membrane types of Ultrafree®-MC series (nominal molecular weight limit
Reagents and Materials

(a) Ethanol.—LC grade (Wako Pure Chem. Ltd., Osaka, Japan).

(b) Distilled water.—LC grade (Wako).

c Veterinary drug standards.—SMZ, SMM, SDM, and SQ (Sigma Chemical Co., St. Louis, MO).

d SA standard solutions.—(1) Stock standard solutions.—500 ppm. Weigh 10 mg of each sulfonamide standard into separate weighing dishes (weights corrected for assayed content) and transfer with LC mobile phase into separate 20 mL volumetric flasks. Dilute to volume with distilled water and mix until dissolved. (2) Mixed working standard solutions (containing 4 SAs).—100 ppm. Pipet 2 mL each stock standard solution into one 10 mL volumetric flask, dilute to volume with distilled water, and mix. Dilute mixed solution to 40, 20, and 10 ppm with distilled water and use these mixed solutions of 4 concentrations as working solutions. These solutions are stable for up to 1 month if stored in a refrigerator.

e Sample extractant.—50% (v/v) ethanol–H₂O.

(f) Egg sample.—Homogenized whole egg.

Procedure

An accurately weighed 2.0 g egg sample was homogenized with 4.0 mL 50% (v/v) ethanol–H₂O for 5 min. Approximately 1 g homogenate was immediately transferred into a preweighed microcentrifuge tube and weighed again. The sample weight was calculated as \( = \) (homogenate + tube) – tube, and centrifuged at 10,000 \( \times g \) for 5 min. A 0.4 mL portion of supernatant liquid was placed in an Ultrafree-MC/PL and centrifuged at 2000 \( \times g \) for 5 min. The ultrafiltrate was injected into the LC system.

Recovery Test

The recoveries of SAs from blank egg samples spiked at 0.1, 0.2, 0.4, and 1.0 ppm were determined. These fortification concentrations were prepared by adding 20 \( \mu L \) of 4 mixed standard solutions of the above drugs (10, 20, 40, and 100 ppm, respectively) to 2.0 g portions of sample. Fortified (all SAs) samples were mixed prior to workup.

In the test, relative standard deviations (RSDs) determined for each spiked concentration were then averaged, which resulted in a mean ± standard deviation (SD). This was defined as interassay variability. Intra-assay variability was defined as the RSD for the mean of 5 replicates of the same sample and represents the variability associated with the analytical procedure used.

Table 1. Comparison of recoveries of SAs from Ultrafree®-MCs

<table>
<thead>
<tr>
<th>Membrane type</th>
<th>SMZ</th>
<th>SMM</th>
<th>SDM</th>
<th>SQ</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ultrafree-MC/Biomax</td>
<td>94.2 (1.8)</td>
<td>95.1 (2.0)</td>
<td>92.8 (1.6)</td>
<td>90.5 (2.0)</td>
</tr>
<tr>
<td>Ultrafree-MC/PL</td>
<td>93.5 (1.0)</td>
<td>96.0 (1.4)</td>
<td>93.5 (1.4)</td>
<td>92.3 (1.6)</td>
</tr>
<tr>
<td>Ultrafree-MC/PT</td>
<td>80.4 (2.3)</td>
<td>87.3 (2.6)</td>
<td>82.3 (2.2)</td>
<td>79.8 (1.9)</td>
</tr>
</tbody>
</table>

* Data are averages \((n = 5)\). Values in parentheses are relative standard deviations (RSD). The extract from an egg was fortified (1.0 ppm of each drug) with a mixed standard solution of SMZ, SMM, SDM, and SQ and applied to the ultrafilter unit.
Results and Discussion

**LC Operating Conditions**

To reduce the amount of toxic solvents required, we used ethanol as a solvent in the extraction and the LC mobile phase. Acetonitrile and methanol are usually used in the mobile phase for the reversed-phase LC analysis of various compounds, including SAs. According to the Swiss toxicity classification (13), these organic solvents are handled as toxic solvents, e.g., acetonitrile: poison class = very strong toxin; LD$_{50}$ oral 200 mg/kg. Methanol is also a toxin. In contrast, the influence of ethanol on the environment and on humans is negligible (poison class = not subject to toxicity).

The absorption spectra of SMZ, SMM, SDM, and SQ standard solutions were measured by a photodiode array detector. The obtained maximum absorptions were SMZ, 266 nm; SMM, 273 nm; SDM, 268 nm; SQ, 267 nm. The wavelength for monitoring the SAs was adjusted to 269 nm, which is an average maximum for all the compounds. The target compounds were detected within 8.40 min (Figure 1C). The solvent (ethanol) consumption per sample was <3 mL.

To determine the effect of ethanol concentrations in the extraction solution on the recoveries of SAs and the forming of emulsions, concentrations over the range of 0–50% were tested; 30% of ethanol gave favorable results.

**Sample Preparation**

The recoveries of SAs from the 3 types of Ultrafree-MC centrifugal filters were examined and compared. Egg extract processed with 50% (v/v) ethanol–H$_2$O was used in this study. The obtained maximum absorptions were SMZ, 266 nm; SMM, 273 nm; SDM, 268 nm; SQ, 267 nm. The wavelength for monitoring the SAs was adjusted to 269 nm, which is an average maximum for all the compounds. The target compounds were detected within 8.40 min (Figure 1C). The solvent (ethanol) consumption per sample was <3 mL.

To determine the effect of ethanol concentrations in the extraction solution on the recoveries of SAs and the forming of emulsions, concentrations over the range of 0–50% were tested; ≥30% of ethanol gave favorable results.

**Table 2. Average recoveries, correlation coefficients ($r$) of standard graphs, inter- and intra-assay variabilities for SAs-fortified eggs**

<table>
<thead>
<tr>
<th>Spiked, ppm</th>
<th>SMZ</th>
<th>SMM</th>
<th>SDM</th>
<th>SQ</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1</td>
<td>80.9 (4.7)</td>
<td>85.2 (3.4)</td>
<td>81.9 (4.4)</td>
<td>86.2 (2.1)</td>
</tr>
<tr>
<td>0.2</td>
<td>81.1 (2.8)</td>
<td>84.8 (2.1)</td>
<td>85.5 (3.4)</td>
<td>88.8 (1.7)</td>
</tr>
<tr>
<td>0.4</td>
<td>85.2 (2.1)</td>
<td>86.4 (2.5)</td>
<td>88.8 (1.6)</td>
<td>86.4 (3.0)</td>
</tr>
<tr>
<td>1.0</td>
<td>90.2 (1.3)</td>
<td>86.9 (1.7)</td>
<td>89.6 (1.9)</td>
<td>89.7 (1.7)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Standard graph</th>
<th>$r$ ($n = 4$) $^{a}$</th>
<th>$r$ ($n = 4$) $^{a}$</th>
<th>$r$ ($n = 4$) $^{a}$</th>
<th>$r$ ($n = 4$) $^{a}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Assay variability, %</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Inter- ($n = 4$)</td>
<td>2.7 ± 1.3</td>
<td>2.4 ± 0.6</td>
<td>2.8 ± 1.1</td>
<td>2.1 ± 0.5</td>
</tr>
<tr>
<td>Intra- ($n = 5$)</td>
<td>2.8</td>
<td>2.5</td>
<td>2.1</td>
<td>3.8</td>
</tr>
</tbody>
</table>

$^{a}$ Data are expressed as averages ($n = 5$). Values in parentheses are RSD.

$^{b}$ Mean of 5 determinations using spiked samples for standard curves; range of concentration was 0.1–1.0 ppm (4 points).

$^{c}$ Data are expressed as average ± standard deviation (SD); $n$ values = number of replicates.

**Calibration**

The calibration graphs obtained by plotting peak area against drug concentration were linear over ranges of 0.02–1.0 ppm (injection volume: 0.4–20 ng) for SMZ, 0.01–1.0 ppm (0.2–20 ng) for SMM and SDM, and 0.03–1.0 ppm (0.6–20 ng) for SQ, and passed through the origin. The correlation coefficients ($r$), ranging from 0.999 to 0.998, were highly significant statistically ($p < 0.01$). The minimum detectable drug concentrations were 0.02 ppm for SMZ, 0.01 ppm for SMM and SDM, and 0.03 ppm for SQ. The precision of the LC procedure was obtained from RSD of areas calculated for 10 replicate injections of 0.1 ppm of each target compound. RSD values between 0.9 and 1.3% were obtained.
Recovery, Variability, and Limit of Quantitation

Table 2 summarizes the recoveries from egg samples at 5 different spiking levels (0.1, 0.2, 0.4, and 1.0 ppm for each drug), correlation coefficients of standard graphs, and inter- and intra-assay variabilities of 4 SAs isolated from spiked egg samples. Average recoveries were >80.9%, with RSDs between 1.3 and 4.7%. Inter- and intra-assay variabilities ranged from 2.1 to 3.8%. The spiked recovery graphs were generated by plotting peak areas of fortified sample extracts ranging from 0.1 to 1.0 ppm. The recovery graph was constructed from 5 points, and each point represented the mean of the 5 injections. The resulting correlation coefficients (r) for all SAs were highly significant statistically (p <0.01; Table 2). For SMZ, SMM, SDM, and SQ, respectively, the spiked recovery graph and its standard (in aqueous solution) graph were pooled statistically, indicating that the slope of the spiked recovery graph was similar to that of the standard. The calibration was performed with a simple procedure using standards. The data in Table 2 indicate that the present method is precise and accurate.

To properly characterize the practical residue monitoring, the limit of detection (LOD) and limit of quantitation (LOQ) for target compounds were calculated in accordance with the CCMAS 1993 (Codex Committee for Methods Analyses and Sampling) and the European Commission’s Decision 93/256/EEC. Based on the peak areas in LC chromatograms, LOD is defined as the average background plus 3 times the SD. LOQ is defined as the average background plus 10 times the SD. Four different blank egg samples were analyzed in duplicate. The LOQs of 4 SAs ranged from 0.044 to 0.093 ppm (Table 3). To ensure the safety of food to consumers, the Joint Expert Committee for Food Additives [JECFA, Codex Alimentarius Commission in Food Agricultural Organization/World Health Organization (FAO/WHO)], European Union (EU), and the U.S. Food and Drug Administration (FDA) have established a maximum residue limit (MRL) or tolerance for SAs in foods of animal origin to 0.1 ppm (1, 14–16). The LOQs in this study were below the MRL and tolerance levels established.

Identification

In LC analysis for residual drug monitoring, a photodiode array gives spectral information and easily confirms peak identity. LC combined with the diode array system detected a wide range of molecules and ensured identification of the target compounds. The retention time and spectrum allowed peak identification. The SAs examined were identified in the egg 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 reliable confirmation. The typical spectra of the SMM and SDM peaks of egg obtained with the diode array detector are given in Figure 2.

The main characteristics of the proposed procedure were shorter analytical time (total < 30 min per sample); highly precise (RSD ≤ 3.8% in the recovery test); no toxic solvents used; and low solvent consumption (total solvent consumption <5 mL ethanol per sample). Therefore, this procedure is useful for routine residue monitoring of SMZ, SMM, SDM, and SQ in eggs.

References


Table 3. Limits of detection (LOD) and quantitation (LOQ) for SAs in eggs

<table>
<thead>
<tr>
<th></th>
<th>SMZ</th>
<th>SMM</th>
<th>SDM</th>
<th>SQ</th>
</tr>
</thead>
<tbody>
<tr>
<td>LOD, ppm</td>
<td>0.035</td>
<td>0.027</td>
<td>0.024</td>
<td>0.066</td>
</tr>
<tr>
<td>LOQ, ppm</td>
<td>0.060</td>
<td>0.045</td>
<td>0.044</td>
<td>0.093</td>
</tr>
</tbody>
</table>

Figure 2. Typical absorption spectra of peaks at 6.60 min (A, for SMM) and 7.57 min (B, for SDM) in chromatograms (see Figure 1). Standards (solid line); spiked (0.2 ppm) egg extract (dashed line).


(15) FAO Food and Nutrition Papers 41/4 (1991) Rome, Italy