Isolation of Sulfonamides from Whole Egg by Supercritical Fluid Extraction

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Abstract

A supercritical fluid extraction (SFE) method is described for the isolation of sulfonamides from chicken eggs. Whole egg is mixed with Hydromatrix and added to an extraction vessel containing neutral alumina. The sample is extracted at 40°C with supercritical CO_2 at 10,000 psi (680 bar) and an expanded gas flow rate of 3.0 L/min to a total volume of 120 L. The sulfonamides are trapped in-line on an alumina sorbent bed. The sulfonamides are eluted post-SFE by using the high-performance liquid chromatographic (HPLC) mobile phase solvent system (phosphate buffer and methanol), followed by separation on an HPLC system using a C_{18} column and ultraviolet detection at 265 nm. Recoveries from fortified liquid whole eggs (six replicates) at the 0.1-ppm level are 99.5% ± 2.2 for sulfamethazine, 87.8% ± 6.0 for sulfadimethoxine, and 97.6% ± 2.5 for sulfaquinoxaline. The detection limit is 0.025 ppm.

Introduction

Sulfonamides (SAs) were initially used in medicine to treat a wide variety of human bacterial infections. Because of their broad range of activity against gram-positive and gram-negative bacteria, SAs are routinely used in veterinary medicine in raising cattle, swine, and poultry. Their uses include the treatment of chronic and acute bacterial infections and their addition to livestock feed at subtherapeutic doses to promote animal growth. Because of their widespread use, there is concern about sulfonamide residues in foods. These concerns can include the acquisition of antimicrobial resistance that makes these drugs ineffective in treating humans (1), the production of allergic responses (2), and the purported carcinogenicity of sulfamethazine (SMZ), one of the most commonly used SAs (3). For these reasons, a tolerance level of 0.1 ppm has been set for these drugs in edible animal tissue (4).

Reviews on methodology indicate that a large number of techniques have been employed for the analysis of sulfonamide residues in meat, milk, and fish (5,6). However, there has been less emphasis on the analysis of sulfonamide residues in eggs (6–10), despite the fact that this class of drugs is widely used for poultry. Most of the published sulfonamide methods are not suitable for routine screening because of the long analysis time caused primarily by the analyte isolation step that usually involves extensive use of organic solvents. This latter aspect is becoming more important with the need to reduce the use and subsequent disposal of halogen-containing solvents in government funded contracts and selected federal laboratories under Environmental Protection Agency (EPA) pollution prevention guidelines (11).

The absence of effective screening or quantitative methods for eggs can be attributed to problems encountered with sample cleanup and purification when traditional liquid–liquid extractions are used (7). Emulsions often occur that require additional sample manipulation to remove proteins, lipids, and pigments that interfere with the analysis. Therefore, it would be desirable to develop an alternate means of isolating analytes from eggs without the need for these time-consuming steps. Although there are many advantages of employing supercritical fluid extraction (SFE) over conventional solvent extraction for isolating drug residues from animal tissue, this technique has not been widely investigated. While several papers have been published using SFE for SAs in meat tissue (12,13), dried milk powder (14), and model sorbent-based systems (15), this technique has not been applied to SAs in eggs. In this paper, we report the results from the SFE isolation of three sulfa drugs from liquid eggs by using supercritical CO_2 (SC–CO_2)with in-line sorbent trapping and without the use of organic modifiers.

Problems associated with the analysis of eggs for SAs include analyte–matrix binding and the need to minimize co-extractants without the loss of analyte. SAs are also not very soluble in nonpolar solvents. Therefore, extractions of SAs from meat, milk, and eggs have been typically carried out with chloroform, methylene chloride, acetone, acetonitrile, or ethyl acetate (6). Acetonitrile appears to be the most effective solvent for the extraction of SAs from eggs because of their solubility and the small amount of co-extracted fat obtained (16). While

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solvent-based extractions have been widely applied to residue analysis, other techniques, such as solid-phase extraction, matrix solid-phase dispersion, and SFE have been used, but to a much lesser extent. The full potential for SFE in residue analysis has not been realized. Although cosolvents such as methanol help in displacing the analytes from bound or adsorbed matrix sites, which increases extraction efficiency, their use in residue analysis results in the co-extraction of other materials that require further sample cleanup. Otherwise, chromatographic separation and detection of the analytes are adversely affected. In one study on SAs in spiked liver tissue, changes in SA retention time and peak shape were observed with SFE CO_2–MeOH (12). Because these authors used an extraction pressure of only 380 atm, a methanol modifier was required to obtain optimum recoveries.

Experimental

Materials

Eggs were obtained from a local supermarket. The eggs were removed from their shells, the yolk and white were thoroughly mixed to insure a representative sample, and they were then stored in a freezer at –20°C until used. The eggs were thawed in a refrigerator at 4°C and kept for two weeks before being discarded. The eggs were analyzed by SFE prior to use to ensure the absence of SAs. SMZ and sulfadimethoxine (SDM) were obtained from Sigma Chemical (St. Louis, MO); sulfaquinoxaline (SQX) was purchased from Pfaltz and Bauer (Waterbury, CT). A stock solution containing 1.0 mg/mL was prepared for each compound (SMZ and SDM in methanol, SQX in tetrahydrofuran). Standard solutions containing 0.005, 0.01, and 0.05 ug/µL of each SA were prepared in methanol from the stock solutions and used to fortify the egg samples at the 0.05-, 0.1-, and 0.5-ppm levels. Hydromatrix (Celite 566, sieved at 30–40 mesh to remove fines) (Applied Separations, Allentown, PA), neutral alumina (Brockman #1, 80–200 mesh) (Fisher Scientific, Malvern, PA), SFC-grade CO_2 without helium headspace (Scott Specialty Gas, Plumsteadville, PA), Burdick & Jackson brand methanol and tetrahydrofuran (Baxter Health Care, Muskegon, MI), 40% aqueous tetrabutylammonium hydroxide (TBAH) (Aldrich Chemical, Milwaukee, WI), and mono (NaH_2PO_4) and dibasic (Na_2HPO_4–$7H_2O$) sodium phosphate (Mallinckrodt, Paris, KY) were used without further purification. The phosphate buffer, which had been used previously for SAs in meat tissue (13), was prepared by first making individual 0.2M monobasic and dibasic phosphate solutions, then combining 97.5 mL of the monobasic solution, 152.5 mL of the dibasic solution, and 3.5 mL of TBAH, and diluting to 1 L. The final pH was 7.2.

Sample preparation

A plug of polypropylene wool (Aldrich Chemical), 2.0 g neutral alumina, and another plug of polypropylene wool were added to a high-pressure (10,000 psi) 24-mL extraction vessel (Keystone Scientific, Bellefonte, PA). A 1.0-g egg aliquot was weighed into a 100-mL beaker, and the sample was fortified with 10 µL of the appropriate SA standard. Fortified samples were allowed to sit at room temperature for 10 min. Hydromatrix (4.0 g) was then added, and the mixture was stirred with a glass rod until it was uniform in appearance. The dry, free-flowing mixture was added to the high-pressure extraction vessel, followed by a plug of polypropylene wool. Finally, 2.0 g Hydromatrix was added to the extraction vessel, topped with another plug of polypropylene wool. All of the material added to the extraction vessel was firmly tamped. The end of the extraction vessel containing the neutral alumina was designated the top, as illustrated in Figure 1.

SFE procedure

The extraction vessels were installed in the supercritical fluid extractor (Applied Separations Spe-ed SFE model 7010). The model 7010 pump was fitted with a chiller assembly, which allowed the pump to be cooled to –15°C using a Neslab RTE110 refrigerated circulator (Fisher Scientific), obviating the need for helium-pressurized CO_2 cylinders. An empty 6-mL solid-phase extraction cartridge (Applied Separations) was attached to the micrometering valve and discarded at the end of the extraction. The closed system was pressurized to 8500 psi, and the oven was heated to 40°C. The micrometering valve was preheated to

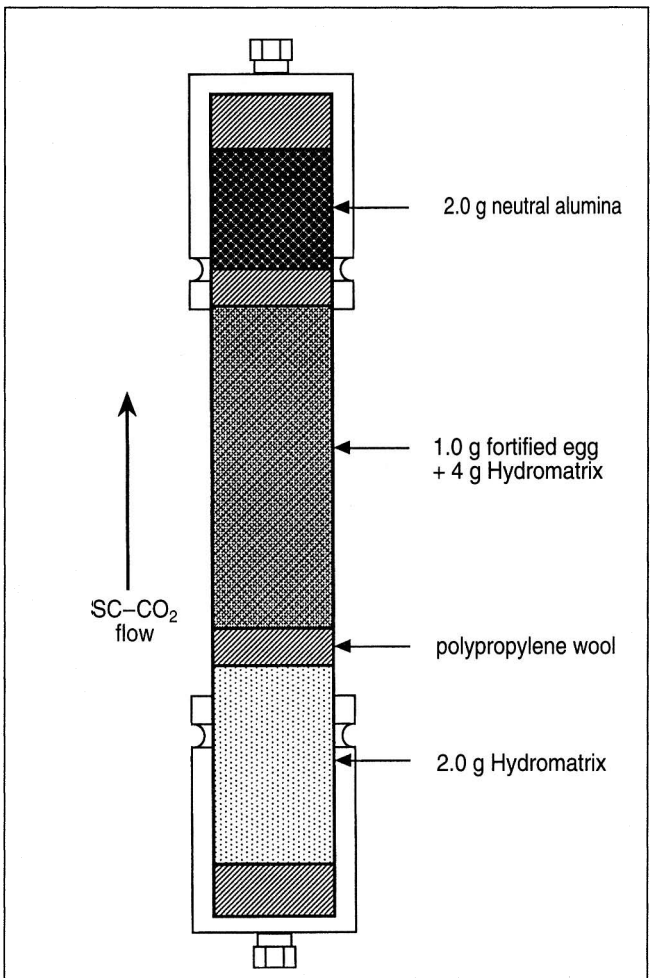

Figure 1. Diagram of the SFE vessel with in-line adsorbent trap.

115°C prior to pressurization. After the extraction vessel reached 40°C, the pressure was adjusted to 10,000 psi (680 bar). To equilibrate the fluid system, a 4-min static holding period was employed prior to the dynamic phase of the extraction. The flow rate of the expanded CO_2 gas was 3 L/min for 40 min (120 L of expanded gas). After post-extraction depressurization, the extraction vessel was removed from the SFE instrument. The upper cap and top layer of polypropylene wool were removed from the vessel, and the in-line alumina sorbent bed was poured into a clean 6-mL SPE cartridge containing 0.5 g alumina. After tapping the cartridge to compact the alumina, 1.5 g of sand was added to the top of the cartridge. The SAs were recovered by eluting the cartridge with 4.0 mL of the HPLC mobile phase solvent and collecting the first 2.0 mL in a 4.0-mL tube (Kontes Glass, Vineland, NJ). A 100-µL amount of the sample was injected into the HPLC. Fortification studies were carried out over several weeks for each fortification level studied.

HPLC analysis

Analyses were carried out using a Beckman Gradient model 334 LC (San Ramon, CA) with a Rheodyne model 7125 injector (Berkeley, CA) connected to a 25-cm × 4.6-mm-i.d. Supelcosil LC-18 column (5-µm film) (Supelco, Bellefonte, PA). SA detection was accomplished at 265 nm using an Applied Biosystems model 1000S diode array detector (Foster City, CA). The HPLC mobile phase, which consisted of phosphate buffer and methanol (65:35), was pumped at a flow rate of 0.9 mL/min. Chromatograms were recorded on a Hewlett-Packard model 3396A integrator (Valley Forge, PA). Under these experimental conditions, the minimum detectable level of SA (signal-to-noise ratio greater than 3) was 0.025 ppm.

Results and Discussion

To avert a large part of the cleanup problem associated with the analysis of tissue samples and to increase solvent strength to attain optimum extraction efficiencies, much of the SFE work conducted in our laboratory explored the possibility of using CO_2 without modifiers to isolate desired analytes. A higher operating SC–CO_2 pressure (680 atm, 10,000 psi) was employed to accomplish this goal (13,17–19). An SFE instrument developed in this laboratory (20) and now in commercial production was used in our investigations because many of the instruments on the market do not have the capability to work at high flow rates and pressures up to 10,000 psi. In initiating the study on SAs in liquid whole eggs, we used the same extraction conditions established by Parks and Maxwell (13) for SAs in fortified chicken tissues: 10,000 psi, 120 L of CO_2 (expanded gas) at 3.0 L/min at an extraction temperature of 40°C. To determine if these conditions were the optimum conditions for extracting the SAs from liquid whole egg, we first

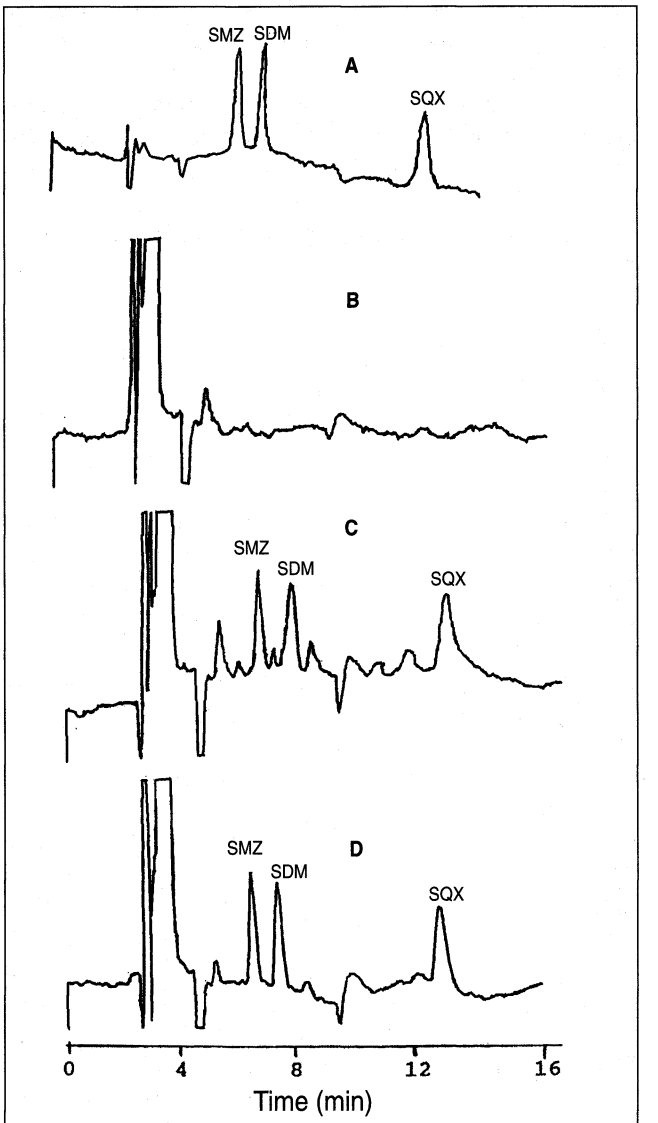

Figure 2. HPLC chromatogram (100 µL of 2.0-mL eluent) of standard (A), control (B), and liquid whole egg fortified with 0.1 ppm SAs and collected off-line (C) and in-line (D).

Table I. Sulfonamide Recoveries from Fortified Liquid Egg Samples

Sulfonamide	Replicates	Recovery (%) and coefficient of variation	Recovery (%) and coefficient of variation	Recovery (%) and coefficient of variation
Sulfamethazine	6	86.7 ± 2.7	99.5 ± 2.2	99.3 ± 2.2
Sulfadimethoxine	6	84.0 ± 2.9	87.8 ± 6.0	90.0 ± 1.0
Sulfaquinoxaline	6	87.0 ± 2.6	97.6 ± 2.5	93.2 ± 3.5
Fortification level (ppm)		**0.05**	**0.10**	**0.50**

looked at the effect of adding water to the sample matrix prior to SC–CO_2 extraction. Parks et al. (17) found that, in fortified chicken liver tissue, dehydration of the sample during SFE resulted in adsorption of the analyte and its metabolites and limited their extractability. They found that adding a small amount of water to the sample prior to SFE increased the recoveries of the analytes by 12–20%. In our comparisons with liquid whole egg, we found less than a 4% difference in recoveries between samples extracted with or without 0.5 mL of water added to the sample matrix. We also tried to determine whether adding methanol to the sample prior to SFE would increase recoveries. For SMZ and SDM, methanol had little or no effect on recovery, but for SQX, recovery decreased approximately 30%. To determine the amount of SC–CO_2 needed to extract the SAs from the egg matrix, recoveries were obtained at different levels of expanded gas. At 50 L, recovery of the SAs was between 60 and 80%, but at 80 L, recoveries were always greater than 85%. However, at 80 L, the HPLC chromatogram showed that the sample contained impurities that could interfere with the quantitation of the SAs. At 120 L of expanded gas, the recoveries of the analytes were still greater than 85%, and the HPLC chromatograms were cleaner.

In their work with SAs in fortified chicken tissues, Parks and Maxwell (13) found that higher recoveries and slightly cleaner HPLC chromatograms were obtained when the SAs were trapped in-line (Figure 1) rather than off-line in a cartridge containing the adsorbent. They found a 20–30% difference in recovery between in-line and off-line trapping methods. This difference in recovery was in part due to the varying amounts of co-extractants (lipids, pigments, etc.), the decrease in density of CO_2 as the flow approached the metering valve, and the analytes being trapped in the system plumbing. We also observed a difference in recoveries between trapping methods, but not as drastic a difference as obtained by these authors (less than 10% difference in recovery for eggs). A difference between trapping methods was observed in the HPLC chromatograms. As can be seen in Figure 2, there are more interfering peaks in the HPLC chromatogram obtained with off-line trapping (Figure 2C) than in the chromatogram from in-line trapping (Figure 2D). These differences result from the co-extractants that pass through the in-line adsorbent but collect after depressurization on the off-line adsorbent cartridge. Typical chromatograms showing the SA standard (Figure 2A), SC–CO_2 extracts from an unfortified egg sample (Figure 2B), and an egg sample fortified at 0.10 ppm (Figure 2D) are also shown in Figure 2. Note that the SA peaks are separated from the void volume and from each other.

Using the SFE conditions listed in the experimental section at 40°C and 10,000 psi, recovery studies of SMZ, SDM, and SQX at the 0.05-, 0.10-, and 0.50-ppm fortification levels were carried out; the results are shown in Table I. We found that at the 0.05-ppm fortification level, average recoveries were still greater than 80%, although somewhat higher recoveries were obtained for 0.10-ppm and 0.50-ppm SA spiking levels. Our recoveries were slightly higher than those obtained by Parks and Maxwell (13) for the same SAs in chicken liver tissue under similar SFE conditions. This was especially true for SQX, which suggests that these drugs were not as tightly bound or that they were bound differently in the egg matrix than in the meat tissue.

Conclusion

This proposed SFE–HPLC method with in-line trapping for SAs in eggs provided excellent recoveries and reproducibility and a clean chromatogram. The amount of sample manipulation and solvent use was minimal, thereby offering a distinct advantage over conventional extraction methods.

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