Quantitative Recovery of Sulfonamides from Chicken Liver, Beef Liver, and Egg Yolk via Modified Supercritical Carbon Dioxide

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Solutions of supercritical CO_2 modified with 5, 10, and 20% methanol, ethanol, acetone, and acetonitrile were compared for the extraction of sulfonamides from fortified chicken liver. The results showed both 20% acetone-modified CO_2 and 20% acetonitrile-modified CO_2 were capable of quantitatively extracting sulfamethazine, sulfaquinoxaline, and sulfadimethoxine, whereas 20% ethanol- and 20% methanol-modified CO_2 were found to be less efficient. Acetonitrile-modified CO_2 however, produced fewer chromatographic interferences than acetone, making quantitation easier. Sub-ppm levels of the three analytes were shown to be quantitatively extracted from chicken liver with 20% acetonitrile modifier. Also, quantitative recovery was obtained with spiked beef liver samples employing 20% acetone modifier, and greater than 90% recovery was obtained for two of the three with 20% acetonitrile-modified CO_2, while either 20% acetone- or 20% acetonitrile-modified CO_2 was found to yield quantitative recovery from egg yolk. In contrast to conventional sample preparation procedures no sample cleanup prior to quantitative analysis was required.

Keywords: *SFE; sulfonamides; extraction; supercritical fluid; chicken; beef; egg*

INTRODUCTION

Sulfonamides are a class of antimicrobial agents that have seen extensive use in medicine. Sulfonamides are often encountered in animal medicine and livestock production. Since wide use is occurring, the presence of certain residues in animal products presents a potential health hazard due to their allergenic properties (Blanchflower and Rice, 1988). Also, some people exhibit hypersensitivity to drug residues, and/or low levels of drug residue may produce genetically altered bacteria that are resistant to existing drug therapy (Kagan, 1974). The current regulatory level for most sulfonamides is 0.1 mg/kg (*Code of Federal Regulations*, 1996). This work focuses on evaluating supercritical fluid extraction as a alternative sample preparation technique for sulfonamide residues in biological tissues.

Traditional analysis of sulfonamide extracts from tissues has involved the use of 75/25 chloroform/ethyl acetate extracted at room temperature (Parks, 1994). It is necessary to centrifuge the extract, pass it through an alumina column, rinse with chloroform, dry under reduced pressure, and elute the analytes from the column with the chromatographic mobile phase. Supercritical fluid extraction (SFE) is able to eliminate the use of chlorinated solvents and to reduce the number of sample preparation steps necessary to produce an extract of sufficient purity to perform quantitative assay. SFE of sulfonamides from silica and biological matrices, to date, has used several different strategies: high pressures, different supercritical fluids, and modified extractions. Parks and Maxwell (1994) extracted sulfonamides using high pressures (10 000 psi) from a variety of chicken tissues including liver, breast, and thigh and obtained greater than 80% recovery for all analytes. Cross et al. (1993) studied the extraction of sulfonamides from inert and animal matrices. With 25% methanol-modified CO_2 and 680 atm, greater than 90% recovery of sulfamethazine from chicken liver was experienced, although an extensive cleanup procedure was necessary to remove interfering fatty materials. Tena et al. (1995) investigated methods to improve supercritical fluid extraction of sulfonamide salts from silica gel and diatomaceous earth. Poor recovery (<36%) was obtained (a) using pure CO_2, (b) the addition of a methanol spike to the matrix, or (c) methylation of the sulfonamides. However, the addition of an ion-pairing agent produced much higher (>80%) recovery. Ashraf-Khorassani and Taylor (1996) have used CHF_3, and methanol-modified CHF_3 to extract sulfonamides from chicken liver tissue. Near-quantitative recovery was obtained for two of three sulfonamides investigated. Carbon dioxide with 10% methanol modifier yielded less than 50% recovery for all three analytes under the same conditions. Combs et al. (1996) extracted sulfonamides from various matrices (sand, nonfat milk powder, egg yolk, and beef liver) using both CO_2 and CHF_3 (pure and methanol-modified). Improved recovery was realized for both sulfamethazine and sulfadimethoxine using CHF_3 compared to CO_2 for each matrix investigated. Unfortunately, the yields were not quantitative.

This paper considers the effect of different modifiers and modifier concentrations upon the extraction efficiency of sulfonamides at both ppm and sub-ppm spike levels from chicken liver and beef liver tissues using supercritical CO_2. Trends in the extraction efficiency of sulfamethazine (SMZ), sulfaquinoxaline (SQX), and sulfadimethoxine (SDM) (Figure 1) upon altering both modifier identity and composition will be discussed in an attempt to obtain complete recovery of all three analtyes. On the basis of previous work, neither high pressure nor polar supercritical fluids alone could yield complete recovery of all three sulfonamides.

EXPERIMENTAL PROCEDURES

An Isco (Lincoln, NE) SFX-3560 automated supercritical fluid extractor equipped with an automatic variable restrictor system was used for extracting sulfonamides from chicken

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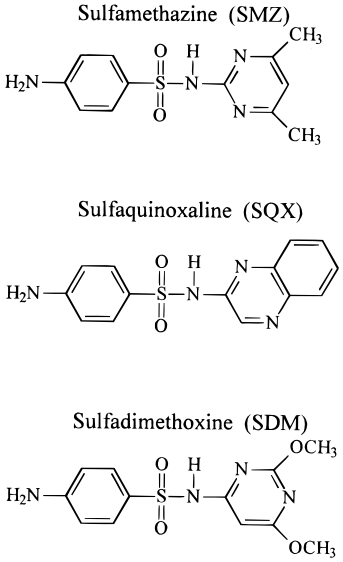

Figure 1. Structures of the target analytes sulfamethazine (SMZ), sulfaquinoxaline (SQX), and sulfadimethoxine (SDM).

liver. The system consisted of a 100DX syringe pump capable of delivering CO_2 and another 100DX pump to deliver modifier. The complete system has been described previously (Isco). HPLC-grade methanol, acetone, and acetonitrile were purchased from EM Science (Gibbstown, NJ). Ethanol modifier was purchased from Aaper Alcohol and Chemical Company (Shelbyville, KY). HPLC-grade water was purchased from Mallinckrodt (Paris, KY). CO_2 pressurized with 2000 psi of helium to minimize pump cavitation was obtained from Air Products and Chemicals, Inc. (Allentown, PA). The sulfonamide standards were provided by the USDA/ARS (Philadelphia, PA).

Modifier (acetone, methanol, acetonitrile, or ethanol) was added in-line to CO_2 at either 5, 10, or 20% by volume. Isco PEEK extraction vessels with 10 mL internal volume were used for all extractions. All extractions involved a single 30 min dynamic step at 490 atm of CO_2 and a temperature of 40 °C. A flow of 1.5 mL/min of liquid CO_2 was used for all extractions. The restrictor was maintained at 50 °C. A liquid trap composed of 85% 8 mM ammonium acetate (NH_4OAc)/15% acetonitrile (3.5 mL) was used for each extraction. The liquid trapping system was maintained at 10 °C during each extraction. In addition, a head pressure of 30 psi was applied to the liquid trap to improve trapping efficiency and to minimize trap solvent loss during the extraction. Following extractions with both 5 and 10% modifier, the trap solvent was diluted to 5 mL with 85/15 8 mM NH_4OAc/acetonitrile. Extracts employing 20% modifier were diluted to 10 mL with the same mobile phase. Following each extraction, the liquid trap contents were passed through a 0.2 μm Teflon filter (Supelco, Bellefonte, PA) to remove particulates.

Chicken liver samples were purchased at a local grocery store. Samples were prepared by spiking 0.5 g of liver tissue with 10 μL of drug standard (0.6 μg/μL each of SMZ, SQX, and SDM in methanol). The spiked matrix was then thoroughly mixed with 1.0 g of Hydromatrix (Varian, Sugar Land, TX), followed by an incubation period of at least 30 min at −10 °C. The entire frozen contents were then added directly to the extraction vessel. To minimize void volume, Ottawa sand standard (Fischer Scientific, Fair Lawn, NJ) was used to completely fill the extraction vessel. Sub-ppm chicken liver samples were prepared by spiking 20 μL of a 24 ng/μL standard of SMZ, SQX, and SDM onto 0.5 g of chicken liver (960 ppb). Hydromatrix was again added to immobilize excess moisture. A similar incubation period was used followed by the addition of Ottawa sand to fill the vessel. The beef liver samples were obtained from USDA/ARS in Philadelphia, PA, and treated in a similar manner (6 μg of each sulfonamide). The egg yolk sample matrix was prepared by first separating the yolk and egg white. A 0.5 g portion of egg yolk was then treated similarly to the chicken and beef liver samples. Each sample matrix extraction was repeated in triplicate.

Table 1. Percent Recovery of Sulfamethazine (SMZ), Sulfaquinoxaline (SQX), and Sulfadimethoxine (SDM) from a Chicken Liver−Hydromatrix Mix Using 5, 10, and 20% Methanol-Modified CO_2[a]

	5% methanol	10% methanol	20% methanol
SMZ	14 (12)	60 (2)	85 (4)
SQX	14 (12)	54 (1)	82 (3)
SDM	24 (6)	59 (4)	86 (4)

[a] Numbers in parentheses are relative standard deviations.

A Hewlett-Packard (Little Falls, DE) series 1050 HPLC equipped with a variable-wavelength UV detector was used to assay all sample extracts. A 250 × 4.6 mm (5 μm dp) Deltabond ODS (Keystone Scientific, Bellefonte, PA) column was used throughout the study. The mobile phase employed was 85% 8 mM NH_4OAc/15% acetonitrile adjusted to pH 6.5 with acetic acid operated at a flow of 1 mL/min. All three sulfonamides were detected at 266 nm.

RESULTS AND DISCUSSION

The objective of this study was to investigate the effect of modifier identity and concentration on the extraction efficiency of SMZ, SQX, and SDM spiked at ppm and sub-ppm levels from chicken liver and beef liver using methanol, ethanol, acetone, and acetonitrile as modifiers. In addition, the applicability of employing a pressurized liquid trap was investigated.

Initially, an Ottawa sand sample spiked with the three drugs was successfully extracted using 10% methanol modified CO_2 to ensure complete liquid trapping efficiency. Due to the nonvolatile nature of sulfonamides, quantitative trapping was achieved both with and without trap pressurization. However, pressurizing the trap reduced trapping solvent loss and was used throughout.

Table 1 shows the recovery of sulfonamides spiked in chicken liver using different concentrations of methanol-modified CO_2. At 5% modifier concentration recovery of all sulfonamides did not exceed 25%. Sample extracts were opaque due to the presence of coextracted fatty material, although chromatographic interference with the analytes was not observed. To ensure integrity of the filtering system, a sample was injected both before and after filtering through a 0.2 μm filter. The results showed no differences due to filtering of the extracts. By increasing the methanol concentration to 10%, recovery of the drugs increased to nearly 60%, which supports the previous findings of Ashraf-Khorassani et al. (1996) under similar conditions. They concluded that appreciable analyte−matrix interactions existed and that 10% methanol was not sufficient to release sulfonamides from the biological matrix. Extractions in this study were extended to 20% methanol. Increased recovery to 85% for SMZ, 82% SQX, and 86% SDM was observed. Cross et al. (1993) extracted sulfonamides from chicken liver using an even higher methanol-modifier content (25%). They used an extensive cleanup procedure after liquid-phase trapping, including evaporation under nitrogen and solid-phase extraction, prior to quantitation. It is possible that the cleanup method was assumed to be necessary although none was needed. The sample extracts obtained in our laboratory with 20% methanol were yellow due to the presence of coextractives, but no chromatographic interference was obtained. Only filtering the sample through a 0.2 μm Teflon filter was necessary to obtain quantifiable extracts.

Table 2. Percent Recovery of Sulfamethazine (SMZ), Sulfaquinoxaline (SQX), and Sulfadimethoxine (SDM) from a Chicken Liver−Hydromatrix Mix Using 5, 10, and 20% Ethanol-Modified CO_2[a]

	5% ethanol	10% ethanol	20% ethanol
SMZ	33 (17)	45 (4)	92 (5)
SQX	34 (10)	41 (3)	78 (6)
SDM	49 (5)	47 (3)	81 (4)

[a] Numbers in parentheses are relative standard deviations.

Table 3. Percent Recovery of Sulfamethazine (SMZ), Sulfaquinoxaline (SQX), and Sulfadimethoxine (SDM) from a Chicken Liver−Hydromatrix Mix Using 5, 10, and 20% Acetone-Modified CO_2[a]

	5% acetone	10% acetone	20% acetone
SMZ	34 (4)	51 (4)	102 (6)
SQX	38 (2)	53 (4)	92 (8)
SDM	53 (5)	63 (4)	106 (4)

[a] Numbers in parentheses are relative standard deviations.

Ethanol modified CO_2 was investigated next (Table 2). Ethanol possesses a polarity index of 5.2 (Phenomenex), whereas methanol is 5.1. It is envisioned that a slightly more polar solvent could possibly increase extraction efficiency of polar sulfonamides from chicken liver. Extractions using 5% ethanol (33% SMZ, 34% SQX, and 49% SDM) were indeed much better than with 5% methanol (14% SMZ, 14% SQX, and 24% SDM). Surprisingly, at 10% modified conditions recoveries were higher using methanol than ethanol. At 20% modifier conditions, ethanol produced almost quantitative recovery (92%) of SMZ, but no greater than 81% for either SQX or SDM. These differences in extractability do not correlate directly with polarity and may be related to each analyte's solubility in the solvent modifier employed. We therefore concluded that ethanol was no better or worse than methanol for this matrix/analyte.

Acetone-modified CO_2 (Table 3) was then investigated since it possesses a polarity index (5.1) similar to methanol and ethanol, but SQX has a 6-fold larger solubility in acetone than in 95% alcohol (Budarari, 1989). Recoveries employing 5% acetone were similar to those obtained with ethanol, 34% SMZ, 38% SQX, and 53% SDM, but precision was improved compared to either 5% methanol or 5% ethanol modifier. The extracts obtained with 5% acetone modifier contained insoluble particulates but were filtered out using the 0.2 μm Teflon filter. Some chromatographic interference was obtained, however, with the use of acetone (Figure 2), especially for SMZ, which eluted first since the sulfonamides were detected at 266 nm and the UV cutoff for acetone is 330 nm. Recoveries increased only slightly upon increasing the modifier to 10% (51% SMZ, 53% SQX, and 63% SDM). The amount of insoluble material present in the 10% acetone extracts actually decreased. This is believed to be due to increased solubility of the coextractive material in the liquid trap since the amount of acetone content increased. Upon increasing the acetone concentration to 20%, quantitative recovery of all three sulfonamides was obtained. The acetone modifier may (a) disrupt the analyte−matrix interactions, which makes the analytes more available for extraction, (b) increase solubility of fatty material, which may release the sulfonamides trapped within the fatty material, or (c) enhance the solubility of the analytes in the extraction fluid. Each mechanism probably aids the extraction, making quantitative recovery possible. Similar chromatographic interference was observed for 20% acetone modifier compared to 10% acetone, and quantitation of the sample extracts was still possible only by filtering the extracts prior to analysis. Coextractives present in the sample did not appear to interfere with the quantitation method.

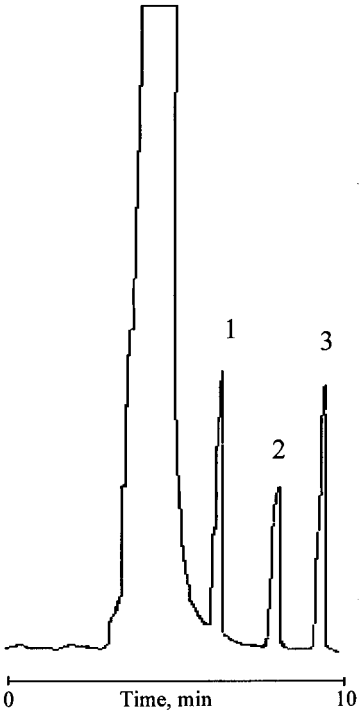

Figure 2. HPLC separation of sulfonamides extracted from chicken liver−Hydromatrix mix. Extraction conditions: 10% acetone-modified CO_2, 490 atm, 40 °C, 1.5 mL/min for 30 min, liquid trap consisting of 3.5 mL of 85/15 8 mM NH_4OAc/acetonitrile pH adjusted to 6.5 using acetic acid. HPLC conditions: Deltabond ODS, 250 × 4.6mm, 5 μm d_p. Mobile phase: 85/15 8 mM NH_4OAc/acetonitrile pH adjusted to 6.5 using acetic acid. Flow 1 mL/min. Detector UV monitored at 266 nm. Elution order: (1) SMZ, (2) SQX, (3) SDM.

Table 4. Percent Recovery of Sulfamethazine (SMZ), Sulfaquinoxaline (SQX), and Sulfadimethoxine (SDM) from a Chicken Liver−Hydromatrix Mix Using 5, 10, and 20% Acetonitrile-Modified CO_2[a]

	5% acetonitrile	10% acetonitrile	20% acetonitrile
SMZ	38 (4)	55 (6)	110 (3)
SQX	32 (1)	50 (7)	104 (2)
SDM	46 (1)	55 (6)	106 (2)

[a] Numbers in parentheses are relative standard deviations.

Lastly, acetonitrile (5.8 on the polarity index)-modified CO_2 (Table 4) was investigated. A more polar solvent was thought to increase the solubility of the polar sulfonamides to a greater extent than the slightly less polar solvents investigated previously. Both 5% and 10% acetonitrile produced recoveries similar to those obtained with acetone (i.e., less than 55% for all analytes). However, since acetonitrile was already present in the mobile phase, less chromatographic interference was obtained (Figure 3). By increasing the modifier concentration to 20%, 110% SMZ, 104% SQX, and 106% SDM was observed within 30 min. The 20% acetonitrile modifier sufficiently disrupted analyte−matrix interaction, and more importantly, neither the coextractives nor modifier produced chromatographic interferences as in the case of acetone.

Next, a sub-ppm (960 ppb) sample of chicken liver was extracted under the optimum conditions determined previously (i.e., 20% acetonitrile-modified CO_2). Recoveries are shown in Figure 4. Lower spiking levels are

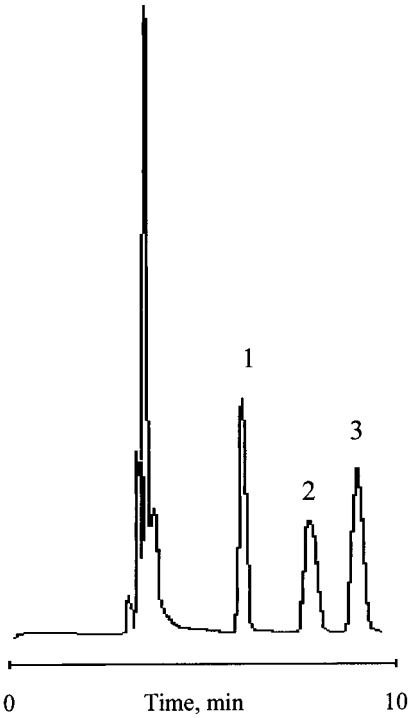

Figure 3. HPLC separation of sulfonamides extracted from chicken liver−Hydromatrix mix. Extraction conditions: 10% acetonitrile-modified CO_2, 490 atm, 40 °C, 1.5 mL/min for 30 min, liquid trap consisting of 3.5 mL of 85/15 8 mM NH_4OAc/acetonitrile pH adjusted to 6.5 using acetic acid. HPLC conditions: Deltabond ODS, 250 × 4.6 mm, 5 μm d_p. Mobile phase: 85/15 8 mM NH_4OAc/acetonitrile pH adjusted to 6.5 using acetic acid. Flow 1 mL/min. Detector UV monitored at 266 nm. Elution order: (1) SMZ, (2) SQX, (3) SDM.

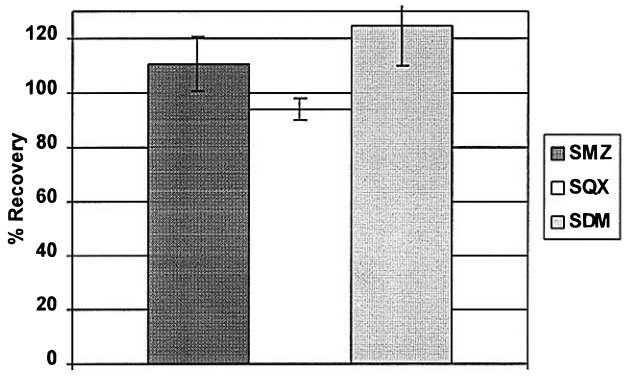

Figure 4. Extraction efficiency of SMZ, SQX, and SDM spiked at 960 ppb onto a chicken liver−Hydromatrix mix using 20% acetonitrile-modified CO_2.

desirable to more closely simulate a sample near the regulatory limit set by the Food Safety and Inspection Service. Quantitative recovery at the lower levels was obtained for the first time for all three analytes using 20% acetonitrile.

Beef liver sample extractions were attempted using the two best conditions found for chicken liver (20% acetone and acetonitrile). Beef liver has been found to be more difficult to extract than chicken tissues. Combs et al. (1996) previously obtained only 41% SMZ, 24% SDM, and less than 2% recovery for SQX from beef liver using 10% methanol-modified CO_2. Table 5 shows that quantitative recovery was obtained for all three analytes within 30 min using 20% acetone. The acetone again caused chromatographic interference (Figure 5). In an attempt to reduce interferences, the sample was evaporated under a stream of nitrogen to a volume of 2−3 mL, thus removing the acetone. The sample was then redissolved in the chromatographic mobile phase (Figure 6). This procedure greatly reduced the amount of interference caused by the residual acetone. Similar peak areas were obtained from both chromatograms (within 5%); therefore, all quantitation was performed without acetone evaporation.

Table 5. Percent Recovery of Sulfamethazine (SMZ), Sulfaquinoxaline (SQX), and Sulfadimethoxine (SDM) from a Beef Liver−Hydromatrix Mix Using 20% Acetone and 20% Acetonitrile-Modified CO_2[a]

	20% acetone	20% acetonitrile
SMZ	100 (9)	93 (6)
SQX	91 (3)	81 (8)
SDM	97 (2)	95 (6)

[a] Numbers in parentheses are relative standard deviations.

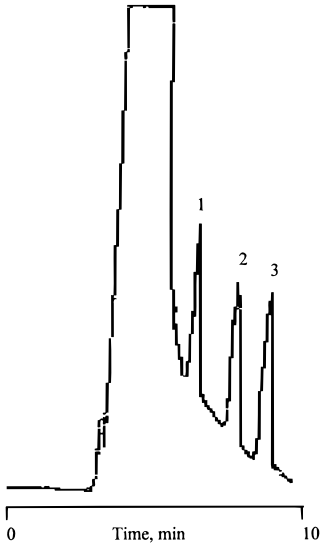

Figure 5. HPLC separation of sulfonamides extracted from beef liver−Hydromatrix mix prior to acetone evaporation. Extraction conditions: 20% acetone-modified CO_2, 490 atm, 40 °C, 1.5 mL/min for 30 min, liquid trap consisting of 3.5 mL of 85/15 8 mM NH_4OAc/acetonitrile pH adjusted to 6.5 using acetic acid. HPLC conditions: Deltabond ODS, 250 × 4.6 mm, 5 μm d_p. Mobile phase: 85/15 8 mM NH_4OAc/acetonitrile pH adjusted to 6.5 using acetic acid. Flow 1 mL/min. Detector UV monitored at 266 nm. Elution order: (1) SMZ, (2) SQX, (3) SDM.

Acetonitrile-modified CO_2 (20%), which was found to be the most efficient modifier for chicken liver, was not able to produce quantitative recovery for all three analytes from beef liver (Table 5). Greater than 90% recovery was obtained for SMZ and SDM, but only 81% recovery was obtained for SQX. It is not surprising that the interaction between the analyte, fluid, and the matrix would be different between beef and chicken liver since sulfonamides are known to reversibly bind to proteins (Seydel, 1971). This matrix interaction is affected differently using different modifiers, as in the case of beef liver where acetone was found to more completely extract the sulfonamides.

Lastly, an egg yolk sample was extracted using both 20% acetone, and 20% acetonitrile-modified CO_2 since each modifier was found to yield complete extraction of each sulfonamide from chicken liver or beef liver depending on the matrix. However, by employing modifiers that possess better characteristics for the analytes (solubility and polarity), both acetone and acetonitrile produced quantitative recovery of all three analytes. Recoveries obtained using acetonitrile (20%)

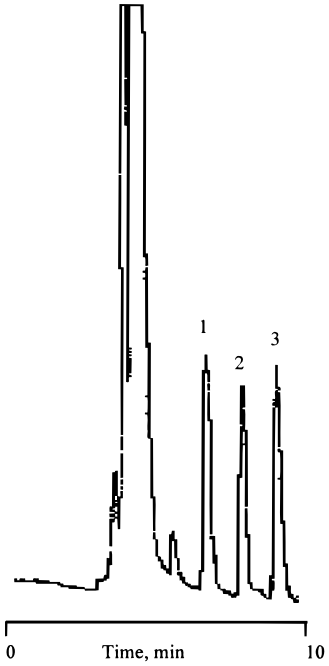

Figure 6. HPLC separation of sulfonamides extracted from beef liver−Hydromatrix mix followed by evaporation of acetone and rediluted with 85/15 8 mM NH_4OAc/acetonitrile. Extraction conditions: 20% acetone-modified CO_2, 490 atm, 40 °C, 1.5 mL/min for 30 min, liquid trap consisting of 3.5 mL of 85/15 8 mM NH_4OAc/acetonitrile pH adjusted to 6.5 using acetic acid. HPLC conditions: Deltabond ODS, 250 × 4.6mm, 5 μm d_p. Mobile phase: 85/15 8 mM NH_4OAc/acetonitrile pH adjusted to 6.5 using acetic acid. Flow 1 mL/min. Detector UV monitored at 266 nm. Elution order: (1) SMZ, (2) SQX, (3) SDM.

Table 6. Percent Recovery of Sulfamethazine (SMZ), Sulfaquinoxaline (SQX), and Sulfadimethoxine (SDM) from an Egg Yolk−Hydromatrix Mix Using 20% Acetone and 20% Acetonitrile-Modified CO_2[a]

	20% acetone	20% acetonitrile
SMZ	99 (0.5)	108 (3)
SQX	103 (2)	107 (4)
SDM	107 (1)	110 (3)

[a] Numbers in parentheses are relative standard deviations.

and 20% acetone are shown in Table 6. Both fluids produced better than 99% recovery for all three analytes. Acetonitrile, although, is the modifier of choice since acetone causes UV detection problems.

CONCLUSIONS

Supercritical CO_2 modified with 5, 10, and 20% methanol, ethanol, acetone, and acetonitrile were compared for the extraction of sulfonamides from fortified chicken liver. The results showed both 20% acetone-modified CO_2 and 20% acetonitrile-modified CO_2 were capable of quantitatively extracting all three sulfonamides from chicken liver, and the analysis could be performed with no sample cleanup. The optimum modifier was found to change with the matrix. Acetone better extracted sulfonamides from beef liver, whereas acetonitrile was slightly better for chicken liver. Either acetone or acetonitrile modifier was found to yield quantitative recovery of all sulfonamides from egg yolk. Acetonitrile-modified CO_2, however, produced fewer chromatographic interferences than acetone, making quantitation easier. In addition, it was demonstrated that liquid trapping of the analytes was efficient and no extensive cleanup procedure was necessary even with high levels of modifier. It would appear that sample preparation via a supercritical fluid affords a viable alternative to conventional sample preparation techniques for the quantitative analysis of sulfonamides in biological matrices.

ABBREVIATIONS USED

NH_4OAc, ammonium acetate; ODS, octadecylsilica; SFE, supercritical fluid extraction; SMZ, sulfamethazine; SQX, sulfaquinoxaline; SDM, sulfadimethoxine.

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