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Rapid high-performance liquid chromatographic determining technique of sulfamonomethoxine, sulfadimethoxine, and sulfaquinoxaline in eggs without use of organic solvents

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Abstract

A determining technique of sulfonamides (SAs) (sulfamonomethoxine (SMM), sulfadimethoxine (SDM), and sulfaquinoxaline (SQ)) in eggs, without use of organic solvents, is developed utilizing a high-performance liquid chromatography (HPLC) interfaced with a photo-diode array detector. The sample preparation was performed by homogenizing with perchloric acid solution using a handy ultrasonic-homogenizer followed by a centrifugal ultra-filtration unit. An analytical column and an isocratic mobile phase for HPLC are a reversed-phase C_4 column (150 mm × 4.6 mm i.d.) and 0.18 mol l^{-1} citric acid solution, respectively. The proposed technique was shown to be linear ($r > 0.998$) over the concentration range 0.1–2.0 $\mu g\, g^{-1}$. Average recoveries of three SAs (spiked 0.05, 0.1, 0.15, and 0.2 $\mu g\, g^{-1}$) ranged from 80.3 to 88.4%, with relative standard deviations (R.S.D.s) between 3.4 and 5.8%. The practical detection limits and total time required for the analysis of one sample were $< 0.05\ \mu g\, g^{-1}$ and <30 min, respectively. In all the processes, no organic solvents were used at all.
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Keywords: Sulfonamides; Handy ultrasonic-homogenizer; No-use of organic solvents; Eggs

1. Introduction

Sulfonamides (SAs) (Fig. 1) are regularly used by veterinarians for therapeutic, prophylactic or growth-promoting purposes in laying hens. In Japan, sulfamonomethoxine (SMM), sulfadimethoxine (SDM), and sulfaquinoxaline (SQ) are mainly used for prevention or treatment of poultry leucocytozoonosis and coccidiosis, and are generally co-administered in feed. The treatment of hens with SAs-supplemented feed may result in SA residues being present in market eggs if these drugs have been improperly administered or if the withdrawal time for the treated hens has not been observed. To assure the food safety for consumers, the European Union (EU) has set a maximum residue limit (MRL, 0.1 $\mu g\, g^{-1}$) for SAs in foods of animal origin such as meat, milk, and eggs [1].

Improper use of these veterinary drugs in laying hens is of great concerns because the drug residues are turning up in eggs, an indispensable food for the consumers because it is highly nutritious, cheap, and readily available. A rigid residue monitoring of SAs in eggs is therefore an important specific activity to guarantee the food safety.

Discharging the waste of organic solvents is also a severe problem on the world scale. From the viewpoint of the affect of organic solvents to environments and analysts, analytical methods for the monitoring should avoid the use of organic solvents [2–7].

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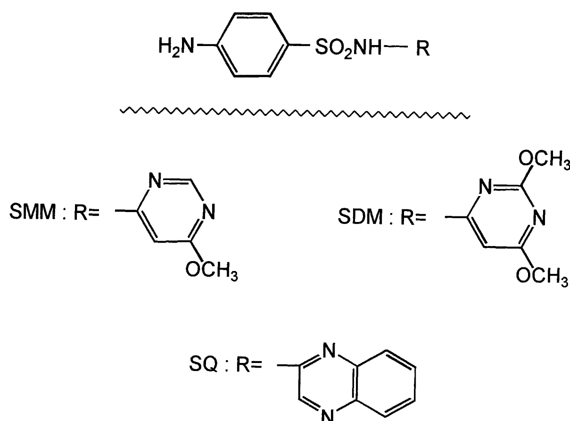

Fig. 1. Structures of sulfamonomethoxine (SMM), sulfadimethoxine (SDM), and sulfaquinoxaline (SQ).

International trade of animal products has become a big business. As a strategy to guarantee the equitable international trade of the products and to assure the food safety for consumers, the Codex Committee on Residues of Veterinary Drugs in Foods (CCRVDF), a sectional meeting under command of Codex Alimentrium Commission (CAC, in FAO/WHO), is advanced developing and harmonizing the international standardized analysis methods for veterinary drug residues in animal products, which are adopted as the Codex standards [8]. At present, regardless of industrial nations or developing states, the "*international harmonized analytical method*" of SA residues is required. The acceptable method must be rapid and precise, economical in cost and time and capable of determining residues below the MRL to permit the monitoring of large numbers of sample, with negligible harm to the environment and analyst.

There have been significant developments in these years in the determining and identifying methods of SA residues involving high-performance liquid chromatography (HPLC) interfaced with UV [9,10], photodiode array detection (DAD) [11], fluorescence detection [12], or mass spectrometery (MS) [13,14] have been described for the determination of some SAs in eggs. However, these methods have the following problems: (1) the extraction and clean-up involves varying analytical steps that are time consuming and can give low resolution; (2) recoveries are sometimes low and variable; (3) most importantly, organic solvents are used as extraction solvents and/or as HPLC mobile phases without fail, which may be harmful to the environment. There is presently no acceptable harmonized analytical method of SA residues in eggs.

For drug residue drug analyses in eggs the difficulties are caused by formation of an emulsion that hinders the recovery, thus interfering with co-extracts when target compounds are isolated (which require more effective cleanup techniques). For determination of target compounds in eggs using HPLC, more effective extraction and deproteinization are required. To improve the problem of techniques involve numerous analytical steps/times and extensive use of organic solvents, the author has recently applied the centrifugal ultra-filtration unit (Ultrafree-MC/PL) to purify sulfadimethoxine (including its hydroxyl metabolites) [14], sulfamethazine [15], or two antibiotics [16,17] (penicillin G and tetracyclines) extracted from animal products. This tool was able to deproteinize the extracted solution easily, in a short time, only with centrifuging.

The present paper describes an epoch-making method that enables rapid/simple determination of sulfamonomethoxine (SMM), sulfadimethoxine (SDM), and sulfaquinoxaline (SQ) residues in laying hen's eggs without use of organic solvents at all. The three SAs are most popularly used by veterinarians. Determination is performed by HPLC equipped with DAD which measures retention time and absorption spectrum simultaneously. The target peak components can be identified instantly. The DAD provides information as to both the identity and purity of chromatographic peaks and can be of readily used routinely.

2. Experimental

2.1. Apparatus

HPLC system was as follows: JASCO Model PU-980 pump and DG-980-50 degasser (Jasco, Tokyo, Japan) equipped with SPD-M10A $_{VP}$ diode array detector (Shimadzu, Kyoto, Japan). Operating conditions: analytical column, Mightysil RP-4 GP column (5 μm, 150 mm × 4.6 mm i.d.) (Kanto Chem., Tokyo, Japan), equipped with a guard column (5 mm × 4.6 mm i.d.) (Kanto Chem.) containing the same packing material; mobile phase, 0.18 mol l^{-1} citric acid; flow-rate of 1.0 ml min^{-1}; column temperature, ambient; injection

volume, 20 μl. The following apparatuses were used in the sample preparation: a handy ultrasonic-homogenizer (model HOM-100, 2 mm i.d. chip, Iwaki Glass, Funabashi, Japan); a microcentrifuge (Biofuge® fresco, Kendo Lab. Products, Hanau, Germany); a micro-centrifugal ultrafilter unit (Ultrafree-MC/PL, regenerated cellulose ultra-filtration membrane, nominal molecular mass limit = 5000, capacity ≤0.5 ml, Millipore, Bedford, MA, USA).

2.2. Reagents

SA standards (SMM, SDM, and SQ) were obtained from Sigma (St. Louis, MO, USA). Each stock standard solution of SAs was prepared by accurately weighing 10 mg and dissolving it in 100 ml distilled water (HPLC grade, Wako, Osaka, Japan). Working mixed standard solutions of these three drugs were prepared by diluting the stock solutions with distilled water. These solutions can be kept in a refrigerator for up to 1 month.

2.3. Egg samples

Eggs from laying hens that were kept in individual cages and given SAs-free basal layer diet continuously were used. These whole eggs that were removed from their shells were uniformed fully and used as blank egg samples.

2.4. Procedure

An accurate 0.2 g of sample was taken into a 1.5 ml micro-centrifuge tube and homogenized in 0.4 ml of 10% (v/v) perchloric acid (PCA) solution (in water) with a handy ultrasonic-homogenizer for 30 s. After 30 s, the capped tube was centrifuged at $12\,000 \times g$ for 5 min. A 0.2 ml portion of supernatant liquid was put into an Ultrafree-MC/PL and centrifuged at $5000 \times g$ for 5 min. The ultra-filtrate was injected into the HPLC system.

2.5. Recovery test

The recoveries of SAs from blank samples spiked at 0.05, 0.1, 0.15, and 0.2 $\mu g\,g^{-1}$, respectively, were determined. These fortification levels were prepared by adding 20 μl of three working mixed standard solutions (5, 10, 15, and 20 $\mu g\,ml^{-1}$), respectively, to separate 2.0 g portions of the samples. Fortified samples were mixed prior to the test.

3. Results and discussion

3.1. Sample preparation

The extraction operation used a handy ultrasonic-homogenizer was especially easy and was able to extract SAs in a little of egg sample (0.2 g) effectively with a little of 10% (v/v) PCA solution (0.4 ml) used as an extracting/deproteinizating solution in a micro-centrifugal tube. The extract did not form an emulsion. The resulting extract was further cleaned up by an Ultrafree-MC/PL as a centrifugal ultrafiltration unit. The procedure allowed rapid and efficient purification of SAs and resulted in high recovery and reproducibility.

3.2. HPLC conditions

In the previous papers [9–13] for the reversed-phase (RP) HPLC analysis of SAs in animal products, the C_{18} or C_8, non-polar sorbents, columns are used the most frequently. In general terms, the C_4 is less retentive than C_{18} and C_8 sorbents when retention is based on non-polar interactions alone; where the C_{18} and C_8 sorbents require larger volume of strong elution solvents, like acetonitrile and/or methanol, as the mobile phase, the C_4 should reduce remarkably the volume of elution solvents required and provide a more concentrated and clear separation as described previously [14–16].

A Mightysil RP-4 GP (C_4) column with an acidic solution as the mobile phase was tested with regard to the separations: among SMM, SDM, SQ, and the interference of the resulting extract origin; their sharp peaks obtained upon injection of equal amounts. Since the pK_a values of SAs in aqueous solution are approximately 6 [18,19], a citric acid solution as the mobile phase was used in order to protonate the SAs. The mobile phases with molarities of citric acid between 0.01 and 0.3 $mol\,l^{-1}$ (pH 2–3) were tested. Under the above acidic conditions, SAs were eluted as sharp peaks with short retention times. A chromatogram

with complete separation of target compounds, their clear/sharp peaks, and their short retention times was obtained by using the present C_4 column and an isocratic mobile phase of 0.18 mol l^{-1} citric acid solution with the SAs monitoring wavelength set at 267 nm (= an average maximum absorption spectrum for SMM, SDM, and SQ) (Fig. 2).

Fig. 2 shows HPLC chromatograms of blank and spiked egg samples obtained under the established procedure. The resulting extracts were free from interfering compounds for detection and identification by HPLC-DAD, indicating that purification was satisfactory for these three SAs.

The chromatographic repeatability was obtained from the relative standard deviations (R.S.D.s) of areas and retention times calculated for 10 replicate injections of a spiked (0.1 $\mu g\, g^{-1}$ each drug) egg sample that was obtained under the procedure established here. The values for SAs were calculated to be ≤0.08% for the areas and ≤0.59% for the retention times, respectively.

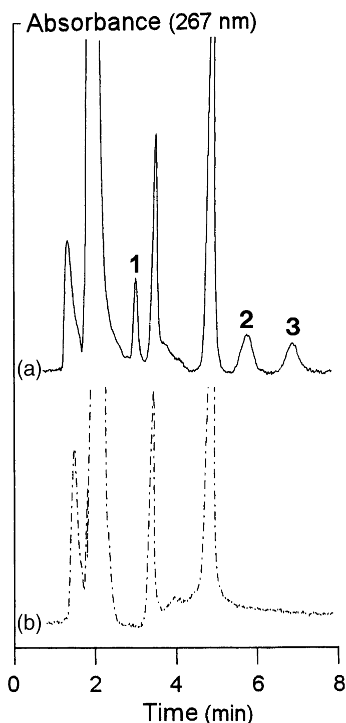

Fig. 2. HPLC chromatograms obtained from egg samples (photo-diode array detector set at 267 nm). (a) spiked (0.1 $\mu g\, g^{-1}$ of each SA) egg sample; (b) blank egg sample. Peaks, 1 = SMM; 2 = SDM; 3 = SQ.

The total time required for the analysis of one sample was <30 min. The rapid and harmless method without use of organic solvents at all could be achieved.

3.3. Method evaluation

Table 1 summaries the recoveries from egg samples at four different spiking levels, correlation coefficients (r) of calibration curves of target compounds isolated from spiked egg samples. Overall, satisfactory results were obtained.

Average recoveries were >80% with R.S.D.s between 3.4 and 5.8%. These values are well "acceptable criteria" for the residue analysis that the Codex setup [8]. The acceptable criteria are given further. Average recoveries of 80–110% with their R.S.D.s <15% when the MRL for the analyte is ≥0.1 $\mu g\, g^{-1}$ and when analytical method can be performed with acceptable precision. Recommended acceptable recoveries are 70–110% with R.S.D.s <20% when the MRL is 0.01–0.1 $\mu g\, g^{-1}$.

Calibration was performed by linear regression analysis of peak areas of spiked sample extracts ranging from 0.1 to 2.0 $\mu g\, g^{-1}$ versus their concentrations. The curve was constructed from five points and each point represented the mean of five injections. The resulting curves were linear in the concentration ranges

Table 1
Accuracy, precision, and sensitivity data for SA-fortified eggs

Spiked ($\mu g\, g^{-1}$)	Recovery[a] (%)		
	SMM	SDM	SQ
0.05	85.9 (5.8)	82.0 (5.0)	80.3 (3.8)
0.1	87.6 (5.2)	83.8 (3.8)	80.9 (5.8)
0.15	88.4 (3.4)	81.9 (4.8)	82.2 (4.9)
0.2	86.4 (5.1)	82.5 (4.7)	81.5 (4.2)
Calibration			
r ($n = 5$)[b]	0.999 ± 0.001	0.998 ± 0.001	0.998 ± 0.002
PDL[c] ($\mu g\, g^{-1}$)	0.02	0.03	0.04

[a] Data are means ($n = 5$); relative standard deviations in parentheses.

[b] Mean of five determinations using spiked egg samples for calibration curves (range of concentration was 0.1–2.0 $\mu g\, g^{-1}$); r, correlation coefficient.

[c] Practical detection limit.

0.1–2.0 μg g^{-1} for all of the SAs, with all values of r greater than 0.998 ($P < 0.01$). The data given in Table 1 demonstrate that the accuracy and precision are well within accepted values for drug residue analyses.

The practical detection limits (PDLs) which give clearly discernible peaks of SAs (signal-to-noise ratio > 5) ranged from 0.02 to 0.04 μg g^{-1} (Table 1). These values were below the MRL (0.1 μg g^{-1}).

4. Conclusions

A rapid and simple method without use of organic solvents for determination and identification of SMM, SDM, and SQ in eggs using HPLC-DAD has developed. The main advantages of proposed procedure are summarized as follows: (1) by extraction using a handy ultrasonic-homogenizer followed by purification using a micro-centrifugal ultrafiltration unit, the sample preparation is especially easy/rapid and is able to recover SAs effectively; (2) shorter analysis time, the total time required for the analysis of one sample <30 min; (3) proving reproducible and repeatable recoveries, the R.S.D.s were 3.4–5.8%, and economical; (4) no organic solvents used at all, harmless to the environment and human. The present procedure may be useful for the international harmonized analytical method for routine residue monitoring of SAs in eggs.

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