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Short communication

Multiresidue supercritical fluid extraction method for the recovery at low ppb levels of three sulfonamides from fortified chicken liver

Robert J. Maxwell*, Alan R. Lightfield

U.S. Department of Agriculture, Agricultural Research Service, Eastern Regional Research Center, 600 East Mermaid Lane, Wyndmoor, PA 19038, USA

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Abstract

A supercritical fluid extraction (SFE) method is proposed for the recovery of three sulfonamides from chicken liver. Samples were extracted at 680 bar and 40°C using unmodified carbon dioxide and were collected free of co-extracted artifactual material on an in-line neutral alumina sorbent bed. High recoveries of sulfamethazine (SMZ), sulfadimethoxine (SDM) and sulfaquinoxaline (SQX) were obtained from chicken liver samples fortified at levels from 1000 to 50 ppm.

Keywords: Sulfamethazine; Sulfadimethoxine; Sulfaquinoxaline

1. Introduction

Many classes of pharmaceuticals are administered to farm animals in the U.S. both for therapeutic and prophylactic purposes, including compounds such as the sulfonamide antimicrobials [1,2]. The responsibility for monitoring the level of these compounds in edible tissues is assigned to the Food Safety Inspection Service (FSIS, USDA). Organic solvent-based methods are typically used by FSIS and other regulatory agencies to monitor for residues of these drugs in tissue [2]. For instance, the current FSIS method for sulfonamides in tissues employs large volumes of both dichloromethane and ethyl acetate [3], which are on the EPA list of hazardous solvents mandated for reduction or elimination [4]. This mandate has spurred interest in alternative technologies such as supercritical fluid extraction (SFE) as potential replacements for conventional sulfonamide isolation methods.

Recently, we reported the use of SFE for the isolation of three sulfonamides; sulfamethazine (SMZ), sulfadimethoxine (SDM) and sulfaquinoxaline (SQX), from fortified chicken liver tissue. The extractions were accomplished using unmodified SF CO_2 on an SFE assembled in our laboratory. Sulfonamides were collected both off-line after carbon dioxide decompression in standard SPE columns and in-line on sorbent beds contained in the extraction vessel containing the sample matrix. The off-line SPE columns and the in-line sorbent beds subsequently were eluted with the HPLC mobile phase to recover the target analytes [5]. Comparison studies demonstrated that sulfonamides collected in-line

*Corresponding author.

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during the dynamic extraction process were obtained in higher recoveries and yielded HPLC chromatograms with lower background interference than similar sulfonamide mixtures collected off-line after carbon dioxide decompression.

In our earlier study, we tested this SFE method with chicken liver samples fortified at the 1.0 ppm level, whereas the current tolerance level for these drugs in poultry is set by the FSIS at 100 ppb [2]. Moreover, our initial investigation was performed on a laboratory assembled apparatus, rather than a commercially available instrument, as would be required for adoption by regulatory agencies.

Since our original investigation, our laboratory assembled SFE has been patented and is now a commercially available product [6]. This achievement has allowed us to test our SFE method on a commercial apparatus at the tolerance levels set by FSIS. Moreover, the original laboratory assembled SFE required the use of carbon dioxide cylinders pressurized with helium, which are not required with the commercial version. In fact, recent reports have suggested that recoveries of some analytes may be affected by the presence of helium in SF CO_2 [7,8], a premise which we also tested in the present investigation.

2. Experimental

2.1. Reagents and chemicals

2.1.1. Chicken liver

Bulk tissue was purchased from a local retail outlet. A food processor was used to blend two pounds of tissue into a homogenous mixture. The homogenous tissue was divided into 60 g portions and placed in 4 ml ziplock bags which were frozen solid and stored at −76°C until needed.

2.1.2. Reagents

Hydromatrix (Celite 566) was obtained from Varian Sample Preparation Products (Harbor City, CA, USA). Aluminum oxide (activated, neutral, Brockman 1, ~150 mesh) and 40% aq. tetrabutylammonium hydroxide (TBAH) were purchased from Aldrich (Milwaukee, WI, USA). Methanol (MeOH), a Burdick and Jackson high-purity solvent, was obtained from Baxter Health Care (Muskegon, MI, USA) and was used in the HPLC mobile phase and in sulfonamide stock solutions. HPLC-grade *N*,*N*-dimethylformamide (DMF) was purchased from Fisher Scientific (Fairlawn, NJ, USA). Sulfamethazine (SMZ, 99% purity) and sulfadimethoxine (SDM, 98% purity) were obtained from Sigma (St. Louis, MO, USA). Sulfaquinoxaline (SQX, 98% purity) was purchased from Pfaltz and Bauer (Waterbury, CT, USA). SFC-grade carbon dioxide was a product of Scott Specialty Gases (Plumsteadville, PA, USA).

2.1.3. Solutions

Stock solutions of each sulfonamide were prepared at concentrations of 2.0 mg/ml each in methanol for SMZ and SDM. The SQX stock solution was 2.0 mg/ml in *N*,*N*-dimethylformamide. Sulfonamide working solutions were prepared at concentrations of 10 and 100 ng/μl each in methanol.

2.1.4. HPLC standard solutions

Each solution contained the three sulfonamides at concentrations of 20, 30, 40 and 50 ppb each in the HPLC mobile phase (see HPLC analysis).

2.2. Pre-SFE sample preparation

Samples (~1.0 g) of frozen, homogenous chicken livers were weighed on tared glassine papers. Samples were maintained at frozen conditions while the top surface of the tissue cubes was scored with a scalpel. A 25 μl Hamilton syringe (Baxter Diagnostic, McGraw Park, IL, USA) was used to deliver the desired amount of fortification solution to the slot in the tissue surface. Each sample was incubated for 1 h at −10°C and then blended in a beaker with 2.0 g of Hydromatrix to disperse the tissue.

A 24 ml (10 000 p.s.i.) SFE vessel obtained from Keystone Scientific (Bellefonte, PA, USA) was fitted with an endcap and sealed. The sealed end was the ''top'' when it was vertically attached in the SFE oven. The vessel was packed in the following order: a plug of polypropylene wool (Aldrich), a 20 μm polyethylene (PE) frit (Cat. No. 7965, Applied Separations), 2.0 g neutral alumina, a 20 μm PE frit, the free-flowing tissue/Hydromatrix mixture, a 20 μm PE frit, 3–4 g Hydromatrix and a final 20 μm

PE frit at the vessel ''bottom''. The vessel contents were tightly packed with a tamping rod after each successive addition of a 20 μm PE frit. The ''bottom'' endcap was threaded in place and sealed. Packed extraction vessels were stored at −4°C until they were installed in the SFE oven module.

2.3. Supercritical fluid extraction

The Spe-ed SFE Model 7010 extractor was purchased from Applied Separations (Allentown, PA, USA) and was configured to parallel extract two vessels. The Model 7010 also was configured with an optional pumphead chiller assembly, which was cooled to 0–5°C using a Neslab RTE-110 refrigerated circulating bath (Fisher Scientific, Malvern, PA, USA) maintained at −15°C and obviating the need for helium pressurized carbon dioxide cylinders. A 6 ml solid-phase extraction (SPE) column filled with 2.0 g of neutral alumina was connected to the instrument at the off-line micrometering valve–SPE interface.

The extraction vessels were installed upright in the SFE with the endcap labeled ''top'' connected to the upper fitting. The micrometering valves were preheated to 110°C and the system was pre-pressurized to 655 bar (9500 p.s.i.) with SF CO_2. To equilibrate the vessels, a static hold period of 5 min at 40°C was used prior to beginning the dynamic extraction, then the system pressure was set at 680 bar (10 000 p.s.i.) and the dynamic flow of carbon dioxide (expanded gas) was adjusted to 2.5–2.7 l/min. Flow-rates were measured on a Floline SEF-51 flow meter-gas totalizer (Horriba, Sunnydale, CA, USA) attached to the off-line SPE column. Flow was maintained until a total of 120 l carbon dioxide (expanded gas) was recorded.

After system depressurization, the vessels were cooled and the endcap labeled ''top'' was unscrewed from each vessel. The polypropylene wool and PE frit were removed and the in-line alumina sorbent bed was carefully transferred into an empty 6 ml SPE column fitted with a 20 μm PE frit. A second frit was placed over the sorbent layer and the bed was tightly compressed with a tamping rod. Analytes were recovered by eluting the SPE column with 4 ml of the HPLC mobile phase and collecting the first 2 ml of the eluate for analysis, a portion of which (100 μl) was injected directly into the HPLC.

2.4. HPLC analysis

The HPLC system was a Hewlett-Packard 1050 Series (Valley Forge, PA, USA) equipped with a HP 1050 diode array detector. The mobile phase was a 68% 0.05 *M* phosphate buffer (pH 7.0) containing 0.1% tetrabutylammonium hydroxide (final pH 7.2) and 32% methanol. The flow-rate was 0.9 ml/min. All instrumental operations, methods development and chromatographic analysis were performed by means of a HP Vectra XM2 computer utilizing HP Chemstation software. The diode array detector was set at the UV maximum for each sulfonamide: SMZ at 263 nm, SDM at 269 nm and SQX at 252 nm. Analytes were separated on a Supelcosil LC18 column (25 cm×4.6 mm I.D.) (Bellefonte, PA, USA). Quantitation was accomplished using external standards to develop a standard curve.

3. Results and discussion

Our goal in this investigation was to develop the in-line sulfonamide collection technique at analyte concentrations of interest to regulatory agencies (50–100 ppb). In our earlier study using the laboratory assembled SFE, the lowest fortification level tested with chicken liver samples was 1000 ppb. Therefore, in our initial experiments, we repeated extractions at this fortification level using a two-vessel commercially available version of the one-vessel laboratory assembled SFE. The percent recoveries of the sulfonamides obtained from the two versions of the same SFE apparatus design are shown in Table 1. In trials on the commercial SFE, the recoveries were slightly higher for SDM (98.2 vs. 89.9) and SQX (80.6 vs. 76.4) and somewhat lower for SMZ (83.2 vs. 89.9) compared to recoveries obtained by Parks and Maxwell [5] using the laboratory assembled apparatus. The laboratory assembled SFE and the commercial model were different in some design features; the commercial model could extract two samples simultaneously and did not require the use of helium pressurized carbon dioxide cylinders, unlike the laboratory assembled apparatus. It would

Table 1
Comparison of sulfonamide recoveries from chicken liver at three fortification[a] levels

Fortification level (ppb)	*n*	Recovery (mean%±SD)		
		Sulfamethazine (SMZ)	Sulfadimethoxine (SDM)	Sulfaquinoxaline (SQX)
1000[b]	5	89.9±2.3	96.9±1.2	76.4±3.0
1000[c]	4	83.2±3.0	98.2±3.0	80.6±4.0
100[c]	23	86.5±5.7	92.2±6.4	78.4±5.9
50[c]	12	88.2±10.2	87.0±6.8	71.6±6.0

[a]SFE conditions: 40°C; 680 bar (density CO_2 1.042 g/ml); flow 2.5–2.7 l/min (expanded gas); duration 40 min.
[b]Laboratory assembled SFE.
[c]Commercial model SFE.

be difficult, however, to ascribe the small differences in recoveries obtained by the two instruments to their vessel capacity or to the presence or absence of helium in the SF CO_2. Previous to this study, we found that the solubility of other pharmaceutical compounds in SF CO_2 was significantly enhanced due to the presence of water and lipids in the tissue samples from which they were extracted [9]. Increases in analyte solubility due to the presence of these tissue components in a supercritical fluid may outweigh any potential losses in recoveries that could occur when helium is present in the SF CO_2.

The SFE results obtained for chicken livers fortified at the 1.0 ppm level suggested that sulfonamide fortification levels could be significantly lowered without affecting the overall method performance. To verify this, liver samples were fortified at 100 ppb per sulfonamide and extracted under the same experimental conditions used at the higher fortification level (Table 1). A total of 23 samples were extracted in five subsets. The 100 ppb sets are compared in Table 1 with those obtained at the 1.0 ppm fortification level, where it may be seen that recoveries and standard deviations are similar even with the 10-fold difference in sulfonamide concentrations.

Additional studies were performed to measure sulfonamide recoveries below the 100 ppb tolerance level in order to determine the minimum level of reliable measurement. Liver samples were fortified at 50 ppb per sulfonamide and extracted using the SFE conditions employed at the higher fortification levels (Table 1). Each extraction set comprised four to six samples. The mean average of the data are similar to those obtained at the 100 ppb fortification level, although the standard deviations at the 50 ppb level are somewhat higher. In contrast to the results presented in this study, other investigators have reported poor recoveries using unmodified SF CO_2 to recover sulfonamides from animal tissues [10–12]. For example, Ashraf-Khorassani et al. reported recoveries of 30, 38 and 21%, respectively, for SMZ, SDM and SQX from chicken liver fortified with 600 ppb of each sulfonamide [11], and Combs et al. reported percentages of 23, 18 and <2, respectively, for the same sulfonamides from beef liver fortified at the 12 ppm level [12]. The poor recoveries obtained by these and other investigators may be due in part to the extraction pressures employed in their studies (<470 bar) and the use of off-line rather than in-line trapping. It has been our experience that operating pressures in the range of 600–680 bar are necessary to ensure acceptable recoveries of sulfonamides from tissue samples. Moreover, we have observed that off-line trapping using unmodified carbon dioxide may decrease target analytes recoveries. In addition, the extracts obtained from off-line traps may produce HPLC chromatograms that are difficult to interpret and quantify, a topic discussed in the next paragraph.

Comparisons were made between HPLC chromatograms of liver samples fortified with sulfonamides at the 100 ppb level obtained by both in-line and off-line trapping to illustrate the selectivity for polar analytes possible when employing the in-line technique. While post-SFE processing remained the same for both in-line and off-line eluates, their chromatograms vary dramatically (Fig. 1). Fig. 1b depicts a control liver eluate from an in-line trap while Fig. 1d is a chromatogram of a similar control eluate from an off-line SPE column. The in-line control liver chromatogram (Fig. 1b) is free of background interference in the retention windows

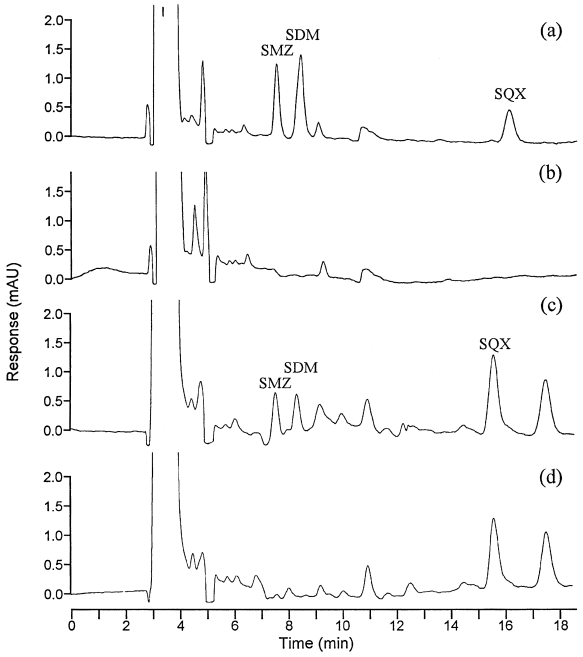

Fig. 1. HPLC chromatograms (UV detection at 269 nm) of post-SFE chicken liver extracts from in-line alumina sorbent beds: (a) liver fortified with three sulfonamides at 100 ppb each; (b) control liver, off-line alumina SPE columns; (c) liver fortified with three sulfonamides at 100 ppb each; (d) control liver.

where the analytes of interest elute. In contrast, the control liver chromatogram (Fig. 1d) from the off-line alumina SPE column is a continuous pattern of eluting peaks. The chromatogram in Fig. 1c is of a fortified liver eluate obtained from an off-line SPE column. The peak for SQX in the chromatogram is twice the area of the peak for the same analyte from an in-line trap (Fig. 1a). The enlarged SQX peak occurred because of the presence of a large artifact in the control liver chromatogram (Fig. 1d) with the same retention time as SQX, a condition not encountered with in-line solute trapping.

4. Conclusions

This study demonstrates that polar analytes can be isolated from biological matrices using unmodified supercritical carbon dioxide. The in-line trapping techniques described permit polar analytes such as sulfonamides to be trapped in the supercritical state free of co-extracted artifacts and prepares solutes eluted from the in-line sorbent beds for facile and rapid post-SFE chromatographic analysis. This technique for sulfonamide recovery from animal tissues has achieved the method development requirements of the FSIS and the solvent reduction and elimination standards set by the EPA.

Acknowledgements

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