

Effect of Sample Matrix Dehydration During Supercritical Fluid Extraction on the Recoveries of Drug Residues from Fortified Chicken Liver

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Abstract

The role of H₂O in the supercritical fluid extraction (SFE) of zoalene (3,5-dinitro-*o*-toluamide) and its metabolites from fortified chicken livers is reported. Dehydration of the sample matrix during SFE results in adsorption of the analytes, limiting their extraction. Adding 0.5 mL of additional water to the sample matrix prior to SFE prolongs the effective extraction period, thereby increasing the recoveries of the metabolites 12–20%. The following recoveries are found for zoalene, 3-amino-5-nitro-*o*-toluamide, 5-amino-3-nitro-*o*-toluamide, and N⁵-acetyl-3-nitro-*o*-toluamide extracted from a liver-Na₂SO₄ mixture with added H₂O at 60°C and 1034 bar (density, 1.069) using 3 L CO₂/min (expanded gas) for 30 min: 87, 92, 77, and 66%, respectively.

Introduction

The use of supercritical carbon dioxide (CO₂) to extract veterinary drug residues from biological matrices, in lieu of solvent extraction techniques, is currently under investigation in our laboratory. Our initial work in this area on the relatively non-polar anticoccidial nitrobenzamides (nitromide, aklomide, and zoalene) (1) was encouraging and led to further supercritical fluid extraction (SFE) studies on anabolic steroids (nortestosterone, testosterone, and methyl testosterone) (A.A.M. Stolker, L.A. vanGinkel, R.W. Stephany, R.J. Maxwell, O.W. Parks, A.R. Lightfield, and M.B. Medina; submitted for publication) and the more polar sulfonamides (sulfamethazine, sulfadimethoxine, and sulfaquinoxaline) (2). In each of these investigations, the chemical structures, functional groups, and polarities of the compounds in each drug class were similar and presented no particular problem with extraction from the biological matrix with neat supercritical CO₂. In the later two investigations, an in-line adsorbent trap was used to collect the analytes from supercritical CO₂. This proved very effective in not only improving recoveries but also eliminating the need for time-consuming post-extraction cleanup procedures that are

performed prior to analysis and are generally required with off-line collection techniques. For the most part, this procedure is not compatible with extractions employing supercritical CO₂ modified with polar organic solvents because of potential breakthrough of analytes from in-line sorbent beds. The approach of in-line trapping of analytes extracted with neat supercritical CO₂ was applied to zoalene (3,5-dinitro-*o*-toluamide) and three metabolites of the drug (3,4): 3-amino-5-nitro-*o*-toluamide (3-ANOT), 5-amino-3-nitro-*o*-toluamide (5-ANOT), and N⁵-acetyl-3-nitro-*o*-toluamide (N⁵-Ac). It became obvious early in the study that maximizing the simultaneous extraction of these four analytes with varying functional groups and polarities would require a better understanding of the SFE process. This was needed because their overall recoveries were poor when the experimental conditions used in earlier investigations were employed. This report summarizes our efforts to better understand the SFE process for these drug components.

Experimental

Materials

Anhydrous sodium sulfate (Na₂SO₄) was obtained from Mallinckrodt (Paris, KY). Neutral alumina (Al₂O₃), Brockman #1, approximately 150 mesh, was purchased from Aldrich (Milwaukee, WI). SFC-grade carbon dioxide was a product of Scott Specialty Gas (Plumsteadville, PA). Methanol (MeOH) was obtained from Baxter Health Care (Muskegon, MI). A standard solution of zoalene (Salisbury Labs; Charles City, IA), 3-ANOT (a gift from Dow Chemical USA; Midland, MI), 5-ANOT (synthesized in our laboratory), and N⁵-Ac (synthesized in our laboratory) containing 0.5 µg of each analyte per microliter of MeOH was prepared and used to fortify samples.

Procedure

For sample preparation, 0.5 g of cubed, frozen, ground chicken liver was fortified with 4 µL of the drug standard solution by depositing the solution on the surface of the tissue; the solution permeated the tissue. Fortified tissue samples were

held at -10°C for 1 h and then added in a frozen state to 15 g anhydrous Na_2SO_4 contained in a 30-mL beaker. Following partial thawing, the tissue was ground into the Na_2SO_4 with a metal spatula until the tissue was dispersed throughout, and the mixture flowed freely. In cases where H_2O was added to liver- Na_2SO_4 mixtures, the H_2O was added in 0.05-mL increments and mixed thoroughly. The H_2O -laden sample matrix was held at 4°C for 10 min to dry the sample prior to adding to the extraction vessel (heating the vessel to 60°C before the dynamic phase of the extraction returned the mixture to a "wet" state).

A high-pressure (20,000 psi) extraction vessel (approximately 26 mL) was assembled from a 25.4- \times 1.15-cm i.d. nipple (CNX12010) and two couplings (20F12463) obtained from Autoclave Engineers (Erie, PA). Each coupling was fitted with 40-mesh i.d. stainless steel wire cloth disk (Small Parts; Miami, FL) which served as frits in the assembled vessel. The vessel was packed in the following sequence relative to the exit portion of the vessel: a large plug of polypropylene wool (Aldrich, Milwaukee, WI); 4 g neutral Al_2O_3 ; a plug of polypropylene wool; tissue- Na_2SO_4 mixture; and sufficient polypropylene wool to fill most dead space of the nipple. All additions to the nipple were packed tightly using a stainless steel tamping rod.

SFE procedure

The SFE apparatus used in these studies was designed and assembled for operation at 15,000 psi. Carbon dioxide was pressurized using a gas booster pump (Model AGT-62/152C, Haskel Engineering; Burbank, CA). The pressurized fluid flowed to a temperature programmable oven (Model LAC 1-67, Despatch; Minneapolis, MN) through a 1-m heat transfer coil and then to a shut-off valve (No. 30VM-4074, Autoclave Engineers) located at the base of the oven. The extraction vessel was vertically connected to this valve through a short length of tubing (the system was configured with $\frac{1}{4}$ -in. o.d. \times 0.083-in. i.d. 316 stainless steel tubing fitted with cone and thread connections). The upper fitting of the vessel was connected to a three-way valve (No. 30VM-4075, Autoclave Engineers) also mounted in the oven. This valve in turn was attached to a micrometering valve (No. 30 VRMM-4812, Autoclave Engineers) mounted on the outside oven wall. The micrometering valve was encased in a heated aluminum block. The original connector nut on the

low pressure side of the valve was replaced by a laboratory fabricated assembly that enabled standard commercial 6-mL solid-phase extraction (SPE) columns to be interfaced directly to the valve for off-line collection of analytes if necessary. A description of a similar heating block and micrometering valve has been reported previously (1). The decompressed gas from the valve passed through the sorbent (Al_2O_3) in the SPE column, and its flow was measured on a Floline SEF-51 flow monitor (Scott Specialty Gases; Plumsteadville, PA).

Following installation of the extraction vessel in the SFE apparatus, the closed system was pressurized to 13,500 psi, and the oven was heated to 60°C . The micrometering valve had been preheated to 110°C prior to pressurization. After the extraction vessel reached the set point temperature (approximately 15 min), the pressure was adjusted to 15,000 psi, and the dynamic phase of the extraction began.

Analyte recovery and chromatography

At the conclusion of the extraction and after depressurization, the extraction vessel was removed from the SFE apparatus. The in-line alumina sorbent bed was removed from the vessel by pouring into an empty 6-mL SPE column. The alumina was compacted by tapping the sides and top of the SPE column with a spatula, and then a 5-mm layer of sand was placed on top. Analytes were recovered by elution with the HPLC mobile phase described below. The first 2.5 mL of eluent was collected, then 100 μL of eluent was injected into the HPLC system.

HPLC analyses were performed with an ISCO LC-5000 syringe pump (ISCO; Lincoln, NE) and a Rheodyne Model 7125 injector (Berkeley, CA) connected to a 25-cm \times 4.6-mm i.d. Supelcosil LC-18 column (5 μM) (Supelco; Bellefonte, PA). Analyte detection was accomplished at 254 nm using an Applied Biosystems Model 1000S diode array detector (Foster City, CA). The HPLC mobile phase was 70% 0.05M phosphate buffer (pH 7.0) and 30% MeOH at a flow rate of 0.8 mL/min. Chromatograms were recorded on a Hewlett-Packard Model 3396A integrator (Valley Forge, PA). Quantitation of analyte recoveries was accomplished by comparison of peak heights, areas, or both with drug control standards injected at concentrations equivalent to fortification levels.

Table I. Effect of Total CO_2 and Added H_2O on SFE Recoveries of Zoalene and its Metabolites from Fortified Liver Tissue*

Total CO_2 (L)	Added H_2O (mL)	No. of determinations	Mean % \pm standard deviation			
			3-ANOT	5-ANOT	$\text{N}^5\text{-Ac}$	Zoalene
30	0	5	77.9 \pm 2.2	60.8 \pm 3.4	36.5 \pm 3.7	91.7 \pm 1.3
60	0	5	77.9 \pm 2.5	62.0 \pm 3.3	47.1 \pm 2.8	88.1 \pm 1.4
90	0	9	78.8 \pm 4.3	62.4 \pm 4.8	45.7 \pm 2.7	88.8 \pm 1.7
30	0.5	5	76.6 \pm 1.8	58.7 \pm 5.5	31.2 \pm 3.8	89.6 \pm 3.1
60	0.5	5	87.9 \pm 3.4	72.5 \pm 0.7	50.2 \pm 2.6	89.6 \pm 2.7
90	0.5	12	91.8 \pm 2.0	77.4 \pm 3.2	65.7 \pm 2.8	87.3 \pm 5.0

* SFE conditions: temperature, 60°C ; pressure, 15,000 psi (density, 1.069); flow rate, 3 L/min (expanded gas). The fortification level was 4.0 ppm/drug.

Results and Discussion

Preliminary studies showed that recoveries of the three zoalene metabolites from liver tissue were greater when the tissue had been "dispersed" in anhydrous sodium sulfate than in diatomaceous earth, which had been employed in previous studies (A.A.M. Stolker, L.A. vanGinkel, R.W. Stephany, R.J. Maxwell, O.W. Parks, A.R. Lightfield, and M.B. Medina; submitted for publication) (2). In addition to the dispersion medium, the recoveries of the metabolites, especially $\text{N}^5\text{-Ac}$, which is the most difficult to extract, were dependent on the weight of the tissue extracted. For

example, the recovery of N⁵-Ac from 0.25 g of tissue was twice that obtained from 1.0 g of tissue (all other parameters being equal). For the purposes of this study, a 0.5 g of tissue sample dispersed in 15.0 g Na₂SO₄ was selected as the ideal combination.

Initially, SFE of zoalene and its metabolites from liver tissue was conducted under conditions similar to those employed in the successful extraction of sulfonamides from chicken tissues (2). The following parameters were used: extraction temperature, 40°C; pressure, 10,000 psi (density, 1,043); CO₂, 120 L (expanded gas) at a flow rate of 3 L/min; and metering valve temperature, 110°C. Drug residues were collected on a 4.0-g neutral alumina in-line trap. Approximate average recoveries were as follows: 3-ANOT, 70%; 5-ANOT, 50%; N⁵-Ac, 15%; and zoalene, 90%. Increasing the extraction pressure to 15,000 psi (density, 1.108) increased the recovery of 3-ANOT, 5-ANOT, and N⁵-Ac an additional 15% each without affecting the recovery of the parent drug. A maximum recovery of approximately 45% for N⁵-Ac was attained at 60°C and 15,000 psi (density, 1,069) using 90 L CO₂. However, increasing the temperature from 40 to 60°C caused a corresponding decrease in density, which had a negative effect on the recoveries of 3-ANOT and 5-ANOT; the recoveries were decreased approximately 10 and 5%, respectively. Extractions at temperatures higher than 60°C resulted in degradation of zoalene; therefore, higher temperature studies were abandoned.

Retention times on reversed-phase chromatographic columns are an indication of the relative polarities of solutes and, in turn, their relative solubilities in supercritical CO₂ (5). Based on the reversed-phase retention times of the analytes in this study (3-ANOT, 5.0 min; 5-ANOT, 6.4 min; N⁵-Ac, 9.0 min; zoalene, 13.4 min), the relative solubilities in supercritical CO₂ and, therefore, the predicted recovery rates of the analytes are the following: zoalene > N⁵-Ac > 5-ANOT > 3-ANOT. The recoveries obtained in this study (zoalene > 3-ANOT > 5-ANOT > N⁵-Ac), however, suggested that a factor(s) other than solubility plays a key role in the recovery of the analytes, especially the 5-ANOT and N⁵-Ac metabolites.

Table I (Rows 1, 2, and 3) summarizes the recovery data obtained at an extraction temperature of 60°C and 15,000 psi

(density, 1.069) after 30, 60, and 90 L CO₂ (expanded gas) at a flow rate of 3 L/min. With the exception of N⁵-Ac, which required more than 30 L CO₂, maximum recoveries of the analytes were achieved with less than 30 L CO₂. No breakthrough of analytes from the in-line adsorption trap occurred, as determined by analyzing effluents of the off-line trap; this suggested that changes in the sample matrix (liver-Na₂SO₄) were occurring during SFE that prevented further extraction of the drugs after 30 L. Visual examination of the sample matrix following SFE with 30 L CO₂ showed dehydration had occurred, suggesting adsorption of the drugs on the dehydrated sample matrix limited any further extraction. Table I (Rows 4, 5, and 6) also summarizes recovery data on samples to which 0.5 mL H₂O was added to the liver-Na₂SO₄ mixture prior to SFE. The data demonstrate that the additional H₂O (liver contains 70% H₂O by weight) retarded the development of a dehydrated state and extended the effective SFE period, thereby increasing the recoveries of the metabolites. The 0.5 mL of added H₂O approached the maximum H₂O content feasible for the amount of liver-Na₂SO₄ mixture employed in this study. Higher amounts of H₂O also resulted in breakthrough of analytes (especially zoalene) from the in-line Al₂O₃ sorbent trap.

Further evidence of changes that occur in the sample matrix during SFE and limit recoveries is presented in Table II. As a first step, 0.5 g of unfortified liver plus 15 g anhydrous Na₂SO₄ were extracted 30 min at 60°C and 15,000 psi (density, 1.069) with supercritical CO₂ at a flow rate of 3 L/min. Following SFE, the sample matrix was recovered from the extraction vessel and spiked with 2.0 µg each of zoalene and its metabolites. Following thorough mixing, the now fortified sample matrix was reextracted in the second step under the same conditions as before. Following reextraction, the sample was once again recovered from the vessel, and 0.85 mL H₂O was added. The sample matrix-H₂O was mixed thoroughly and reextracted for a third time. The results presented in Table II demonstrate the role of H₂O in the SFE of zoalene and its metabolites from liver tissue. The recovery of the analytes from previously extracted tissue-Na₂SO₄, void of H₂O and other supercritical CO₂ extractables (e.g., lipid, pigments), are only a fraction of that reported in Table I. The addition of H₂O to the twice extracted sample matrix resulted in the further extraction of increased amounts of the analytes; the total analytes recovered approached the recoveries presented in Table I. The lower total recovery of 5-ANOT may be the result of the relatively lower recoveries of the analyte from solely anhydrous Na₂SO₄ and Na₂SO₄ with added H₂O (Table II), which served as controls in these studies.

The incomplete extraction of the analytes from liver tissue, especially 5-ANOT and N⁵-Ac, cannot be explained solely on the basis of adsorption following dehydration during SFE. For example, the recoveries of 5-ANOT and N⁵-Ac from preextracted tissues without added H₂O (Table II) differs little from that of 3-ANOT, but the recoveries from fresh tissue (Table I) differ significantly. Other factors,

Table II. Effect of H₂O on SFE Recoveries of Zoalene and its Metabolites from Fortified Na₂SO₄ and Previously Extracted Liver*

Sample	Mean % ± standard deviation (n = 4)			
	3-ANOT	5-ANOT	N ⁵ -Ac	Zoalene
15 g Na ₂ SO ₄	80.7 ± 2.5	68.7 ± 2.2	81.9 ± 1.4	88.5 ± 1.8
Na ₂ SO ₄ + 0.5 mL H ₂ O	88.2 ± 2.6	81.9 ± 2.5	91.8 ± 1.4	90.7 ± 2.6
SF Extracted Liver [†]	19.1 ± 4.1	13.1 ± 3.0	17.3 ± 4.7	42.0 ± 5.3
+ 0.85 mL H ₂ O [‡]	67.2 ± 5.6	52.6 ± 3.9	47.3 ± 4.2	46.3 ± 3.3
Total extracted	86.3 ± 3.5	65.7 ± 5.8	64.6 ± 5.0	88.3 ± 4.3

* SFE conditions: temperature, 60°C; pressure, 15,000 psi (d = 1.069 g/mL); flow rate, 3 L/min (expanded gas) for 30 min. The fortification level was 2.0 µg of each analyte.
[†] 0.5 g liver + 15.0 g Na₂SO₄ subjected to SFE prior to fortification and reextraction.
[‡] 0.85 mL H₂O added to sample prior to reextraction.

including solubility of the analytes in H₂O-saturated supercritical CO₂, effect of coextractants other than H₂O on solubility, loss of other coextractants during SFE, and reversible or irreversible adsorption, may alter the extractability of analytes by supercritical CO₂ and need further investigation.

Tolerance levels of zoalene residues in chicken livers, set by the U.S. Code of Federal Regulations (6) are relatively high at 6 ppm and include only the parent compound and 3-ANOT. The SFE parameters outlined here (40°C, 15,000 psi, 90 L or 60°C, 15,000 psi, 90 L with 0.5 mL added H₂O) can allow detection of these levels in chicken tissues. The results compare favorably with recoveries of zoalene (99.2 ± 2.2%) and 3-ANOT (90.1 ± 2.5%) from fortified liver tissue by a chloroform-ethylacetate extraction technique (tissues with incurred residues were not available for analysis) (4). The findings demonstrate that SFE may offer an adequate alternative to solvent extraction techniques currently employed for isolating these regulated analytes and help federal regulatory agencies meet the EPA directive mandating a 50% reduction of specified organic solvents (8).

Conclusion

This study on the SFE of zoalene and its metabolites demonstrated that solubility in supercritical CO₂ alone does not determine the extent of extraction of analytes from a sample matrix. Specifically, these studies show that dehydration of the sample matrix during the SFE process limited the recovery of the metabolites, presumably as a result of their adsorption on the dried matrix. Increasing the H₂O content of the sample matrix prior to SFE, in effect prolonging the effective extraction time, resulted in improved recoveries of the analytes.

These observations suggest that modifying the sample matrix prior to SFE is a feasible approach that needs to be investigated to improve recoveries of analytes from biological systems.

Notice

Mention of brand or firm names does not constitute an endorsement by the U.S. Department of Agriculture over others of a similar nature not mentioned.

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