OPTIMAL ANALYSIS OF SULFONAMIDES FROM BIOLOGICAL MATRICES USING SUPERCRITICAL FLUIDS

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(ABSTRACT)

The objective of this research was to develop new sample preparation procedures for the isolation of sulfonamides, as well as, to determine the applicability of employing on-line nitrogen selective and mass spectrometric detection methods.

The first phase of this research investigated the effect of temperature and pressure on the supercritical fluid extraction (SFE) of sulfonamides from a spiked sand matrix. Temperature effects were either positive or negative with respect to extraction rate and total recovery, depending on the pressure and extraction fluid employed.

The second portion of this research compared trifluoromethane (CHF_3) and carbon dioxide (CO_2) as fluids for the extraction of sulfonamides from spiked non-fat dry milk, beef liver, and egg yolk were found to be more selective using CHF_3 than CO_2. The polar trifluoromethane improved the extraction efficiency of the polar sulfonamides from the biological matrices and also reduced the amount of co-extractives.

The next phase of this research considered the effect of organic modifier and CO_2 in the SFE of sulfonamides from chicken liver, beef liver and egg yolk. Methanol, ethanol, acetone, acetonitrile were compared to determine optimum conditions. A SFE method employing 20% acetonitrile modified CO_2 yielded quantitative recovery of sulfonamides from chicken liver, but 20% acetone modified CO_2 was required to obtain quantitative recovery from beef liver. Either 20% acetone or 20% acetonitrile yielded quantitative recovery from egg yolk.

The last phase of this research focused on the evaluation of selective detection methods for sulfonamide analysis. Chemiluminescence nitrogen detection (CLND) parameters were optimized for use with packed column supercritical fluid chromatography (SFC) yielding a

minimum detectable quantity (MDQ) of 5 ng of sulfamethazine, on column. Improvements in the detector design decreased the MDQ to 0.5 ng, while, decreasing the column diameter further reduced the MDQ to 125 pg. The second part of this phase evaluated HPLC/Atmospheric pressure chemical ionization (APCI) mass spectrometry for the detection of sulfonamides. Sensitivity in selective ion mode was found to be as low as 50 pg on column for sulfamethazine. Supercritical fluid extracts of sulfonamides spiked at 100µg/kg in chicken liver were found to be readily detected by this method.

This dissertation is dedicated to my wife, Amanda, and my parents,
Robert and Brenda Newsome

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Chapter I

INTRODUCTION

Sulfonamides are a class of anti-microbial agents that have seen extensive use in medicine. They were the first agents to be used for the treatment of bacterial infection (1). During the 1940's sulfonamides were considered wonder drugs for the treatment of bacterial infections for both human and animal disease. Use of sulfonamides today is limited to specific disease treatment in human medicine, such as urinary tract infections. However, sulfonamides are more often encountered in animal medicine. Since wide use is occurring, the presence of certain residues in animal products presents a potential health hazard due to their allergenic properties (2). 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 (3). In addition, a study by the National Center for Toxicological Research indicated that sulfamethazine (SMZ) may be a thyroid carcinogen (4).

History

Sulfonamides were first synthesized by Gelmo et al. (5) in 1908 while doing research into azo dyes. Directly following this work, Hoerlein et al. (6) discovered dyes containing the sulfanyl group had affinity for proteins of silk and wool. This led to the discovery by Eisenberg, in 1913, that chrysolidine, one of the azo dyes studied, had pronounced bactericidal action *in vitro* (6). However, it was not until 1932 that the therapeutic properties of sulfonamides were recognized. Dogmagk et al. (7) found Prontisil, ((p-[2,4-diaminophenyl) azo]sulfanilamide), had pronounced *in vivo* antibacterial activity. Sulfanilamide was later found to be the active portion of the Prontisil molecule (8) and this led to considerable research into sulfonamide drug investigation. Many different sulfonamides were synthesized during the late 1930's A great number of those were discovered to possess considerable antibacterial activity for a variety of streptococci and pneumonococci bacteria. Several sulfapyrimidines which were introduced in 1941 (9) were found to possess considerable antibacterial activity as well as lower toxicity than previous sulfonamides. This advancement led to many new sulfonamides being synthesized. Today there are over 5000 sulfa drugs in existence (1), but only 33 of those have been introduced for general medical use.

Mechanism of Action

The mechanism of action for sulfonamide drugs is well known. The key chemical involved is para-aminobenzoic acid (PABA), which is an essential component in the synthesis of dihydrofolic acid. Dihydrofolic acid (FAH_2) is required for normal growth of both bacterial and mammalian cells. Many higher animals are incapable of biosynthesis of FAH_2 and must rely on a pre-formed exogenous source that is able to cross the cell membrane by an active transport mechanism. Bacteria, however, cannot absorb preformed FAH_2 and must synthesize it by a reaction involving PABA, 2-amino-4-hydroxyl-6-hydroxylmethyl dihydropteridine diphosphate, and glutamic acid.

Sulfonamides interfere with bacterial growth by affecting FAH_2 production through two mechanisms, enzyme inhibition and pseudometabolite formation, where enzyme inhibition is the principle mode of action. **Figure 1** (1) shows the synthetic pathways for FAH_2 production. Sulfonamides interfere in the enzyme pathway between PABA or p-aminobenzoylglutamic acid (PABG) with 2-amino-4-hydroxyl-6-hydroxymethyl dihydropteridine diphosphate (AHHDD) both of which lead to the production of FAH_2. Antimetabolite action also arises from the condensation of sulfonamides with AHHDD. This sulfonamide condensation product cannot undergo the biosynthesis necessary to produce FAH_2 thereby resulting in antibacterial activity.

An additional important aspect of the mechanism of action for sulfonamide type drugs is that they are not bactericidal. By interfering with the normal production of FAH_2 bacterial reproduction is stopped and growth is prevented. As a result they are termed bacteriostatic. Since, mammalian cells allows passage of preformed FAH_2 through the cell wall, while bacteria do not, the specificity for limiting formation of FAH_2 results in reduced toxicity for the host animal.

Bacteria can acquire resistance to sulfonamides by any of the three following mechanisms: 1) genetically altering the cell wall permeability to preformed FAH_2, 2) increasing essential enzyme production and 3) increasing production of an essential metabolite. Thus, sulfonamides can become ineffective in drug therapy (1). Sulfonamides, as a result of the inductive properties of the SO_2 group, exhibit amphoteric behavior (**Figure 2**). This behavior has been found to play an extremely important role in the antibacterial activity. Bell and Romblin (10) found that therelationship between acid dissociation constant and bacteriostatic activity possessed a parabolic shape and passed through a maximum between pKa 6 and 7.5. They conclude that the ionic form of the drug is more active than the neutral form, but very acidic sulfonamides have decreased activity because the SO_2 group is less electronegative than moderately acidic sulfonamides.

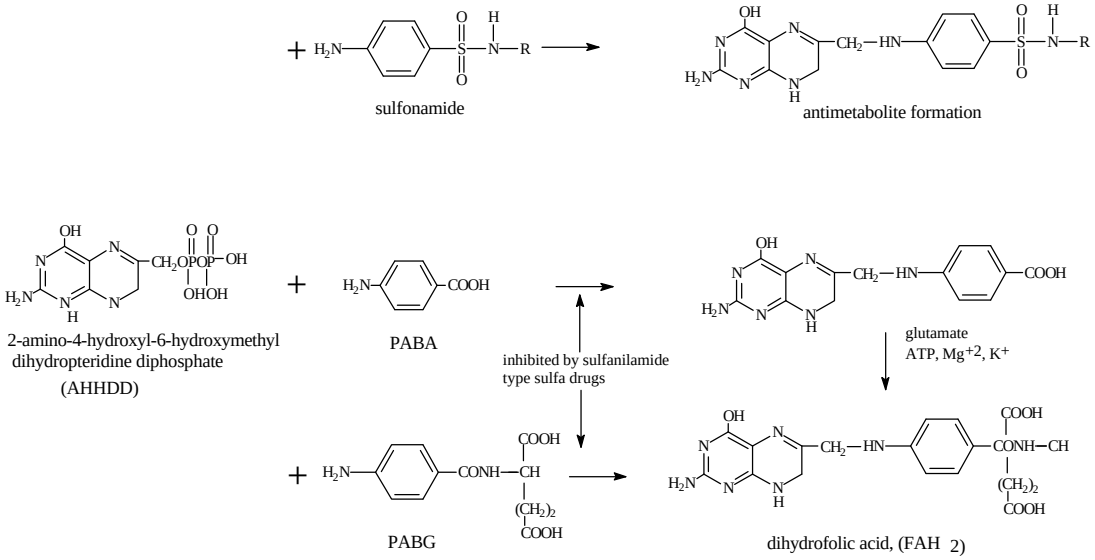
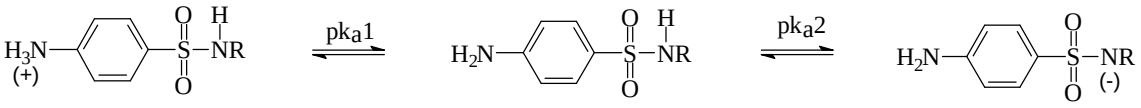


Figure 1:Dihydrofolic acid synthesis and inhibition by sulfa drugs.
(Taken from Ref. 1)



Cationic Form Neutral Form Anionic Form

Figure 2: Amphoteric Nature of Sulfonamides

Therefore, the less negative the SO_2 group in either the ionic or molecular form, the less bacteriostatic activity is exhibited.

Another important aspect of sulfonamide activity and ultimately for monitoring drug tissue levels is protein binding. Since most sulfonamides are weakly acidic, binding to basic amino acids can occur. Several studies have shown: 1) plasma albumin binding is important (11), 2) hydrophobic properties and steric factors, especially at N^1, are extremely important in binding affinities (12), and 3) specific amino acids have been implicated in binding with sulfonamides (13). Protein binding affects the release rate of active drug into the host system and also slows the metabolism. As a result, the sulfonamide may remain in the host system longer than anticipated which may lead to contamination of animal products for human consumption.

Uses in Veterinary Medicine

Sulfonamides, as well as many other antimicrobial and antibacterial agents, have found extensive use in veterinary medicine. The drugs are typically administered at two dosages 1) therapeutic and 2) subtherapeutic. Therapeutic use entails administering drugs, often at high levels, to diseased animals. Sulfonamides are used to treat a multitude of diseases in livestock. For example, sulfonamides are used to treat atrophic rhinitis in swine and heartwater in cattle, in addition to many other diseases in a variety of animals (14).

However, one of the major uses of sulfonamides in animal therapy is the prophylactic administering of subtherapeutic dosages. Often antibiotics and sulfonamides are added to feedstocks and/or water for livestock (15). Subtherapeutic dosing is used to control disease, increase feed efficiency, and increase weight gain of domestic animals (16). In the United States nearly 100% of chickens and turkeys, 90% of swine and veal calves, and 60% of beef cattle receive antimicrobial drugs (antibiotics and sulfonamides) (17). Typical dosing levels for some sulfonamides are: 1) sulfadimethoxine (113.5 g/ton of feed) in conjunction with oremetoprim (68.1 g/ton) administered to chickens, 2) sulfamethazine (100 g/ton) mixtures with chlortetracycline (100 g/ton) and penicillin (50 g/ton) administered to swine, and sulfamethazine in conjunction with chlortetracycline, 350 mg of each drug fed to cattle each day, 3) sulfaquinoxaline continuously fed to chickens at 227 g/ton or fed at higher levels during intermittent feeding periods (18). This high level and continuous use presents ample opportunity for residues to be present in meat samples. A typical withdrawal period of two weeks before slaughter is required to reduce the possibility of contamination. However, the Food Safety

Inspection Service (FSIS) found violative sulfonamide residues in nearly 10% of all tissue samples investigated from 1978-1981, where sulfamethazine (93.3%), sulfaquinoxaline (3.7%) and sulfadimethoxine (2.5%) were the most common residues discovered (19). The current regulatory level for most sulfonamides in edible animal tissues is 0.1 parts-per-million (20).

Sample Preparation and Analysis

Due to the widespread use of sulfonamides in veterinary practice a multitude of sample preparation and analysis methods have been developed using chromatography, spectroscopy and immunoassay. However, the sample preparation portion has often complicated sample assay by requiring difficult and time consuming sample cleanup steps prior to analysis.

Paulson et al. (21,22) extracted tissue samples with methanol, took then to dryness and reconstituted with water. The fatty material was then removed from the water with hexane. Samples were passed through a solid phase XAD-2 column for further purification. Compounds were then eluted with methanol. Followed by purification and separation by reverse HPLC. Parks (23) described a preparation method for sulfamethazine and the N^4-metabolite from swine liver using a water extraction. The extract was to be filtered, passed through two different neutral alumina columns before being analyzed by HPLC/UV at 265 nm. A method by Haagsma et al. (24) extracted swine tissue in an ultrasonic bath using dichloromethane. A solid phase C_{18} cartridge was required to provide a sample of sufficient purity to analyze by HPLC. Using this latter method 89.5% recovery of sulfamethazine was reported. Ten sulfonamides (5 gram sample) were extracted from meat and fish tissue with ethyl acetate followed by cleanup on an amino solid phase cartridge by Ikai et al. (25). The sulfonamides were retained on the column during the ethyl acetate rinse and were eluted from the column with 5 mL of acetonitrile.

One of the most common methods of sample preparation for meat tissue samples was developed by Tishler et al. (26). Their procedure used 50:50 acetone: chloroform to extract tissue samples. The total extract (approximately 350 mL) was placed in a rotary evaporator. The residue was then transferred to a centrifuge tube and four 25 mL portions of hexane, two 3 mL portions of acetone, and two 25 mL portions of hexane were added in order. A 10 mL aliquot of 1 N HCl was added to the extract followed by gentle shaking and centrifugation. The acid phase was collected and the process was repeated with three additional 5 mL aliquots of acid followed by filtering the extract and diluting to 30 mL. Colorimetric assay at 545 nm utilizing diazotization-coupling with N-(1-napthyl) ethylenediamine dihydrochloride was used as the detection method. Recoveries ranging from 76-100% were found in calf, chicken and swine tissues. Parks et al. (27) used a 75/25 ethyl acetate/chloroform solution to extract sulfonamides

from swine tissues. It was 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.

Due to the amphoteric nature of sulfonamides, several sample preparation methods utilize pH to assist in the cleanup of sample extracts. Haagsma and VanDeWater (28) extracted five sulfonamides using a 1:1 mixture of chloroform:acetone. The extract was then acidified and passed through a cation exchange column followed by analysis on a reversed phase C_8 HPLC column. Ion-pairing strategies were employed by Weiss et al. (29) and Parks (30,31). Weiss et al. extracted tissue samples at pH 10 with dichloromethane and tetrabutyl ammonium hydroxide (TBAH). TBAH is an ion pairing agent and is known to extract anions of weak acids in chloroform or dichloromethane (32). A liquid-liquid extraction method was proposed by Petz (33). Muscle and liver tissue were extracted with acetonitrile at pH 8.5. Water was separated from the extract by the addition of sodium chloride. The extract was evaporated and the residue was partitioned between aqueous methanol and hexane. The aqueous layer, adjusted to pH 2, was used for HPLC analysis employing fluorescence excitation at 275 nm and emission at 340 nm.

Many other chromatographic and spectroscopic methods have been employed for the analysis of sulfonamide residues. Using similar sample preparation techniques sulfonamide extracts have been assayed by gas chromatography. Derivativization of the drug residues was required prior to analysis. Methylation of sulfonamides made GC analysis possible. Gas chromatography is attractive since the detectors available are often more sensitive than those for HPLC. Several groups (34-39) used methylation to accomplish GC detection. Finlay et al. (40) introduced crude sample extracts directly into a hybrid mass spectrometer via direct inlet probe or a moving belt interface and Brumley et al. (41) used collision-induced dissociation-mass analyzed ion kinetic energy spectrometry to analyze tissue extracts without employing chromatography. Coupled chromatographic/spectroscopic techniques have also been employed. Henion et al. (42) used HPLC/atmospheric pressure chemical ionization MS to analyze for sulfonamide residues in racehorse urine. Horie et al. (43) and Pleasance et al. (44) employed HPLC/thermospray MS and HPLC/ion spray MS, respectively, to measure residues in swine and salmon tissues at levels from 25 µg/kg to 1 mg/kg.

Other non-chromatographic analysis methods have appeared recently. Dixon-Holland and Katz (45) used a direct competitive enzyme linked immunosorbent assay (ELISA) for detecting sulfamethazine in urine and muscle tissue. With this technique concentrations as low as 20 µg/kg of sulfamethazine could be detected from muscle tissue. Singh et al. (46) and Fleeker et al. (47) both described an enzyme immunoassay method for detecting sulfamethazine in swine blood.

Concentrations to 10 µg/kg could be detected by this method, however both studies found cross reaction occurring with sulfamethazine metabolites and sulfamerazine which reduced the reliability of these methods for analysis. There has been an excellent review published by Guggisberg et al. (48) on methods for the determination of sulfonamides from meat tissues.

Supercritical fluid technology has recently received increased attention for both sample preparation and analysis of sulfonamide drug residues in animal tissues. Supercritical fluids (SFs) offer several distinct advantages compared to conventional liquid solvents used for sample preparation: 1) SFs have tunable solvating characteristics, in that the solvating power (e.g. density) can be adjusted by changing pressure and/or temperature, 2) many SFs are gases at ambient conditions which reduce the disposal costs of conventional extraction solvents, and 3) SFs are typically more environmentally friendly than the chlorinated solvents often used in sample preparation.

There have been several methods reported which describe the analysis of sulfonamides by supercritical fluid chromatography. Games et al. (49) reported analysis of drug residues from freeze-dried pig kidney by combined SFE-SFC/MS/MS employing a moving belt MS interface. Perkins et al. (50,51) have further investigated the utility of SFC and SFC/MS for the analysis of sulfonamides. These workers discovered that separation of the eight currently regulated sulfonamides could not be achieved due to peak coelution on both an amino and silica column (51). Berger (52), commenting on the density of methanol-carbon dioxide mixtures, found that sulfamethoxypyridizine and sulfimidazole exhibited distinctly different elution behavior with changing temperature. Specifically, chromatographic peak reversal occurred with increasing temperature.

Supercritical fluid extraction (SFE) has also been used for the sample preparation of sulfonamide drug residues from animal tissues. The SFE methods have been able to eliminate the use of the chlorinated solvent and to reduce the number of sample preparation steps required in the Tishler method (26). SFE of sulfonamides from silica and biological matrices, to date, has used several different strategies to achieve high recovery of drugs from animal matrices: high pressures, different supercritical fluids, and modified extractions. Parks and Maxwell (53) extracted sulfonamides using high pressure CO_2 (10,000 psi) from a variety of chicken tissues including liver, breast, and thigh and they obtained greater than 80% recovery for all analytes. Their system involved placing activated alumina inside the extraction vessel. The alumina served to trap the polar sulfonamides while allowing the fatty coextractives to pass through. Following extraction the alumina was rinsed with the HPLC mobile phase and assayed without further cleanup. Cross et al. (54) 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 clean-up procedure including sonication and rinsing the extract through an alumina trap with multiple rinse solvents was necessary to remove interfering fatty materials. Tena et al. (55) investigated methods to improve supercritical fluid extraction of sulfonamide salts from silica gel and diatomaceous earth. Poor recovery (<36%) was obtained in each case by (a) using pure CO_2, (b) using CO_2 with the addition of a methanol spike to the matrix or, (c) using CO_2 with methylation of the sulfonamides. However, the addition of an ion pairing agent produced much higher (> 80%) recovery.

Ashraf-Khorassani and Taylor (56) have used CHF_3 and methanol modified CHF_3 to extract sulfonamides from chicken liver tissue. Near quantitative recovery was obtained for sulfamethazine and sulfadimethoxine, whereas, sulfaquinoxaline yielded only 30% recovery using 10% methanol modified CHF_3. Carbon dioxide with 10% methanol modifier yielded less than 50% recovery for all three analytes under the same conditions. No sample cleanup was necessary following extraction. They concluded that selective rinsing of the sulfonamides from the solid phase trap via the aqueous based HPLC mobile phase was occuring and that the fatty coextractives remained on the nonpolar trap. None of the SFE methods developed in these studies have been able to produce quantitative recovery of all target sulfonamides.

The focus of our research is therefore to develop methods for sample preparation and analysis of sulfonamides utilizing supercritical fluids. The goal is to develop a sample preparation method for extraction of sulfonamides from biological matrices that is both efficient and requires little to no additional sample cleanup, while maintaining the advantage of short preparation time. The development and optimization of a specific and sensitive detection method would provide accurate quanitation of sulfonamide residues. Also, by employing specific detection the interference of sample coextractives during sample preparation would be reduced.

Chapter II describes a fundamental study of the effect of temperature and pressure on the supercritical fluid extraction of sulfonamides from a sand matrix. Evaluation of dynamic extraction profiles yielded information on the kinetic behavior and optimum conditions for sulfonamide extraction. Carbon dioxide (CO_2), a nonpolar fluid, and trifluoromethane (CHF_3), a polar fluid, were compared with respect to kinetic (recovery vs. time) and total extraction efficiency. Results from the sand matrix were used to develop optimum conditions for extractions from biological matrices.

Chapter III investigates the differences in extractability of polar sulfonamides from biological matrices using a polar and nonpolar supercritical fluid. The objective of this study was to compare CHF_3 and CO_2 as extraction fluids for the extraction of sulfonamides from a variety of

spiked biological matrices including; 1) non-fat dry milk, 2) beef liver and 3) egg yolk with respect to both extraction efficiency and extraction selectivity. Three sulfonamides, sulfamethazine (SMZ), sulfaquinoxaline (SQX) and sulfadimethoxine (SDM) were used as target analytes for this study.

Chapter IV investigates the effect of the identity and concentration of organic modifier used for SFE of sulfonamides. The objective of this study was to determine the concentration and identity of modifier which best extracts the target analytes in hopes of achieving quantitative recovery from three spiked biological matrices 1) chicken liver 2) beef liver and 3) egg yolk. Four modifiers (methanol, ethanol, acetone, acetonitrile) at three different concentrations (5, 10, and 20% (v/v)) were examined to determine optimum recovery.

Chapter V describes the optimization and evaluation of selective detection methods for sulfonamides including chemiluminescence nitrogen detector (CLND) and HPCL/atmospheric pressure chemical ionization (APCI) mass spectrometry. CLND detector parameters were optimized for use with packed column supercritical fluid chromatography (SFC) employing methanol modified CO_2. The SFC-CLND system was optimized for sulfonamides with respect to ozone flow rate, decompressed CO_2 flow rate, and modifier concentration. Detector design improvements and the effect of column diameter upon the system sensitivity were also evaluated. Minimum detectable quantities of eight regulated sulfonamides using HPLC/APCI-MS were compared in full scanning and single ion modes. Supercritical fluid extracts of sulfonamides from chicken liver were then assayed to test system performance.

Chapter II

Temperature and Pressure Effects on the Supercritical Fluid Extraction Profiles of Sulfonamides from a Spiked Sand Matrix using CHF_3 and CO_2

INTRODUCTION

Temperature and Pressure Effects on Supercritical Fluid Extraction

There has been considerable research effort concerning the effect of temperature, CO_2 pressure, and modifier addition on supercritical fluid extraction behavior of a multitude of analytes including lipids (57,58), caffeine (59), pesticides (60), PAHs, and PCBs (61,62). Increasing the extraction pressure at fixed temperature results in an increase in fluid density, which in many cases improves extraction efficiencies (63-64). The effect of temperature on extraction efficiency, however, has proven to be analyte and matrix dependent. Although increasing the temperature at fixed pressure lowers the fluid density, certain extractions have been shown to be enhanced by an increase in temperature at fixed pressure (65-67). The addition of polar modifier can also aid the extraction efficiency by either 1) increasing the solvating strength of the fluid or 2) improving desorption of analyte from a sample matrix (68-70).

Pressure, temperature, and sample matrix also play an important role in extraction kinetics. Kinetic treatment of data allows large amounts of information to be obtained from extraction profiles. The hot ball model (71) is a theoretical model for the extrapolation of continuous extraction data which is unlimited by solubility. This treatment allows quantitative results to be estimated for an extraction in much less time than required for exhaustive extraction, as well as the calculation of approximate diffusion coefficients. Extraction profiles are necessary to obtain the experimental variables needed for the mathematical model used for quantitative treatment.

Trifluoromethane as a supercritical fluid solvent

Literature regarding the use of trifluoromethane as an extraction solvent has received some attention recently. Howard et al. (72) studied the SFE of environmental analytes and sulfonyl ureas using CHF_3. In their work, CHF_3 was a better extractant than CO_2 or $CHClF_2$ for sulfonyl urea herbicides spiked onto celite, but was least effective for the extraction of three- and

four ring PAHs coated onto onto sodium montmorillonite clay. Ashraf-Khorassani and Taylor (56) have also used CHF_3 and methanol modified CHF_3 to extract sulfonamides from chicken liver tissue. Near quantitative recovery was obtained for sulfamethazine and sulfadimethoxine with 10% methanol-modified CHF_3. However, only 30% of sulfaquinoxaline was obtained under the same conditions.

This chapter considers the effect of temperature and pressure upon the extraction profiles of several polar sulfonamides from a sand matrix using supercritical CO_2, methanol-modified CO_2, CHF_3, and methanol-modified CHF_3. Extraction profiles are usually plots of percent recovery versus extraction time or amount of fluid used, and they may yield information regarding the kinetics of extraction. General trends in extraction behavior upon altering temperature and pressure, as well as the addition of methanol modifier will be discussed with respect to the profile and recovery of sulfamethazine (SMZ), sulfaquinoxaline, sodium salt (SQX), and sulfadimethoxine (SDM) (**Figure 3**).

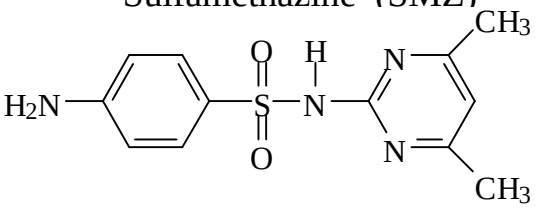
EXPERIMENTAL

Supercritical Fluid Extraction and Quantitation

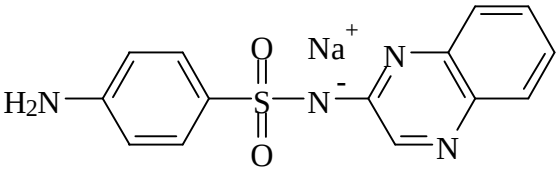
A Suprex Autoprep 44^{TM} (Pittsburgh, PA) supercritical fluid extractor equipped with an Accutrap electronically controlled variable restrictor was used for all extractions in this study. The system was outfitted with an in-line Suprex modifier pump. The HPLC grade methanol and acetonitrile were purchased from EM Science (Gibbstown, NJ). HPLC grade water was purchased from Mallinckrodt (Paris, KY). Both CO_2 and CHF_3 were obtained from Air Products and Chemicals, Inc. (Allentown, PA) and each was pressurized with 2000 psi of helium.

Methanol modifier (10 percent) mixed in-line was employed with both CO_2 and CHF_3. Extractions were conducted in 1mL Suprex vessels. All of the extractions were monitored at 5 minutes, 15 minutes, 30 minutes, 45 minutes, and 60 minutes for a total dynamic time of 60 minutes at the desired conditions. No static extraction period was used. All extractions were accomplished at either 300 atm or 450 atm using CO_2, methanol-modified CO_2, CHF_3, and methanol-modified CHF_3. A flow of 1.5mL/min (liquid CO_2 measured at the pump) was used for all extractions. For this study, the extraction temperatures were 40^{o}C, 70^{o}C and 100^{o}C. All extractions employed a solid sorbent trap of octadecyl silica (Applied Separations, Allentown, PA) at 10^{o}C using pure fluids. For modified-fluid extractions, the solid phase trap temperature was increased to 80^{o}C. Each extraction was completed in triplicate and the extraction profiles

Sulfamethazine (SMZ)



Sulfaquinoxaline, sodium salt (SQX)



Sulfadimethoxine (SDM)

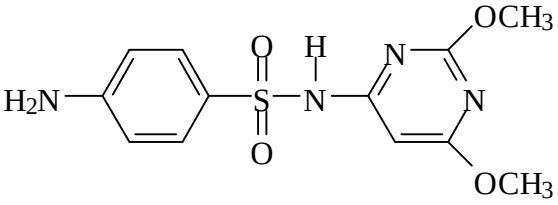


Figure 3: Structure of target analytes; sulfamethazine (SMZ), sulfaquinoxaline, sodium salt (SQX), and sulfadimethoxine (SDM).

reported are the average of three extractions. After completion of each extraction, the solid phase trap was rinsed at 40°C with 2mL of HPLC grade methanol.

Samples for extraction were prepared by spiking 10µL of drug standard (0.6µg/µL each of SMZ, SQX, and SDM in methanol) directly onto Ottawa sand standard (Fischer Scientific, Fair Lawn, NJ). The spiking solvent was then allowed to evaporate for 5 minutes prior to extraction to avoid modification of the fluid.

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 x 4.6mm (5µm dp) Deltabond™ ODS (Keystone Scientific, Bellefonte, PA) column was used throughout the study. The mobile phase employed was 85% 8mM ammonium acetate/ 15% acetonitrile at a flow rate of 1 mL/min. All three sulfonamides were detected at 266 nm. Samples were quantified based upon an external calibration curve using linear regression analysis. Standards were prepared by spiking 2 mL of methanol with 6µg, 3µg, 0.6µg and 0.06µg of each sulfonamide. This corresponds to 100, 50, 10 and 1% recovery, respectively. The equations for the regression lines for each compound is as follows: SMZ: $y=5915x + 9754$, $r^2= 0.995$; SQX: $y= 5611x + 7913$, $r^2= 0.996$; SDM: $y= 6329x + 8581$, $r^2= 0.996$.

RESULTS AND DISCUSSION

The objective of this chapter was a fundamental investigation on the effect of temperature and pressure upon the extraction profiles of SMZ, SQX, and SDM from a sand matrix using both pure and methanol modified CO_2 and CHF_3. This was performed to evaluate the extraction behavior of the three sulfonamides from a relatively inert matrix in order to better understand how experimental parameters alter extractibility. The ultimate goal was to determine the best conditions to first evaluate the extraction of sulfonamides from a biological tissue samples.

Pure Carbon Dioxide

Figures 4 and 5 show the course of extraction for SMZ, SQX, and SDM at both 300 atm and 450 atm using pure CO_2 at variable temperatures. Extraction profiles continually declined which is representative of lower recoveries as the temperature increased. This trend was expected since the density decreased from 0.92g/mL to 0.67g/mL (73) going from 40°C to 100°C at 300 atm. Recoveries did not exceed 50% for any analyte under any of these conditions after 60 minutes. The poor recoveries probably reflect the relatively poor solvating ability of pure CO_2 for sulfonamides since a minimal matrix effect was expected. At 70°C and 100°C the extraction profiles were almost identical for each sulfonamide. At 40°C where density is greatest,

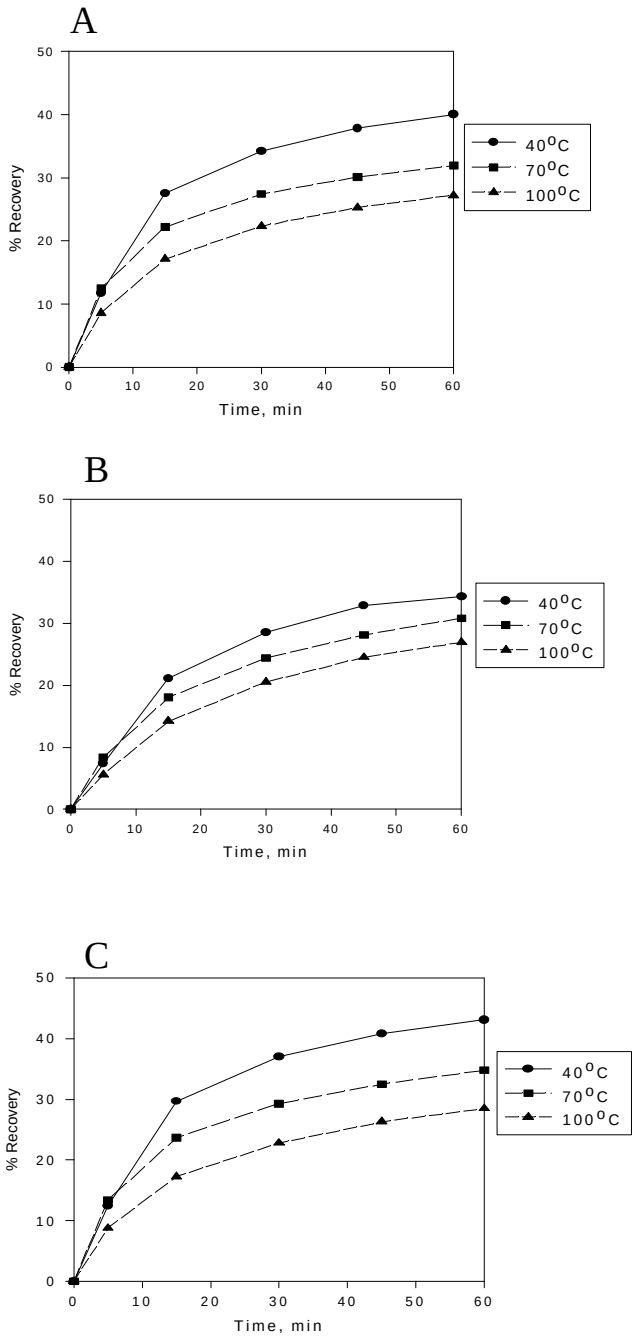


Figure 4. a) Extraction profile (sum of three replicate extractions) of sulfamethazine at 300 atm using pure CO_2, b) Extraction profile of sulfaquinoxaline at 300 atm using pure CO_2, c) Extraction profile of sulfadimethoxine at 300 atm using pure CO_2.

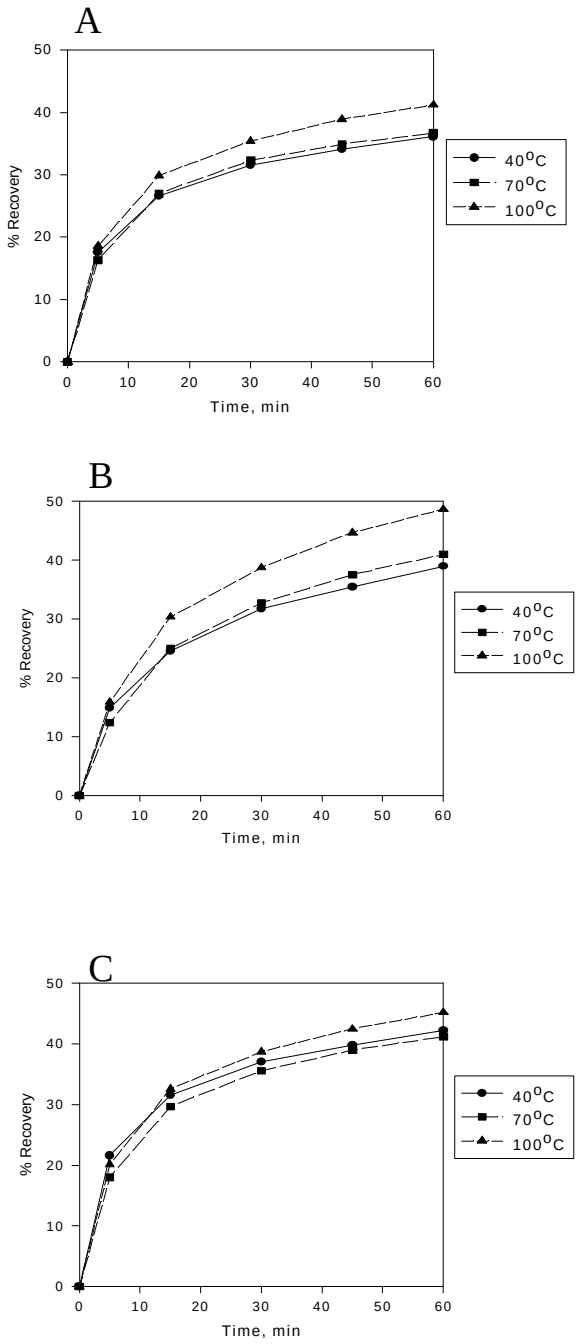


Figure 5. a) Extraction profile of sulfamethazine at 450 atm using pure CO_2, b) Extraction profile of sulfaquinoxaline at 450 atm using pure CO_2, c) Extraction profile of sulfadimethoxine at 450 atm using pure CO_2.

recovery of SMZ and SDM exceeded that of SQX. The change in recovery with increasing temperature at 450 atm was in the opposite direction (**Figure 5**) and not as dramatic. This phenomenon may arise because although sand is thought to be an inert matrix, it is a silica particle. Silica is known to have adsorptive properties. In an extraction both fluid solvating power and analyte- matrix desorption are important factors. The desorption effect from increasing temperature may become dominate in the extraction at 450 atm rather than the decrease in solvent density, resulting in an apparent temperature inversion in the extraction profiles. At 450 atm the density decreases from 0.98g/mL to 0.79g/mL, at 40°C and 100°C respectively, although recoveries were greater at 100°C than at 40°C. Overall recoveries still fell below 50%. All the extraction profiles obtained at either 300 or 450 atm with pure CO_2 exhibit linearity. This is usually indicative of a solvating power limited extraction, which is not surprising since sulfonamides contain several polar functionalities, whereas, CO_2 is non-polar.

Methanol Modified Carbon Dioxide

Results obtained using 10% methanol-modified CO_2 are shown in **Figure 6**. As one would expect, the addition of a polar modifier greatly improved extraction efficiency. At either pressure greater than 70% recovery was obtained within the first 5 minutes with any temperature. Similar extraction profiles were obtained at 450 atm, although an increase in pressure at constant temperature caused considerable increase in recovery. At 450 atm (**Figure 7**) quantitative recoveries (>90%) of all three analytes was obtained with the addition of modifier at both 40°C and 70 °C, but at 300 atm (**Figure 6**) quantitative recovery was not obtained at any temperature. Quantitative recovery was not obtained at 450 atm and 100°C (e.g. less than 86% recovery was found for all analytes). An increase in temperature at constant pressure did not appear to show any significant effects at either pressure investigated, whereas, increasing temperature increased recovery at 450 atm using pure CO_2, but decreased recovery at 300 atm.

Pure Trifluoromethane

Figure 8 compares the extraction behavior with respect to temperature and pressure using pure CHF_3 and pure CO_2 for SMZ, SQX and SDM. It is important to notice differences in the extraction profile and improved final recoveries of SMZ and SDM for CHF_3 relative to CO_2 at both pressures. The extraction profiles with trifluoromethane exhibited a much larger portion extracted during the initial 5 minutes, especially at 450 atm. Extraction profiles for CO_2 are much flatter indicating a difference in sulfonamide extractability for the two fluids. Improved recoveries were obtained by Ashraf-Khorassani et al. (56) with CHF_3 for the extraction of identical

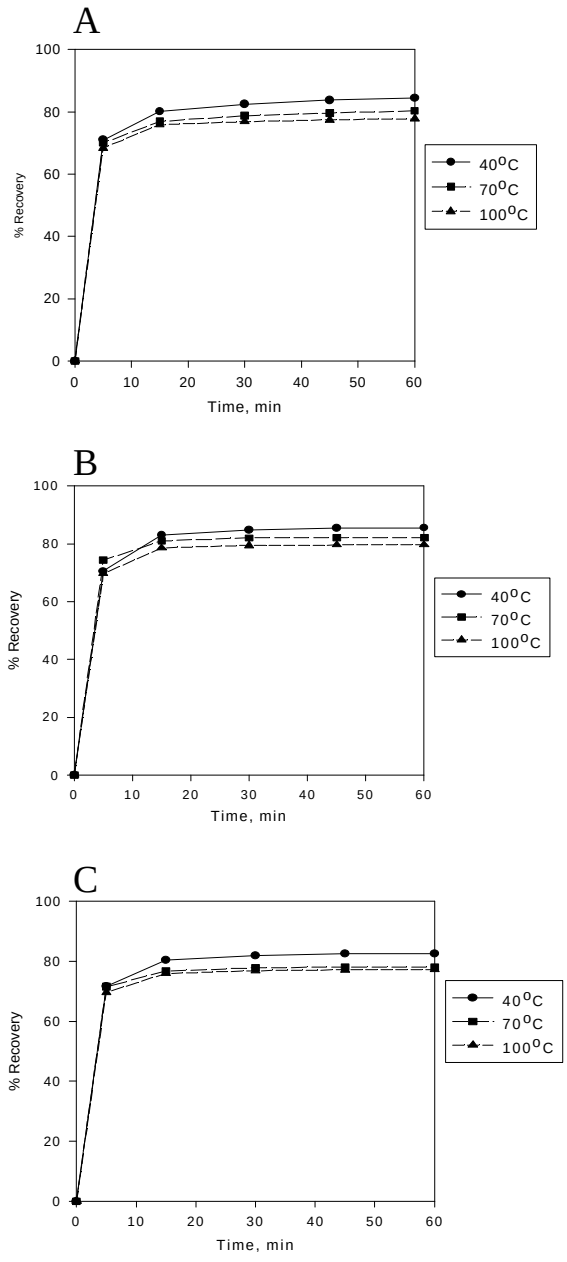


Figure 6. a) Extraction profile of sulfamethazine at 300 atm using 10% methanol-modified CO_2, b) Extraction profile of sulfaquinoxaline at 300 atm using 10% methanol-modified CO_2, c) Extraction profile of sulfadimethoxine at 300 atm using 10% methanol-modified CO_2.

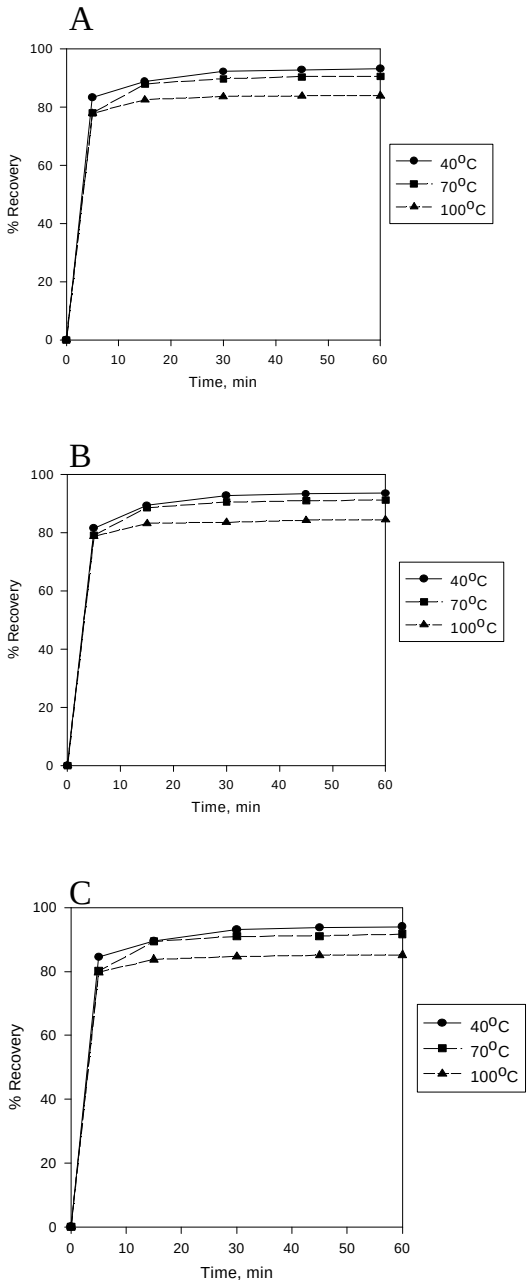


Figure 7. a) Extraction profile of sulfamethazine at 450 atm using 10% methanol-modified CO_2, b) Extraction profile of sulfaquinoxaline at 450 atm using 10% methanol-modified CO_2, c) Extraction profile of sulfadimethoxine at 450 atm using 10% methanol-modified CO_2.

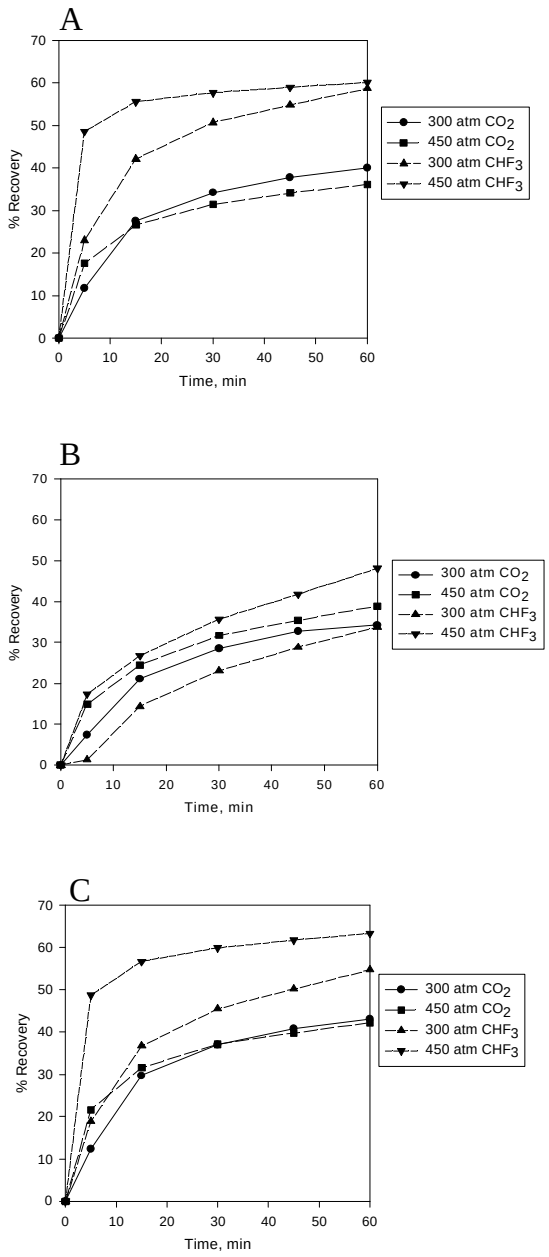


Figure 8. a) sulfamethazine extraction profile with pure CO_2 and CHF_3 at 300 and 450 atm and 40^{o}C, b) sulfaquinoxaline extraction profile with pure CO_2 and CHF_3 at 300 and 450 atm and 40^{o}C, c) sulfadimethoxine extraction profile with pure CO_2 and CHF_3 at 300 and 450 atm and 40^{o}C.

sulfonamides from chicken liver. They found SMZ and SDM both exhibit large improvements in recovery using CHF_3 as the extraction fluid compared to CO_2. It is believed here that both the steeper extraction curves and improved recovery are possible due to the polar nature of CHF_3 (dipole moment of 1.6 D, similar to methanol) (74) and the ability of CHF_3 to hydrogen bond with analytes containing high electron density (75,76). Further evidence to illustrate that CHF_3 is a better solvent than CO_2 for sulfonamides would be the solubility of these compounds in each fluid. Ashraf-Khorassani et al. (77) developed an apparatus to measure the solubility of analytes in supercritical fluids. The system used a recirculating pump to ensure saturation of the analyte in the fluid. Following the saturation period 1μL of the dissolved analyte was diverted into a HPLC mobile phase stream via an internal loop. Peak areas from the HPLC-UV system were directly compared to external standards injected into the same sample loop. This system was used to measure the solubility of sulfamethazine and sulfadimethoxine in CO_2, CHF_3 and 1,1,1,2-tetrafluoroethane (Freon 134a) at 40^{o}C and pressures from 100 to 400 atm. Sulfaquinoxaline was not studied in this manner since the compound was obtained as the sodium salt and had been shown to exhibit similar recovery regardless of the fluid.

Table 1 shows the results obtained for each fluid at the various conditions. Clearly, Freon 134a is a much better solvent than either CHF_3 or CO_2. Freon 134a is a very polar fluid (2.2 Debye) and should have more interaction with the polar sulfonamides. However, under these conditions freon 134a is not supercritical but is a liquid (T_c = 101^{o}C). Nonetheless, it is important to observe that both sulfonamides possess a higher solubility in CHF_3 than in CO_2 at each pressure investigated. This higher solubility directly correlates with improved extraction kinetics ultimately resulting in higher extraction efficiencies. Higher solubility is believed to arise since the polar nature of CHF_3 (1.6 Debye) allows stronger interaction between the fluid and the sulfonamides.

Not surprisingly, the extraction profile and overall recovery of SQX are similar regardless of the fluid used at constant pressure for each temperature investigated. The pH of a SQX solution in methanol was found to be approximately 11 under ambient conditions because the standard was obtained as a sodium salt, whereas sulfamethazine and sulfadimethoxine were both obtained as the neutral compounds. However, the sodium salt of sulfaquinoxaline is known to absorb carbon dioxide to yield neutral sulfaquinoxaline, although this implies the presence of moisture in order to transfer a proton. Sulfonamides are amphoteric, thus can act as an acid and lose a proton (pK_a=3-12 for most sulfonamides (78)). Chemical structure obviously plays an important role in the dissociation constant. Since SQX is partially ionic (SQX converted to the neutral form by absorbing CO_2) the extraction would probably be limited by the amount of non-

Table 1: Solubility (mole/fraction) of sulfonamides in Different Fluids

Sulfamethazine

Pressure, atm	CO_2 Density	CHF_3 Density	$C_2H_2F_4$ Density	CO_2	CHF_3	$C_2H_2F_4$
100	0.638	0.806	1.22	2.23E-06	2.43E-06	1.71E-05
150	0.785	0.91	1.24	2.18E-06	2.52E-06	1.74E-05
200	0.845	0.97	1.27	2.04E-06	2.52E-06	1.79E-05
250	0.854	1.006	1.29	2.09E-06	2.80E-06	1.87E-05
300	0.914	1.038	1.31	2.08E-06	2.83E-06	1.85E-05
400	0.96	1.094	1.34	2.11E-06	3.17E-06	1.91E-05

Sulfadimethoxine

Pressure, atm	CO_2 Density	CHF_3 Density	$C_2H_2F_4$ Density	CO_2	CHF_3	$C_2H_2F_4$
100	0.638	0.806	1.22	2.69E-06	3.64E-06	1.11E-05
150	0.785	0.91	1.24	2.40E-06	3.47E-06	1.12E-05
200	0.845	0.97	1.27	2.32E-06	3.37E-06	1.11E-05
250	0.854	1.006	1.29	2.27E-06	4.06E-06	1.02E-05
300	0.914	1.038	1.31	2.14E-06	4.00E-06	9.53E-06
400	0.96	1.094	1.34	2.26E-06	3.96E-06	7.85E-06

ionized analyte present, thus limiting the amount of analyte available during each extraction step regardless of the fluid employed for extraction. SMZ and SDM being less ionized are more completely extracted and extraction kinetics represent actual extraction behavior of sulfonamides.

It is interesting to note that when using pure CHF_3 as the extraction fluid the total recovery for SQX remained constant as the temperature increased at a constant pressure. At 300 atm (**Figure 9b**) the total recovery was 34%, 37%, and 36% at 40°C, 70°C, and 100°C, respectively. While at 450 atm (**Figure 10b**) total recovery was 48% at 40°C, 46% at 70°C, and 51% at 100°C. It is also important to observe the difference in extraction rates for SQX at different temperatures and constant pressure. At 450 atm very little change in extraction rate was observed regardless of the temperature. All extraction profiles appeared approximately linear, indicative of a solubility limited process. At 300 atm (**Figure 9b**) temperature exhibited a considerable effect on extraction rate for SQX. Although the density was greater, at 40°C, less analyte was extracted during the initial portion, but a steeper slope was present later in the extraction profile, thus producing similar total recoveries. The minimal effect of temperature observed on SQX recovery with CHF_3 could result from the ionization of the analyte discussed previously, since only a fixed amount is available in each step the recovery is limited by the kinetics of removal of the non-ionized analyte and the rate of replacement of neutral analyte following extraction.

An intriguing trend was observed in **Figure 9a** and **9c** with an increase in temperature at 300 atm for both SMZ and SDM. Temperature plays an obvious role with extraction kinetics. The extraction profiles obtained at 40°C were intermediate between those obtained at 70°C and 100°C. Although a large portion of the total recovery of SMZ and SDM was obtained within the first 15 minutes of extraction at 70°C, similar total recoveries were obtained for both at 40°C and 70°C (60% SMZ, 55% SDM and 60% SMZ, 60% SDM , respectively). It is therefore evident that the extractability passes through an optimum with respect to temperature at 300 atm and 70°C. However, the extraction profiles at 450 atm using pure CHF_3 did not show the same behavior as 300 atm. At 450 atm steep initial portions of the extraction profiles were observed for SMZ and SDM (**Figure 10a**,**c**) at any temperature. An increase in temperature served to decrease the amount of SMZ and SDM obtained, but SQX recovery was similar regardless of the temperature (**Figure 10b**). Total recovery dropped from 59% for SMZ and 55% for SDM at 300 atm and 40°C to 41% SMZ and 45% SDM at 300 atm and 100°C (**Figure 9a**,**c)**. At 450 atm and 100°C recovery decreased to 45% SMZ and 52% SDM from 60% SMZ and 63% SDM at 40°C (**Figure 10a**,**c**).

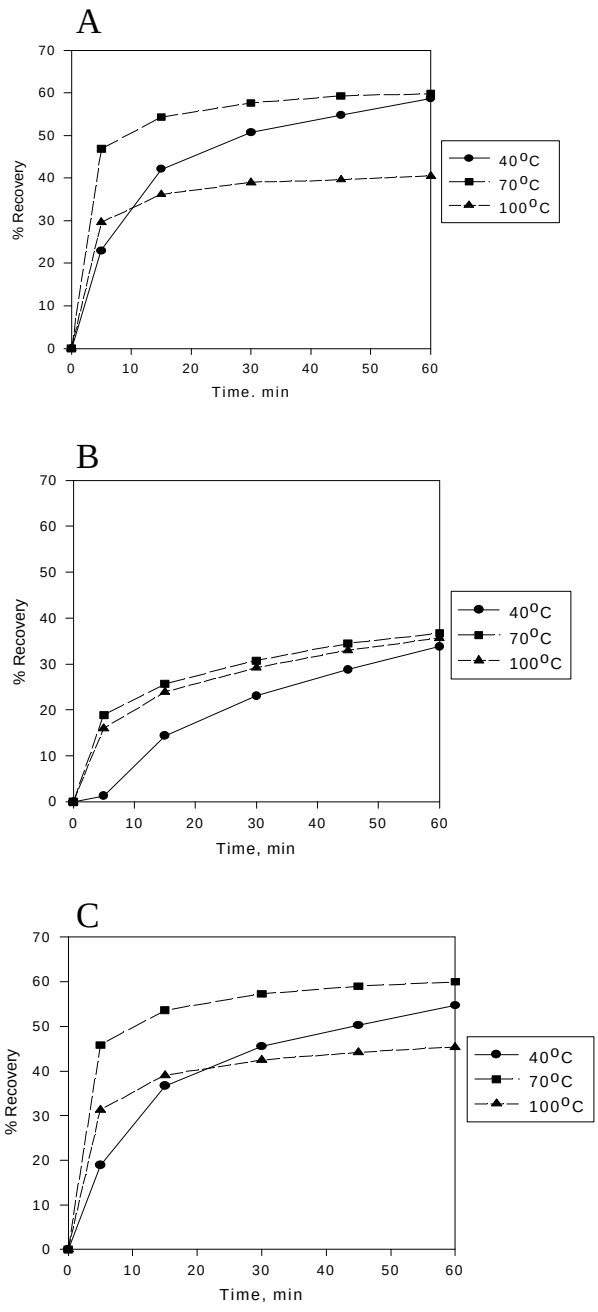


Figure 9. a) Extraction profile of sulfamethazine at 300 atm using pure CHF_3, b) Extraction profile of sulfaquinoxaline at 300 atm using pure CHF_3, c) Extraction profile of sulfadimethoxine at 300 atm using pure CHF_3.

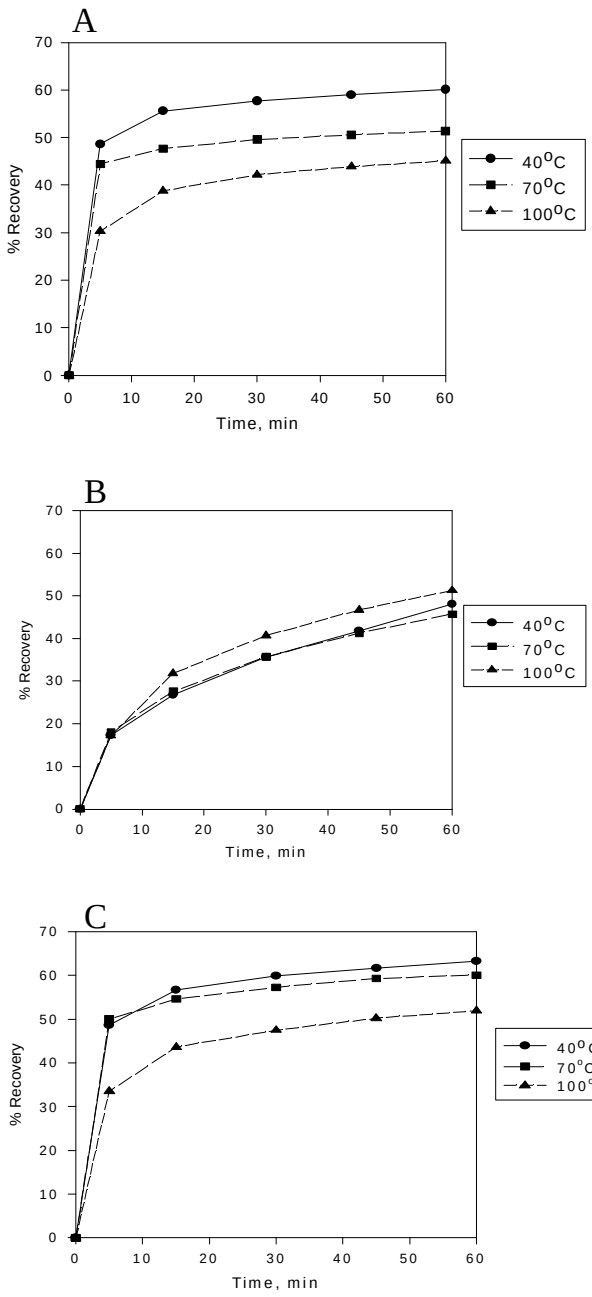


Figure 10. a) Extraction profile of sulfamethazine at 450 atm using pure CHF_3, b) Extraction profile of sulfaquinoxaline at 450 atm using pure CHF_3, c) Extraction profile of sulfadimethoxine at 450 atm using pure CHF_3.

Methanol Modified Trifluoromethane

Figures 11 and 12 show the extraction profiles with the addition of 10% methanol modifier to CHF_3. As expected, the addition of modifier increased the recovery of each analyte with over 75% of the total recovery for every compound at every condition occurring within the first 5 minutes of extraction. An important observation was that quantitative recovery was not obtained for any of the compounds at either pressure, although quantitative recovery was obtained using 10% methanol-modifier with CO_2 at 450 atm. At higher pressures it is believed there is an attraction between methanol and trifluoromethane (e.g. dipole-dipole interaction between F_3C-H--O(H)CH_3) which limits the ability of the fluid to interact with the analyte, therefore decreasing the overall recovery. However, at lower pressures recovery may decrease because of a decrease in the solvating power of the fluid due to a decrease in fluid density. Another interesting trend was observed upon increasing the temperature at 450 atm (**Figure 12**). As the temperature increased the recovery of all three sulfonamides increased. This at first seemed puzzling since at 300 atm an increase in temperature showed very little effect on total recovery. However, if fluid-fluid interaction (methanol- CHF_3) is limiting the extraction, it appears that at higher temperatures there is more energy in the system which should be able to disrupt some of the fluid-fluid interaction thereby increasing the fluid-analyte interaction and increasing the extraction efficiency.

SUMMARY

The effect of temperature and pressure on the extraction profiles of sulfonamides was investigated using supercritical CO_2, methanol-modified CO_2, supercritical CHF_3 and methanol-modified CHF_3 at 300 atm and 450 atm at temperatures of 40°C, 70°C, and 100°C. The effect of temperature upon the extraction profile and extraction efficiency of sulfonamides using both CO_2 and CHF_3 has been shown to be complicated and can be positive or negative. Increasing density (e.g. decreasing temperature or increasing pressure) does not always mean increasing recovery. Pressure also plays an important, although somewhat less complicated role than temperature. Increasing pressure served to aid extractibility of each of the sulfonamides in most cases. In addition, pure CHF_3 is consistently better than CO_2 for the extraction of SMZ and SDM, this is attributed to the much more polar of CHF_3 compared to CO_2. Two of the sulfonamides (SMZ and SDM) have been shown to be much more soluble in CHF_3 than in CO_2. Higher solubility leads directly to increased overall recovery as well as faster extraction. However, for polar amphoteric analytes an ionic form should not be as extractable as a neutral form of the analyte, as

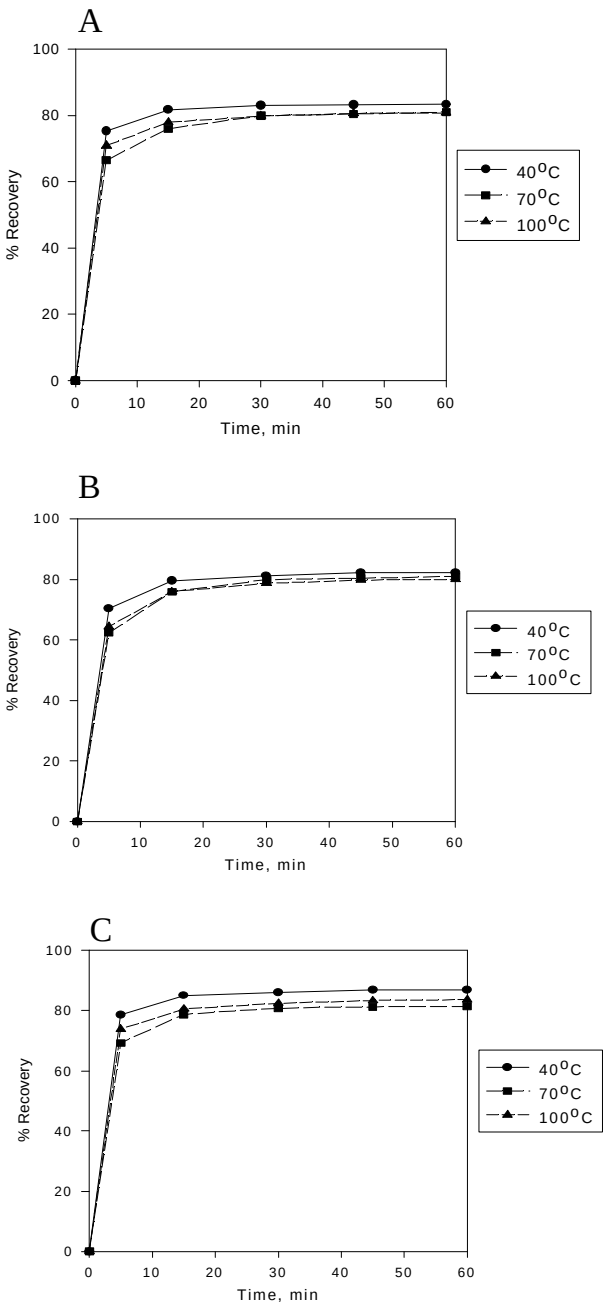


Figure 11. a) Extraction profile of sulfamethazine at 300 atm using 10% methanol-modified CHF_3, b) Extraction profile of sulfaquinoxaline at 300 atm using 10% methanol-modified CHF_3, c) Extraction profile of sulfadimethoxine at 300 atm using 10% methanol-modified CHF_3.

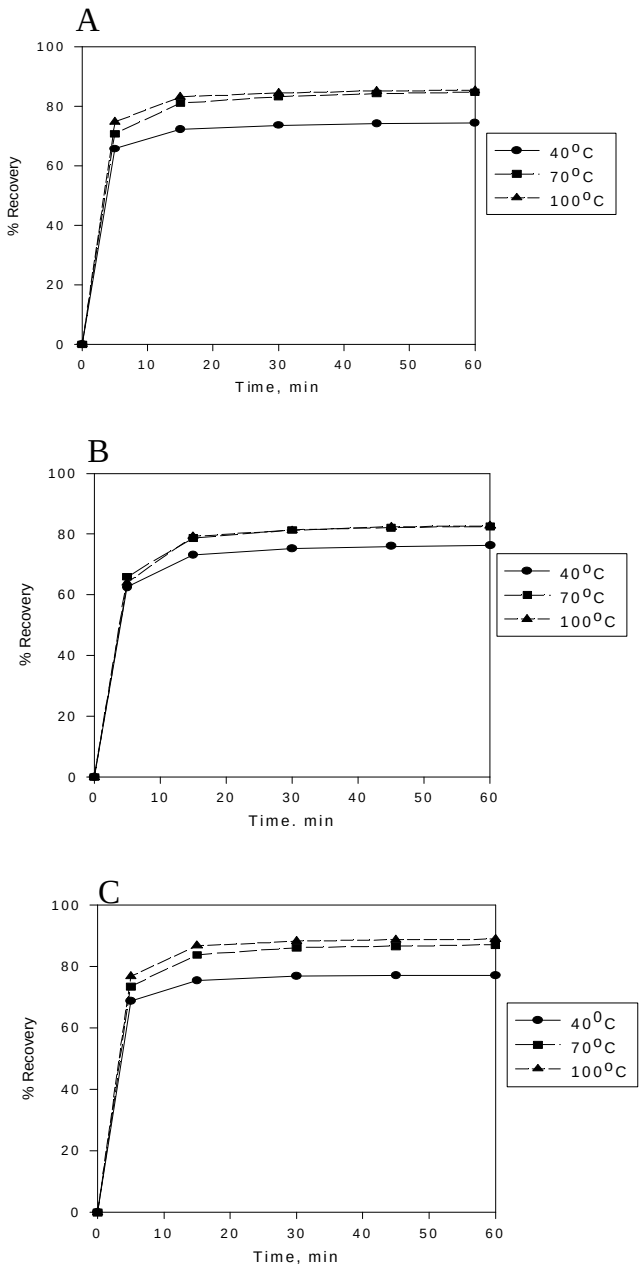


Figure 12. a) Extraction profile of sulfamethazine at 450 atm using 10% methanol-modified CHF_3, b) Extraction profile of sulfaquinoxaline at 450 atm using 10% methanol-modified CHF_3, c) Extraction profile of sulfadimethoxine at 450 atm using 10% methanol-modified CHF_3.

in the case of SQX because of weak ion-fluid interactions. In order to obtain quantitative recovery (>90%) of these sulfonamides from sand methanol modified CO_2 must be used. Methanol modified CHF_3 however, did not produce quantitative recovery at any of the conditions investigated. This is believed to arise from a high degree of fluid-fluid interaction.

Chapter III

Comparison of Supercritical CHF_3 and CO_2 for Extraction of Sulfonamides from Various Biological Matrices

INTRODUCTION

The work in the previous chapter demonstrated the kinetic extraction behavior of sulfamethazine, sulfaquinoxaline and sulfadimethoxine from a spiked sand sample. For the analysis of sulfonamides this method needs to be evalutated for biological samples in which these drugs may be found. This chapter deals again with comparing CHF_3 and CO_2 as extraction fluids but for the extraction of sulfonamides from non-fat milk powder, beef liver, and egg yolk. The goal was to evaluate the fluids but ultimately to achieve quantitative recovery of each sulfonamide from the biological sample with a minimum amount of post-extraction cleanup.

Alternate Fluids

Other supercritical fluids such as ammonia, water, nitrous oxide, pentane and sulfur hexafluoride are not suited for routine analytical use owing to either their unfavorable (a) physical properties, (b) reactivity, (c) solvating power, or (d) incompatibility with modern instrumentation. However, applications of these non-ideal supercritical fluids do (SFs) exist. Smith et al. (79-81) have employed supercritical ammonia coupled with mass spectrometry for the direct extraction and analysis of diesel fuel. They were able to extract nearly all of the polar components in the fuel. Additionally, Hawthorne et al. (82) used sub- and supercritical water for the extraction of a variety organic pollutants from environmental solids. They concluded that the polarity of water can be continuously altered by adjusting the temperature from 50 to 400^{o}C. Ashraf-Khorassani et al. (83) have compared CO_2 and nitrous oxide (N_2O) for extraction of primary, secondary, and tertiary amines using on-line SFE/GC. Their results showed that N_2O exhibited higher selectivity for extraction of amines. McNally et al. (84) used CO_2, methanol-modified CO_2, N_2O, and amine-modified N_2O for extraction of different aromatic amines. Extraction of aromatic amines with either CO_2 or N_2O from different matrices yielded acceptable recovery. Onuska et al. (85) and Levy (86) compared N_2O, CO_2, and mixtures of these fluids with different percentages of methanol for extraction of 2,3,7,8-tetrachlorodibenzo-p-dioxin and polynuclear aromatic hydrocarbons (PAHs) from aquatic sediment and soil. Onuska et al. (85) also compared sulfur

hexafluoride (SF_6) as an extraction fluid with CO_2 and N_2O. Greater than 90 percent recovery was reported using pure or methanol modified N_2O. Similar results were reported by Levy et al. for extraction of PAHs. Lowest recovery of analytes was obtained with SF_6 as an extraction fluid. The use of N_2O as an extraction solvent has been "refuted" by Raynie (87). In his research, an extraction vessel filled with N_2O and coffee exploded and resulted in destruction of the extraction vessel. He concluded that N_2O should not be used as an extraction fluid in SFE, especially when the sample is organic.

This chapter describes the use of supercritical CO_2, methanol-modified CO_2, CHF_3 and methanol-modified CHF_3 as solvents for the extraction of several polar drugs (sulfonamides) from various matrices including, non-fat dry milk, egg yolk, and beef liver. In the first part of this chapter, the effect of pressure and addition of modifier upon extraction efficiency of sulfamethazine (SMZ), sulfadimethoxine (SDM), and sulfaquinoxaline (SQX) using both CHF_3 and CO_2 is investigated. It was necessary to evaluate the pressure dependence since increasing the pressure to 450 atm from 300 atm in the previous chapter improved the recovery for most conditions. The instrumental maximum allowed was 490 atm and it is believed that increasing the pressure further may produce further gain in extractability. Since kinetic information was not necessary only total recovery was needed to evaluate the pressure effects.

In the second part of this chapter, the selectivity of CHF_3 and CO_2 is compared for the extraction of the sulfonamides from non-fat dry milk, egg yolk, and beef liver.

EXPERIMENTAL

Supercritical Fluid Extraction and Quantitation

A Suprex (Pittsburgh, PA) Prepmaster™ equipped with an Accutrap automatic variable restrictor system was used for extracting sulfonamides from each biological matrix. A Suprex Autoprep 44™ automated SFE also equipped with an Accutrap automatic variable restrictor was used for all extractions in the pressure study. Both systems were outfitted with an in-line SSI microbore reciprocating pump (Alltech Associates, Deerfield, IL). The modifier pump continuously adjusted the delivery rate of liquid modifier to compensate for fluctuating flows in the SFE system. The HPLC grade methanol and acetonitrile were purchased from EM Science (Gibbstown, NJ). HPLC grade water was purchased from Mallinckrodt (Paris, KY). CO_2 and CHF_3 were both obtained from Air Products and Chemicals, Inc. (Allentown, PA). Both were pressurized with 2000 psi of helium to minimize pump cavitation.

Methanol modifier (10 percent) was employed with both CO_2 and CHF_3. The extraction vessels used for the pressure study were 1mL, and for the matrix extractions were 3 mL in volume (Keystone Scientific, Bellefonte, PA). All of the extractions involved a single 60 minute dynamic step at the desired pressure. All matrix extractions were accomplished at 490 atm using CO_2 and methanol-modified CO_2 or 400 atm using CHF_3 or methanol-modified CHF_3. A flow of 1.5 mL/min of liquid solvent measured at the pump was used for all extractions. For the pressure study, the pressure was varied from 360 atm to 490 atm. Trapping for matrix extractions consisted of a tandem solid sorbent trap of octadecyl silica (Applied Separations, Allentown, PA) at 10^{o}C and a liquid trap of 5 mL methanol using pure fluids. During modified-fluid extractions, the solid phase trap temperature was increased to 80^{o}C. No tandem trap was needed for extractions in the pressure study, and the solid phase trap was cryostated at either 10^{o}C or 80 ^{o}C with the use of modifier. After completion of each extraction, the solid phase trap was rinsed at 40^{o}C with 5mL of the HPLC mobile phase (85% 8mM ammonium acetate (NH_4OAc)/ 15% acetonitrile solution) into a 5mL volumetric flask. The liquid tandem trap was also diluted to 5mL with 85% 8mM NH_4OAc/ 15% acetonitrile.

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 x 4.6mm (5µm dp) Deltabond^{TM} ODS (Keystone Scientific, Bellefonte, PA) column was used throughout the study. The mobile phase employed was 85% 8mM NH_4OAc/ 15% acetonitrile operated at a flow of 1mL/min. All three sulfonamides were detected at 266 nm. Samples were quantified based upon an external calibration curve as in the previous chapter. Standards were prepared by spiking 5 mL of the mobile phase with 6µg, 3µg, 0.6µg and 0.06µg of each sulfonamide. This corresponds to 100, 50, 10 and 1% recovery, respectively.

Sample Preparation

Pressure study samples were prepared by spiking 10 µL of drug standard (0.6 µg/µL each of SMZ, SQX, and SDM in methanol) directly on to Ottawa sand standard (Fischer Scientific, Fair Lawn, NJ). The solvent was then allowed to evaporate prior to extraction to avoid modification of the fluid.

The non-fat dry milk powder was purchased at a local grocery store. Samples were prepared by spiking 10 µL of the drug standard directly onto 0.2 g of the milk matrix (30 ppm). The solvent was then allowed to evaporate prior to extraction. The vessel was then sealed and extracted under specified conditions.

The egg yolk sample matrix was prepared by first separating the egg white and yolk. Next, the egg yolk was mixed 1:3 with Celite 545 (Supelco, Bellefonte, PA) and spiked to 6 ppm of each sulfonamide per 1 gram of egg/Celite mixture. The mixture was then allowed to incubate at -10^{o}C for a minimum of 30 minutes. The incubation period was used to allow time for analyte-matrix interactions to occur to more closely mimic incurred samples.

Beef liver samples were prepared by spiking 0.5 gram of liver tissue with 10 µL of the drug mixture (12 ppm). The liver was provided by the USDA/ARS located in Philadelphia, PA which was ground prior to receipt. The spiked matrix was allowed to incubate for a minimum of 30 minutes at -10^{o}C. Following frozen storage, 1 gram of Celite was added to the liver and mixed thoroughly. The entire contents were added directly to the extraction vessel and extracted under specified conditions.

RESULTS AND DISCUSSION

The objective of the first part of this study was to investigate the effect of fluid pressure and methanol modifier addition upon the extraction efficiency of SMZ, SQX and SDM using both CHF_3 and CO_2. The second part of the study was concerned with comparing CO_2, methanol-modified CO_2, CHF_3, and methanol-modified CHF_3 as fluids for the extraction of SMZ, SQX, and SDM from non-fat milk powder, egg yolk, and beef liver.

Evaluation of High Pressure on Extractability

Figure 13 shows the effect of raising the pressure from 360 atm to 490 atm upon the extraction efficiency of SMZ, SQX, and SDM from a sand matrix for pure CO_2. The pressures investigated (360 atm, 400 atm, 440 atm, and 490 atm) exhibited little effect on recoveries of the three sulfonamides. At 360 atm recoveries were 38%, 28%, and 40% for SMZ, SQX, and SDM, respectively. Increasing the pressure to 490 atm shows an insignificant increase in recovery to 43% SMZ, 34% SQX, and 44% SDM. At intermediate pressures similar recoveries were obtained. With the addition of 10% methanol to the CO_2, quantitative recoveries could be obtained from sand for all three sulfonamides (**Figure 14**). At 490 atm, 104 %, 104%, and 105% recovery was observed for SMZ, SQX, and SDM, respectively. Again, altering the pressure showed a minimal effect upon extraction efficiency. Decreasing the pressure to 360 atm only decreased recovery to 92% for SMZ, 90% for SQX and 94% for SDM. At 400 atm and 440 atm the recoveries lie between that obtained at 490 and 360 atm. Extraction efficiencies obtained under similar conditions for pure CHF_3 and 10% methanol modified CHF_3 are shown in

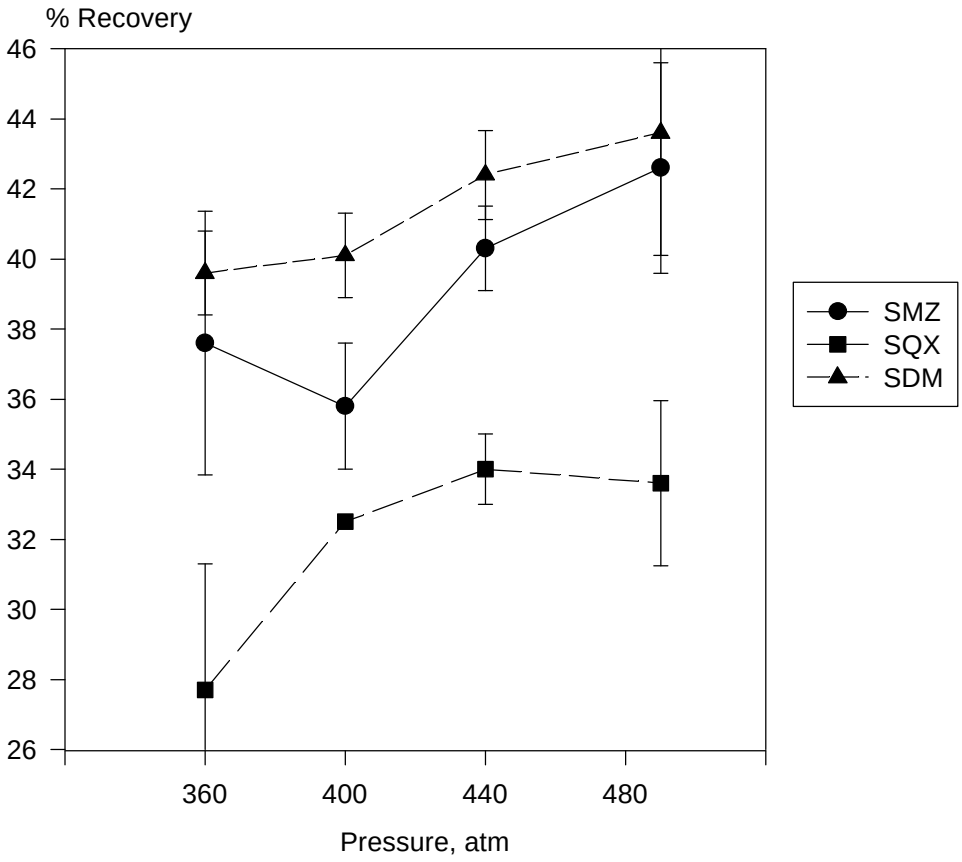


Figure 13. Extraction Behavior of Sulfonamides from Sand using Pure CO_2.

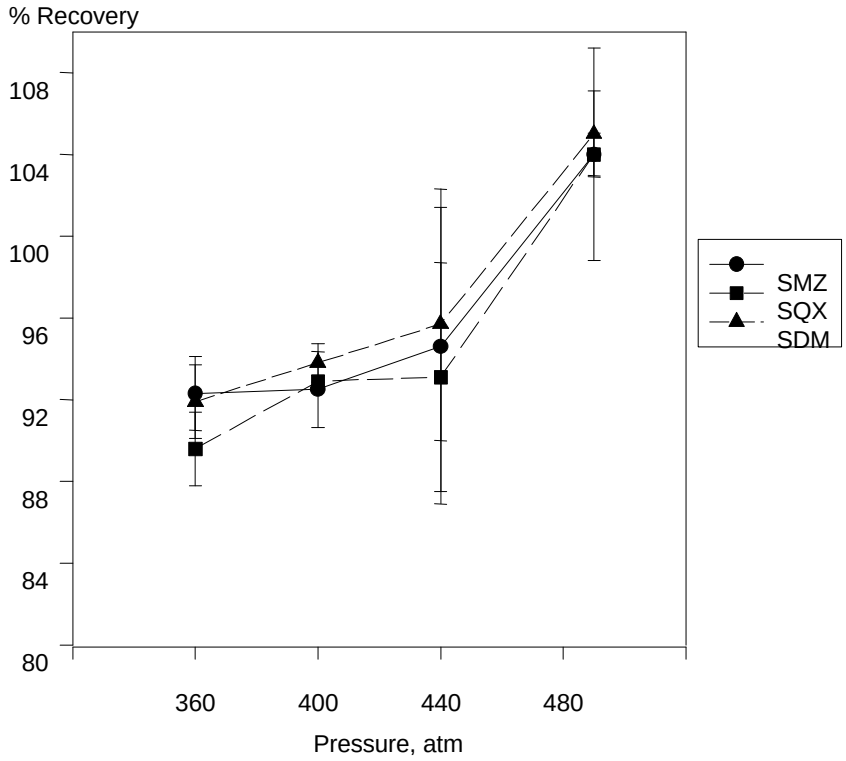


Figure 14. Extraction Behavior of Sulfonamides from Sand using 10% Methanol Modified CO_2.

Figures 15 and **16**. In comparing **Figure 13** and **Figure 15**, there is a distinct difference in percent recovery obtained for SMZ and SDM for CHF_3 relative to CO_2. At 360 atm, 69% of SMZ and 61% of SDM was extracted with CHF_3, whereas, with CO_2 only 38% and 40% was extracted for SMZ and SDM, respectively. This corresponds to an 82% increase in extractability for SMZ and a 53% increase for SDM on going from CO_2 to CHF_3. However, SQX showed no enhanced extractability for CHF_3 relative to CO_2. Due to the hydrogen bonding character of trifluromethane we wanted to compare pKa values to determine whether the trends in extractability were related to base strength. Since CHF_3 has an acidic proton and a dipole moment of 1.6 Debye (74) it follows that a basic analyte should be able to bond with the fluid, making the extraction more efficient. Sulfamethazine was has a pKa of 7.4, sulfaquinoxaline a pKa of 5.5, but since it was obtained as a sodium salt the pKa is not relevant and sulfadimethoxine has a pKa of 6.2 (48). Sulfamethazine is the strongest base and therefore should hydrogen bond better with trifluoromethane. This trend is confirmed within experimental error using pure CHF_3 which strengthens the belief that the polar character and ability of CHF_3 to hydrogen bond increases the extractability of more basic analytes with CHF_3. For the remaining three pressures investigated, 400 atm, 440 atm, and 490 atm, the same trend was observed. At each pressure, the extraction efficiency of SMZ was at least 80% higher than the corresponding CO_2 extraction, in addition SDM was at least 45% higher. Extraction efficiency for SQX was similar regardless of the fluid used for these three pressures.

With the addition of 10% methanol modifier strikingly different behavior could be observed (**Figure 16**). At 360 atm recoveries obtained for SMZ and SDM were similar to those obtained for pure CHF_3 (e.g. 78% and 65%, for SMZ and SDM, respectively with pure CHF_3, and 69% and 72% using 10% methanol-modified CHF_3, but the recovery of SQX was increased to 73% compared to 41% with pure CHF_3. At both 400 atm or 440 atm, quantitative recovery could be obtained for all sulfonamides with methanol modifier. The behavior of CHF_3 at 490 atmospheres was the most puzzling. Recoveries for each of the three sulfonamides were lower than that obtained at lower pressures. Solvating strength is usually believed to increase as the density of the fluid is increases (88) (e.g. increase pressure at constant temperature). However, recovery was found to decrease at pressures above 400 atm. Similar declines in recovery with increasing pressure were observed by Hedrick et al. (89). They found that extraction efficiency of phenols from an aqueous environment increased with pressure to a point at a fixed temperature, but at pressures above 300 atm yields decreased with increasing pressure. They theorized that at higher pressures there was less mixing with the aqueous phase due to increased fluid-fluid interactions.

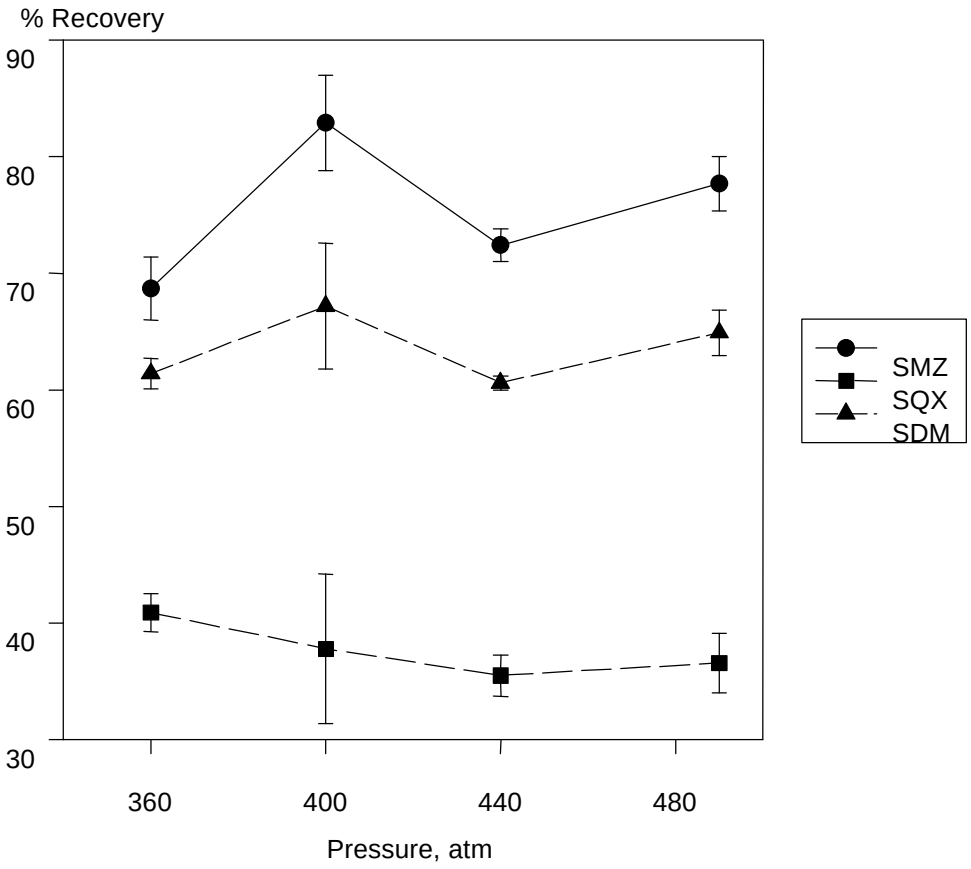


Figure 15. Extraction Behavior of Sulfonamides from Sand using Pure CHF_3.

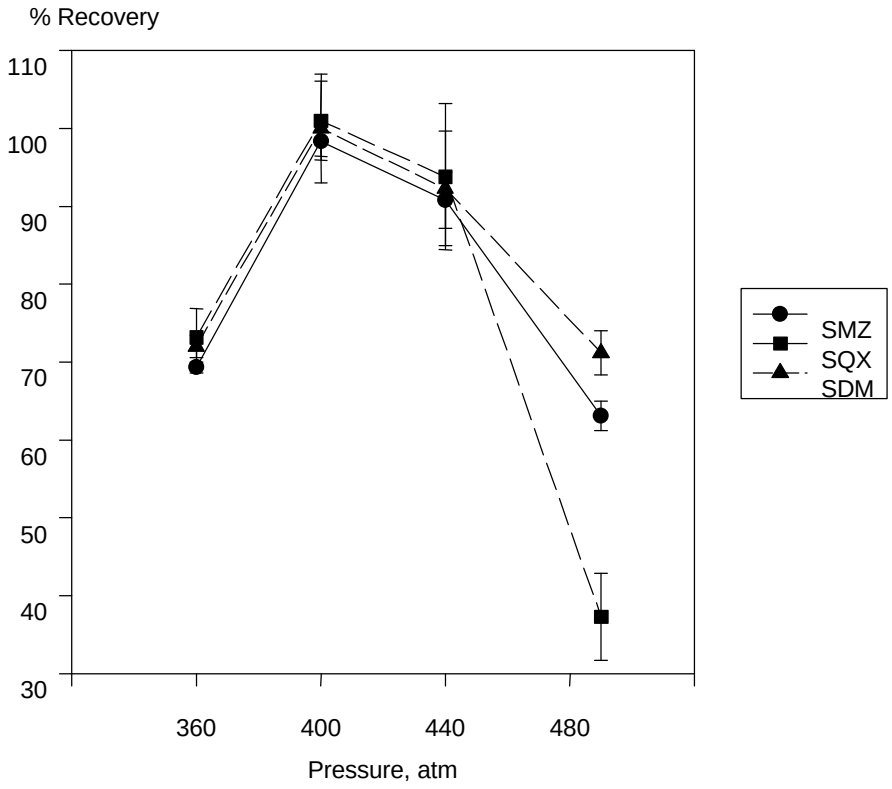


Figure 16. Extraction Behavior of Sulfonamides from Sand using 10% Methanol Modified CHF_3.

It is also important to notice that similar recoveries were obtained at 490 atm whether modifier was absent or present. The addition of modifier should aid the extraction by 1) increasing the solvating strength of the fluid and/or 2) disrupting matrix/analyte interactions. However, the presence of a polar modifier with a polar fluid may produce different behavior. Johnston et al. (76) have found that as pressure of SF_6 increases the degree of hydrogen bonding between perfluoro-tertiary butanol and dimethyl ether decreases. CHF_3 was not used in this study since it could hydrogen bond with the model analytes, therefore sulfur hexafluoride was used. Studies using CHF_3 by Johnston et al. (75) have found the hydrogen bond donor strength of CHF_3 is comparable to that of $CHCl_3$. In addition, hydrogen bonds between CHF_3 and phenol blue were well developed at 70 bar, but did not change significantly with an increase in pressure. The degree of hydrogen bonding between analytes or analyte-and-fluid may be envisioned to decrease due to increased intermolecular interaction between the fluid molecules, thereby decreasing the tendency of the fluid to hydrogen bond with the analyte. A similar phenomenon could occur between the polar fluid and a polar modifier. Strong hydrogen bonding and dipole-dipole interaction can be envisioned to occur between F_3C-H--O(H)CH_3 (methanol-trifluoromethane). If this fluid-fluid interaction occurs to a greater extent at higher pressures, a decrease in recovery at 490 atmospheres would be expected. Increased interaction is entirely resonable since at higher pressures more molecules occupy the same space. In this situation the molecules have closer approach to one another. It is well established that the dipole-dipole interaction is related to $1/r^3$, where r is the internuclear distance. Since the internuclear distance decreases stronger interaction should occur between CHF_3 and methanol since they are the most abundant species relative to the analytes present. Extraction at 400 atmospheres was found to yield optimum recovery and it was used throughout for all CHF_3 extractions of biological matrices.

Extraction from Biological Matrices

The second part of this chapter is concerned with comparing CO_2, methanol-modified CO_2, CHF_3, and methanol-modified CHF_3 for the extraction of SMZ, SQX, and SDM from non-fat milk powder, egg yolk, and beef liver tissues.

Non-fat Milk Powder

The first matrix investigated was a commercial non-fat milk powder sample. **Table 2** shows the results from this study. As can be seen the highest recovery obtained using pure CO_2 was 72% for SMZ and 70% for SDM, whereas greater than 90% of both were obtained using

Table 2: Percent recovery of sulfamethazine (SMZ), sulfaquinoxaline (SQX), and sulfadimethoxine (SDM) from non-fat milk powder. Numbers in parentheses are relative standard deviation. n = 3 for all samples

	100% CO_2	90% CO_2 10% MeOH	100% CHF_3	90% CHF_3 10% MeOH
SMZ	72 (7)	95(4)	90(3)	102(3)
SQX	63(9)	100(5)	64(9)	103(1)
SDM	69(7)	99(6)	92(7)	107(4)

pure CHF_3. It is important to note that the recovery of SQX is the same regardless of the fluid obtained (63% with CO_2 and 64% using CHF_3), which is similar to results obtained on sand during the pressure study and the kinetic study of chapter II. This is believed to occur since the SQX is partially ionic. In addition, recoveries with both fluids are greater than those obtained in the pressure study, which may be due to less interaction between the non-fat milk matrix and the sulfonamides than is present with a sand matrix. In the previous chapter, matrix interaction with the silica sand particle was thought to be responsible for a negative effect on extraction efficiency. If less matrix interaction is occuring better recoveries would be expected. Upon the addition of 10% methanol modifier, quantitative recovery could be achieved with either CO_2 or CHF_3. Non-fat milk powder appears to be a relatively inert matrix that can be easily extracted with the addition of modifier.

Egg Yolk

A matrix consisting of egg yolk and Celite was investigated next. The egg yolk was separated from the egg white prior to spiking the matrix. Celite was added to the matrix to decrease the mobility of the matrix as well as absorb the free moisture content of the sample. Very poor recoveries (**Table 3**) were obtained when using non-modified CO_2. Only 10% of SMZ and less than 2% of SDM and SQX were extracted under the experimental conditions. The egg yolk presents a more difficult matrix to extract than the non-fat milk powder. Comparing **Tables 2** and **3**, greater than 60% of each sulfonamide could be extracted from non-fat milk using non-modified CO_2, whereas, less than 15% was obtained for these from egg yolk. The addition of methanol modifier should aid in disrupting analyte-matrix interaction. This effect was demonstrated by obtaining recoveries of 29% SMZ, 29% SDM, and 28% SQX. Although some matrix interaction was alleviated, only poor recoveries could be obtained, suggesting considerable matrix interaction still exists. Sulfonamides are known to reversibly bind to proteins, it is possible that the egg yolk sample possess more proteins to which the sulfonamides can bind (basic amino acids) resulting in more matrix-analyte interaction than was evident in the non-fat milk powder.

Using CHF_3 as the extraction fluid showed an enhancement of extractability for SMZ and SDM from the egg yolk (**Table 3**). With non-modified CHF_3, 58% SMZ and 36% SDM could be extracted; whereas, no SDM and only 10% SMZ were extracted with CO_2. No recovery was obtained for SQX with either non-modified fluid. The egg yolk still showed considerable matrix interaction with the sulfonamides. Minimal gains in recovery were obtained upon the addition of 10% methanol to the fluid. Recovery for SMZ remained the same at 57%, SDM increased

Table 3: Percent recovery of sulfamethazine (SMZ), sulfaquinoxaline (SQX), and sulfadimethoxine (SDM) from egg yolk/celite mix. Numbers in parentheses are relative standard deviation. n = 3 for all samples

	100% CO_2	90% CO_2 10% MeOH	100% CHF_3	90% CHF_3 10% MeOH
SMZ	10(11)	29(19)	58(6)	57(2)
SQX	<2	28(7)	<2	24(30)
SDM	<2	29(15)	36(13)	46(5)

slightly to 46%, however, SQX was increased to 24%. It appears from these results that trifluoromethane may serve to disrupt some matrix-analyte interaction without the addition of modifier due to its polar nature.

Another interesting consideration is the notion that addition of polar modifier to nonpolar CO_2 results in clusters that present areas of localized "superpolarity", whereas, trifluoromethane (dipole moment of 1.6 Debye; similar to methanol) may mix better with methanol thereby producing no such "pockets." This idea may explain the observation that the addition of methanol modifier exhibited only minimal effect on the extraction of the sulfonamides from egg yolk with CHF_3 but a large relative increase for CO_2. Similarly, the ionic nature of SQX results in weak solvation even with polar CHF_3 whereas, the addition of methanol to the CHF_3 enhances the extractability of SQX by increasing the solubility.

An additional important aspect in the extraction of the egg yolk matrix is the presence of co-extractives, namely fatty material. Upon extraction with either modified CO_2 or non-modified CO_2 a yellow "gooey" substance believed to be fatty material and pigments was observed on the solid phase trap. The material was present to such an extent so as to require replacement of the solid sorbent trap after each extraction to ensure high trapping efficiency. However, when CHF_3 was used as the extraction fluid much less of this material was extracted. With the addition of modifier some selectivity was lost and more fatty material was extracted with CHF_3. Similar results were reported by Ashraf-Khorassani et al. (56,90) for the extraction of sulfonamides from chicken liver and pesticides from rendered chicken fat.

Preliminary studies by Taylor and King (91) have reported similar results on chicken tissue. They showed that the amount of chicken fat extracted with supercritical CHF_3 is approximately 100 fold less than the fat extracted with supercritical CO_2. Percent recovery of fat from poultry adipose tissue using pure CO_2 was reported to be 54.4, while recovery from the same matrix using pure CHF_3 was 0.45 percent. Parks and Maxwell (53) have encountered co-extractives in attempts to extract sulfonamide from chicken liver. Their system consisted of an alumina trap that collected the polar analytes inside the vessel; while the fatty material passed through the vessel to be collected off-line. They employed pure CO_2 at pressures of 10,000 psi with at a temperature of 40^oC. Cross et al. (54) also extracted sulfamethazine and other drugs from chicken liver. They employed 25% methanol-modified CO_2 and a liquid solvent trap. An additional clean-up step was necessary following the extraction due to the presence of co-extractives. The extract solution was dried under a nitrogen atmosphere and then redissolved in the HPLC mobile phase and eluted from an SPE column. In our study, we believe no clean-up is

necessary even for CO_2 extracts, because the sulfonamides were selectively rinsed from the solid phase trap with the HPLC mobile phase.

Beef Liver

The data for sulfonamide recovery from a beef liver matrix is presented in **Table 4**. Again very poor recoveries were obtained with pure CO_2. Recovery ranged from 23% for SMZ and 18% for SDM to zero for SQX. The inability to extract SQX using pure CO_2 from beef liver again suggests very strong affinity between analyte and matrix. It is not surprising that the matrix plays an important role in extraction efficiency. Since sulfonamides are know to bind to proteins and each matrix consists of different proteins, the degree of binding is logically dependent upon the matrix. Addition of 10% methanol to the CO_2 improved the recovery of SMZ to 41%, but the recovery of SDM remained low at 24%. Less than 2% SQX is extractable even during modified conditions.

Results for extractions with pure CHF_3 are shown in **Table 4**. It is clearly evident that a large enhancement in extractability of SMZ and SDM has resulted. Recovery of SMZ was 74% and SDM was 69% with pure CHF_3. Pure CO_2 was able to extract only 23% and 18% for SMZ and SDM, respectively. Upon extracting beef liver with CHF_3 an over 200% increase in the extraction of SMZ and SDM was observed. The enhancement in extractability was believed to be due to the hydrogen bonding character of trifluoromethane with SMZ and SDM. It may also be possible that CHF_3, in addition to being better able to solvate the analytes, may serve to disrupt some of the matrix analyte interaction (i.e. emulating the effect of methanol) without the addition of the organic modifier. The behavior with methanol-modified CHF_3 is somewhat puzzling. The percent recovery for SMZ and SDM decreased under modified conditions, 24% for SMZ and 27% for SDM. Recovery for SQX increased with modifier addition to 16%. Multiple extractions (n=8) were performed to ensure the accuracy of these results. High relative standard deviations from 24 to 42 percent were obtained, but lower recoveries were consistent. The reason for the decrease is not well understood at present.

Beef liver also contains a large amount of fatty material that is co-extracted using CO_2 and methanol-modified CO_2. A large amount of yellow material was observed in the solid trap following extraction. Such a large amount was co-extracted, in fact that upon rinsing the solid trap directly with methanol a yellow rinse was obtained. However, if the liver was extracted with pure CHF_3, no yellow color was present in the rinse. The addition of modifier to fluoroform again diminished some of the selectivity in limiting fatty co-extractives. The differences in selectivity are clearly demonstrated in **Figure 17**, which is a blank extraction of 1 gram of beef

Table 4: Percent recovery of sulfamethazine (SMZ), sulfaquinoxaline (SQX), and sulfadimethoxine (SDM) from beef liver/celite mix. Numbers in parentheses are relative standard deviation. n = 3 for all samples

	100% CO_2	90% CO_2 10% MeOH	100% CHF_3	90% CHF_3 10% MeOH*
SMZ	23(14)	41(10)	74(9)	29(23)
SQX	<2	<2	<2	21(40)
SDM	18(18)	24(23)	69(6)	30(22)

* n = 8

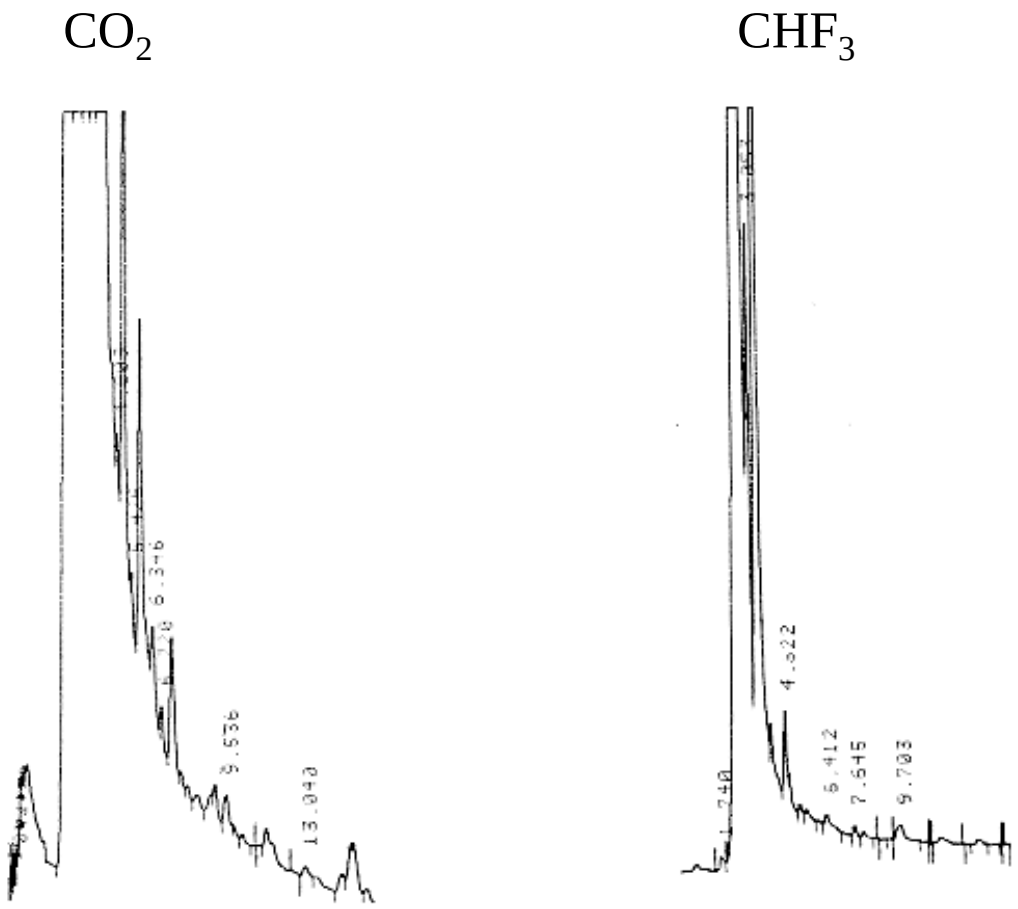


Figure 17: HPLC trace of 1 gram of beef liver blank extracted with 10% methanol modified CO_2 and 10% methanol modified CHF_3

liver extracted with methanol modified CO_2 and CHF_3. The CHF_3 extraction produces a much cleaner chromatogram than that obtained using CO_2. However, by employing a solid phase trap it is believed that the sulfonamides are selectively rinsed from the non-polar trap leaving the fatty co-extractives behind by using a polar rinse solvent (HPLC mobile phase). Both CO_2 and CHF_3, with or without modifier, upon selective rinsing yield extracts that have little interference from co-extractives and allows easy quantitation.

SUMMARY

Supercritical CHF_3 and methanol-modified CHF_3 were compared with supercritical CO_2 and methanol-modified CO_2 for the extraction of sulfonamides from a spiked sand, fortified non-fat milk powder, fortified egg yolk, and fortified beef liver. The results showed CHF_3 has higher solvating power and selectivity for extraction of the two neutral of the three sulfonamides compared to CO_2. This behavior is believed to be mostly due to the polar nature and strong hydrogen bonding afforded by CHF_3 with polar drug analytes, especially with basic sites. Extraction efficiency of SMZ and SDM using pure CHF_3 was more than 45% higher than with CO_2 from spiked sand samples and over 200% higher from fortified beef liver samples, where matrix effects are more prevalent. Fortified non-fat milk powder appeared to be a relatively inert matrix producing quantitative recoveries using either 10% modified CO_2 or CHF_3. But egg yolk and beef liver exhibited strong matrix-analyte interactions resulting in poor recoveries even with the addition of methanol modifier. Also, visual observation of the extract collected on a solid sorbent trap showed that CHF_3 yields a much cleaner extract compared to CO_2, although by selectively rinsing the sulfonamides from the trap, quantifiable extracts were obtained with either fluid.

Chapter IV

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

INTRODUCTION

In the previous chapter CHF_3 was shown to be a better extractant for both SMZ and SDM from each of the biological matrices investigated. However, this enhancement produced quantitative recovery of all analytes from only dry non-fat milk powder and in this case quantitative recovery was obtained with methanol modifier using either CO_2 or CHF_3. Since CHF_3 does not produce satisfactory results, further optimization of the extraction method is needed to yield complete extraction of all three analytes from each of the matrices investigated. This will be attempted by investigating different polar organic solvents as co-solvents (modifiers) with CO_2.

The effect of modifier addition on supercritical fluid extraction of analytes with CO_2 has received much attention. The addition of polar modifiers can aid the extraction by either 1) increasing the solvating strength of the fluid or 2) improving desorption of the analyte from a sample matrix. Acetonitrile modified CO_2 has been used, for example, to elute rotenone from an extraction disk (92) with greater than 90% recovery. Acetonitrile has also been used to extract phenoxy acids from water (64). Greater than 81% recovery was obtained for both phenoxyacetic and phenoxybenzoic acid using 10% acetonitrile, whereas, 10% methanol modifier yielded only 53% recovery of phenoxybenzoic acid. Near quantitative recovery (>90%) of three phenols was achieved by Ashraf-Khorassani et al. (63) with both acetonitrile and methanol modified CO_2. Levy et al. (93) found methanol modified CO_2 better extracted crude oil contaminated soil compared to acetone (94% recovery compared to 83% for acetone). Comparable recoveries were obtained for most members of a series of organophosphorus pesticides using either ethyl acetate, acetone, or methanol modified CO_2 (94).

Alcoholic modifiers have found the most widespread applications. Ethanol modified CO_2 has been used to fractionate egg yolk (95), extract Taxol from *Taxus Brevifolia* bark (96), defat corn distillers' grain for use as a high neutral detergent fiber content food ingredient (97), and for the extraction of PCBs from sewage sludge (98). Many environmental analytes have been extracted with methanol-modified CO_2 including pesticides (72, 99), herbicides (100-101), PAHs

(102-103), organometallics (104-105), and dioxin (106). Methanol modified CO_2 has also been used in the extraction of Aflatoxin B1 from food matrices (107).

This chapter 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, beef liver and egg yolk matrices using modified supercritical CO_2. Trends in the extraction efficiency of the same target sulfonamides as the previous chapters upon altering both modifier identity and composition will be discussed in an attempt to obtain complete recovery of all three analtyes. Based upon previous work neither high pressure (53) nor polar supercritical fluids (chapter III) alone could yield complete recovery of all three sulfonamides. Therefore, this work will attempt to utilize the characteristics of the organic solvent modifier to achieve complete extraction from all three targeted biological matrices.

EXPERIMENTAL

Supercritical Fluid Extraction and Quantitation

An Isco (Lincoln, NE) SFX-3560 automated supercritical fluid extractor equipped with an automatic variable restrictor system was used for extracting sulfonamides from chicken liver. The system consisted of a 100DX syringe pump capable of delivering CO_2 and another 100DX pump to deliver modifier. 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).

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 minute dynamic step at 490 atm and a temperature of 40^{o}C. A flow of 1.5 mL/min of liquid CO_2 was used for all extractions. The restrictor was maintained at 50^{o}C. A liquid trap composed of 85% 8mM ammonium acetate (NH_4OAc) / 15% acetonitrile (3.5 mL) was used for each extraction. The liquid trapping system was maintained at 10^{o}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 5mL with 85/15 8mM 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 0.2µm Teflon filter (Supelco, Bellefonte, PA) to remove particulates.

A Hewlett-Packard (Little Falls, DE) series 1050 HPLC equipped with a variable wavelength UV detector at 266 nm was used to assay all sample extracts. A 250 x 4.6mm (5µm dp) Deltabond^{TM} ODS (Keystone Scientific, Bellefonte, PA) column was used throughout the study. The mobile phase employed was 85% 8mM NH_4OAc/ 15% acetonitrile operated at a flow of 1mL/min. Sample quantitation was performed as described previously (chapter 3).

Sample Preparation

Chicken liver samples were purchased at a local grocery store. Samples were prepared by spiking 0.5 gram of liver tissue with 10µL of drug standard (0.6µg/µL each of SMZ, SQX, and SDM in methanol). The spiked matrix was then thoroughly mixed with 1.0 gram of Hydromatrix (Varian, Sugar Land, TX), followed by an incubation period of at least 30 minutes at -10^{o}C. Hydromatrix was used in place of Celite for this study because it possesses better dispersion characteristics. Celite is in a powder form, whereas, Hydromatrix is pellicular. This afforded much easier sample preparation and sample handling. Hydromatrix is similar to Celite, but is a mixture of diatomaceous earth and silica, whereas Celite is only diatomaceous earth. 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 20µL of a 24ng/µL standard of SMZ, SQX, and SDM onto 0.5g of chicken liver (960ppb). 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 obtained from USDA/ARS in Philadelphia, PA were ground prior to receiving the sample and were treated in a similar manner (6µg 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.

RESULTS AND DISCUSSION

The objective of this chapter 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, beef liver and egg yolk using methanol, ethanol, acetone, and acetonitrile as modifiers. In addition, the applicability of employing a pressurized liquid trapping system was investigated.

Liquid Trapping

Initially, we wanted to ensure the integrity of the trapping system. An Ottawa sand sample was spiked with the three sulfonamides. Trapping efficiency was determined using methanol and 85/15 8mM NH_4OAc:Acetonitrile both with and without positive pressure applied to the trap during extraction. These analytes proved to be easily trapped in that all conditions investigated produced greater than 90% recovery of all three analytes using 10% methanol modified CO_2. Since quantitative recovery was obtained for each analyte the system was considered efficient. The conditions producing the best recovery used the buffered mobile phase and trap pressurization, therefore identical liquid trapping conditions were used for all subsequent extractions.

Chicken Liver

We first decided to investigate chicken liver samples. Extraction of sulfonamides from chicken tissues has been shown to be less difficult. Parks et al. (53) were able to achieve near quantitative recovery of SMZ, SDM, and SQX from chicken breast, thigh, and liver using pure CO_2 at very high pressures (10,000 psi) and 40^{o}C. Similarly, Ashraf-Khorassani et al. (56) achieved near quantitative recovery of SMZ and SDM from chicken liver employing 10% methanol-modified CHF_3 at 6500 psi and 40^{o}C. A less difficult matrix was attractive in that the effect of modifier composition and identity should be more readily observed.

Methanol Modified CO_2

Table 5 shows the recovery of sulfonamides spiked in chicken liver mixed with Hydromatrix using different methanol modified CO_2 concentrations. 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. In order to remove the coextractive particulates a 0.2µm Teflon filter was used. To ensure the integrity of the filtering system a sample was injected both before and after filtering through a 0.2µm Teflon filter. The results showed no differences due to filtering of the extracts and filtering was used for all subsequent analyses. By increasing the methanol concentration to 10%, recovery of the drugs increased to nearly 60% which supports previous findings of Ashraf-Khorassani et al. (56) 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.

Table 5: 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. Numbers in parenthesis are relative standard deviations.

	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)

Cross et al. (54) extracted sulfonamides from chicken liver using an even higher methanol-modifier content (25% v/v). They found that an extensive clean up procedure after liquid phase trapping, including evaporation under nitrogen and solid phase extraction, was necessary to obtain extracts in which quantitation was possible. The sample extracts obtained with 20% methanol were yellow due to the presence of co-extractives, but no chromatographic interference was observed. Only filtering the sample through a 0.2µm Teflon filter was necessary to obtain quantifiable extracts. This is a significant improvement in the sample preparation of sulfonamides compared to the conventional methods discussed previously.

Ethanol Modified CO_2

Ethanol modified CO_2 was investigated next (**Table 6**). Ethanol possesses a polarity index of 5.2 (108), whereas methanol is 5.1. The polarity index is a measure of the relative polarity of different solvents (i.e. the hydrogen bond donor, hydrogen bond acceptor, and dipole characteristics of the solvent). A higher value on the polarity index indicates a more polar solvent. 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 modifier polarity, however, methanol and ethanol possess very similar dipole moments. Therefore, it was concluded that ethanol was no better or worse than methanol for this matrix/analyte. Again, the only sample cleanup necessary was filtering the sample.

Acetone Modified CO_2

Acetone modified CO_2 (**Table 7**) was then investigated since it possesses a polarity index (5.1) similar to methanol and ethanol, but SQX has a six-fold larger solubility in acetone than in 95% alcohol (109). 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 0.2µm Teflon filter. Some

Table 6: 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. Numbers in parenthesis are relative standard deviations.

	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)

Table 7: 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. Numbers in parenthesis are relative standard deviations.

	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)

chromatographic interference was observed, however, with the use of acetone (**Figure 18**) especially for SMZ, which eluted first. This interference arises because 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 co-extractive material in the liquid trap since the amount of acetone increased in the sample extract. Upon increasing the acetone concentration to 20%, quantitative recovery of all three sulfonamides was obtained. The acetone modifier may (a) better disrupt the analyte-matrix interactions, which makes the analytes more available for extraction, (b) increase solubility of fatty material which may better release sulfonamides trapped within the fatty material, or (c) enhance solubility of the analytes. Each mechanism probably aids the extraction, making quantitative recovery possible. Although some interference was observed, quantitation of the sample extracts was still possible only by filtering the extracts prior to analysis. Co-extractives present in the sample did not appear to interfere with the quantitation method.

Acetonitrile Modified CO_2

Lastly, acetonitrile (5.8 on the polarity index) modified CO_2 (**Table 8**) was investigated. A more polar solvent was thought to better solubilize the polar sulfonamides 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 observed (**Figure 19**). By increasing the modifier concentration to 20%, 110% SMZ, 104% SQX and 106% SDM was recovered within 30 minutes. The 20% acetonitrile modifier sufficiently disrupted analyte-matrix interaction and more importantly neither the co-extractives nor modifier produced chromatographic interferences, as in the case of acetone.

Sub-ppm Extraction of Chicken Liver with Acetonitrile and Acetone Modified CO_2

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

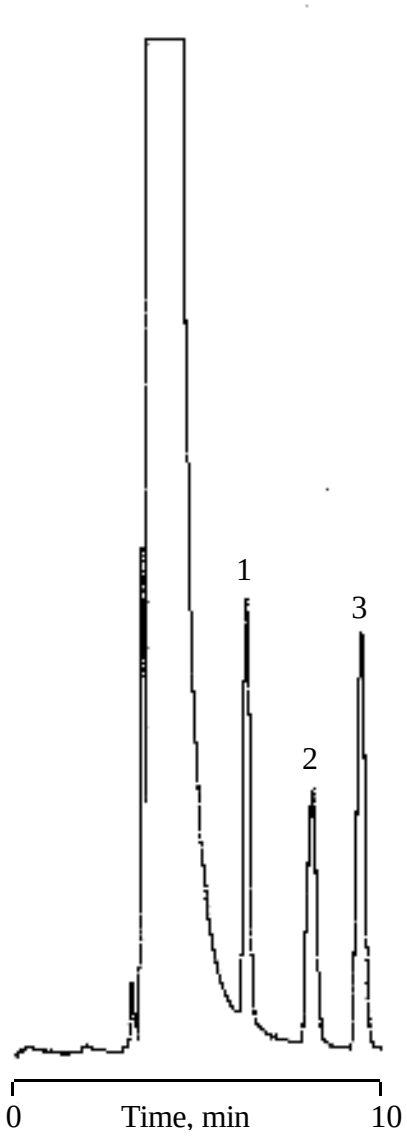


Figure 18. HPLC separation of sulfonamides extracted from chicken liver-Hydromatrix mix. Extraction conditions: 10% acetone modified CO_2, 490 atm, 40°C, 1.5 mL/min for 30 min, liquid trap consisting of 3.5 mL 85/15 8mM NH_4OAc/acetonitrile pH adjusted to 6.5 using acetic acid. HPLC conditions: Deltabond ODS, 250 x 4.6mm, 5µm d_p. mobile phase: 85/15 8mM NH_4OAc/acetonitrile pH adjusted to 6.5 using acetic acid. Flow 1 mL/min. Detector UV monitored at 266nm.
Elution order: 1) SMZ, 2) SQX, 3) SDM

Table 8: 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. Numbers in parenthesis are relative standard deviations.

	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)

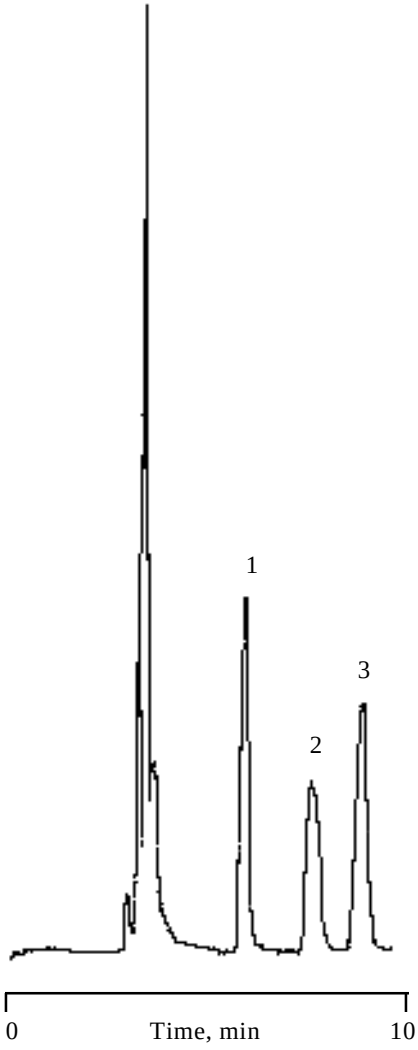


Figure 19. HPLC separation of sulfonamides extracted from chicken liver-Hydromatrix mix. Extraction conditions: 10% acetonitrile modified CO_2, 490 atm, 40^{o}C, 1.5 mL/min for 30 min, liquid trap consisting of 3.5 mL 85/15 8mM NH_4OAc/acetonitrile pH adjusted to 6.5 using acetic acid. HPLC conditions: Deltabond ODS, 250 x 4.6mm, 5µm d_p. mobile phase: 85/15 8mM NH_4OAc/acetonitrile pH adjusted to 6.5 using acetic acid. Flow 1 mL/min. Detector UV monitored at 266nm.
Elution order: 1) SMZ, 2) SQX, 3) SDM

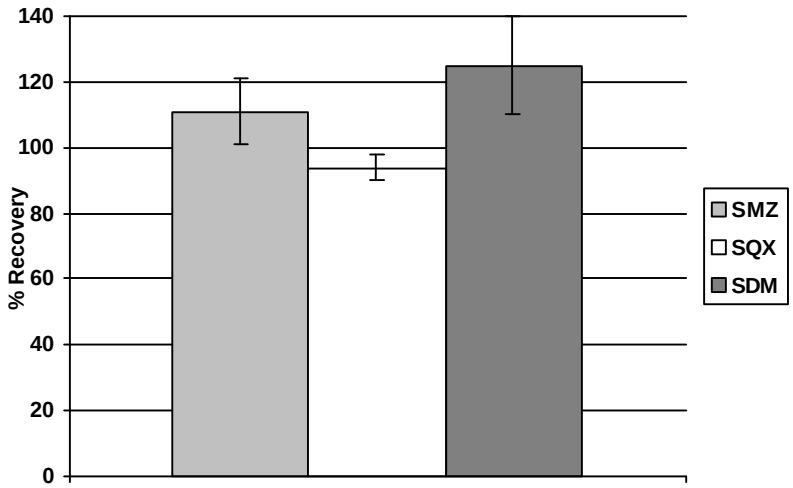


Figure 20. Extraction efficiency of SMZ, SQX, and SDM spiked at 960 ppb onto a chicken liver-Hydromatrix mix using 20% acetonitrile modified CO_2. (n = 3)

%RSDs were below 12% for all three analytes.

Beef Liver Extraction with Acetonitrile and Acetone Modified CO_2

Since quantitative recovery was obtained for chicken tissues, the method was applied to study the matrices that presented difficulty in the previous chapter. Beef liver sample extractions were attempted using the two best conditions found for chicken liver (20% acetone and acetonitrile). In chapter III only 41% SMZ, 24% SDM and less than 2% recovery for SQX was obtained from beef liver using 10% methanol modified CO_2. **Table 9** shows that quantitative recovery was obtained for all three analytes within 30 minutes using 20% acetone modifier. The acetone again caused chromatographic interference (**Figure 21**) . 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 re-dissolved in the chromatographic mobile phase (**Figure 22**). This procedure greatly reduced the amount of interference caused by the residual acetone. Similar peak areas although were obtained from both chromatograms (within 5%), therefore all quantitation was performed without acetone evaporation. Acetonitrile modified CO_2 (20%) which was found to be the best modifier for chicken liver was not able to produce quantitative recovery for all three analytes from beef liver (**Table 9**). Greater than 90% recovery was obtained for SMZ and SDM, but only 81% recovery was obtained for SQX.

Egg Yolk Extraction with Acetonitrile and Acetone Modified CO_2

Lastly, the 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. In chapter III 10% methanol modified CO_2 was found to only yield 29 % recovery of SMZ, 28% SQX and 29% of SDM. However, by employing modifiers that possess better characteristics for the analytes, both acetone and acetonitrile produced quantitative recovery of all three analytes. Recoveries obtained using acetonitrile (20%) and 20% acetone is shown in **Table 10**. Both fluids produced better than 99% recovery for all three analytes. Acetonitrile, although, is the modifier of choice since acetone causes UV detection problems.

Table 9: 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. Numbers in parenthesis are relative standard deviations.

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

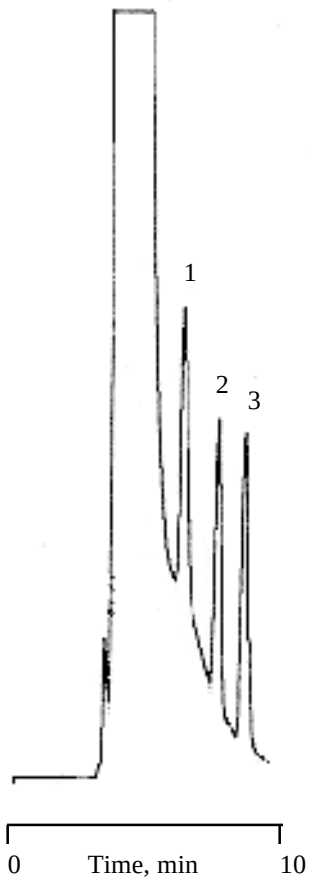


Figure 21. HPLC separation of sulfonamides extracted from beef liver-Hydromatrix mix prior to acetone evaporation. Extraction conditions: 20% acetone modified CO_2, 490 atm, 40°C, 1.5 mL/min for 30 min, liquid trap consisting of 3.5 mL 85/15 8mM NH_4OAc/acetonitrile pH adjusted to 6.5 using acetic acid. HPLC conditions: Deltabond ODS, 250 x 4.6mm, 5µm d_p. mobile phase: 85/15 8mM NH_4OAc/acetonitrile pH adjusted to 6.5 using acetic acid. Flow 1 mL/min. Detector UV monitored at 266nm.
Elution order: 1) SMZ, 2) SQX, 3) SDM

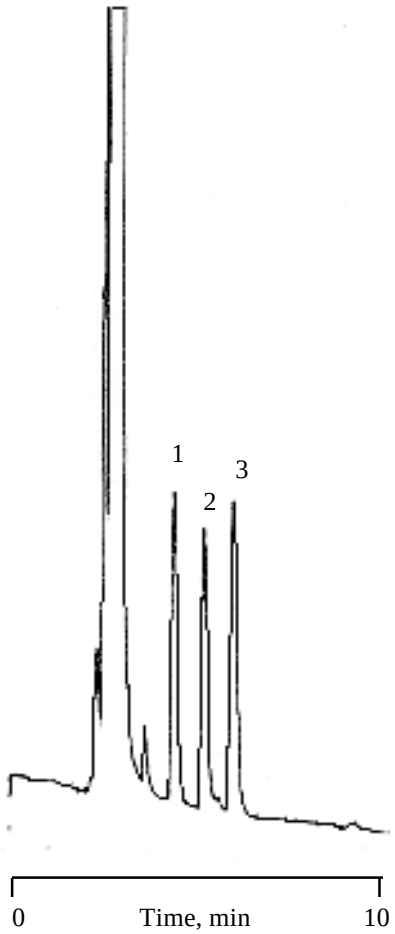


Figure 22. HPLC separation of sulfonamides extracted from beef liver-Hydromatrix mix followed by evaporation of acetone and rediluted with 85/15 8mM NH_4OAc/ acetonitrile. Extraction conditions: 20% acetone modified CO_2, 490 atm, 40^oC, 1.5 mL/min for 30 min, liquid trap consisting of 3.5 mL 85/15 8mM NH_4OAc/acetonitrile pH adjusted to 6.5 using acetic acid. HPLC conditions: Deltabond ODS, 250 x 4.6mm, 5µm d_p. mobile phase: 85/15 8mM NH_4OAc/acetonitrile pH adjusted to 6.5 using acetic acid. Flow 1 mL/min. Detector UV monitored at 266nm.
Elution order: 1) SMZ, 2) SQX, 3) SDM

Table 10: Percent recovery of sulfamethazine (SMZ), sulfaquinoxaline (SQX), and sulfadimethoxine (SDM) from a egg yolk-Hydromatrix mix using 20% acetone and 20% acetonitrile modified CO_2. Numbers in parenthesis are relative standard deviations.

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

SUMMARY

By employing high levels (20%) of polar organic solvents as modifiers complete, quantitative extraction was obtained for all three sulfonamides. This was the first time complete recovery of these three sulfonamides has been reported from either chicken liver, beef liver or egg yolk. Previously quantitative recovery was possible for only two of the three target sulfonamides from chicken liver with beef liver and egg yolk being more difficult to extract. Importantly, the only sample cleanup necessary to perform quantitative analysis by HPLC-UV was passing the sample through a 0.2 µm Teflon filter that removed co-extractive particulates and non-dissolved fatty material. The filtering of the sample was shown to have no effect on the analysis of the sample extracts.

Both 20% acetone modified CO_2 and 20% acetonitrile modified CO_2 were capable of quantitatively extracting all three sulfonamides from chicken liver. The optimum modifier however, was found to change with the matrix. Acetone better extracted sulfonamides from beef liver, whereas acetonitrile was better for chicken liver, while both obtained complete recovery from egg yolk. Acetonitrile modified CO_2 however, produced fewer chromatographic interferences than acetone.

This work demonstrates the usefulness of SFE for the sample preparation of sulfonamides from biological matrices and is the first report of quantitative (>90%) recovery of these three sulfonamides from chicken liver, beef liver or egg yolk by SFE. In addition, it was demonstrated that liquid trapping of the analytes was both efficient and rugged even with high levels of modifier. It would appear that sample preparation via a supercritical fluid affords a more viable alternative to conventional methods which often require extensive cleanup routines before quantitative analysis.

Chapter V

Evaluation and Optimization of Selective Detection Methods for Sulfonamides

INTRODUCTION

In the previous three chapters the supercritical fluid extraction of sulfonamides from various spiked biological matrices was investigated, and complete extraction was obtained from each matrix. Next, selective detection systems were evaluated for the analysis of sulfonamides. Nitrogen selective detection was attractive due to its high sensitivity and because sulfonamides contain several nitrogen atoms. Mass specific detection was also attractive due to its high selectivity and the ability to obtain limited molecular information as well. This Chapter evaluates and optimizes both a nitrogen selective detector interfaced to supercritical fluid chromatography and HPLC/atmospheric pressure chemical ionization (APCI) mass spectrometry as assay methods for sulfonamides. The use of selective detectors for chromatography is a key approach in analytical chemistry. Valuable information about complex samples is more easily obtained with selective detectors than with conventional detectors which often require extra time and skill to achieve efficient separation of interfering analytes.

Chemiluminescene detectors which are specific for nitrogen and sulfur are especially attractive. These detectors were initially developed for gas chromatography (110-118) but they are beginning to find application in supercritical fluid chromatography and liquid chromatography(119-121). A flow diagram of the chemiluminescent nitrogen detector (CLND) is shown in **Figure 23**. It operates first by combusting the nitrogen-containing sample that elutes from the column in an oxygen atmosphere. The combustion product, nitric oxide (NO), then reacts with ozone to produce an excited NO_2 molecule which results in chemiluminescence (**Figure 24**). An equimolar response for all nitrogen compounds has been observed to date (119,120).

Mass spectrometers are versatile detectors for chromatographic separations offering both selective and molecular information from the same chromatographic separation during scanning conditions. Atmospheric pressure ionization, either electrospray or atmospheric pressure chemical ionization (APCI), is rapidly becoming one of the primary ionization methods for both SFC and HPLC. APCI uses a heated nebulizer which vaporizes both the mobile phase and sample. Ionization is accomplished with a corona discharge pin that emits electrons which

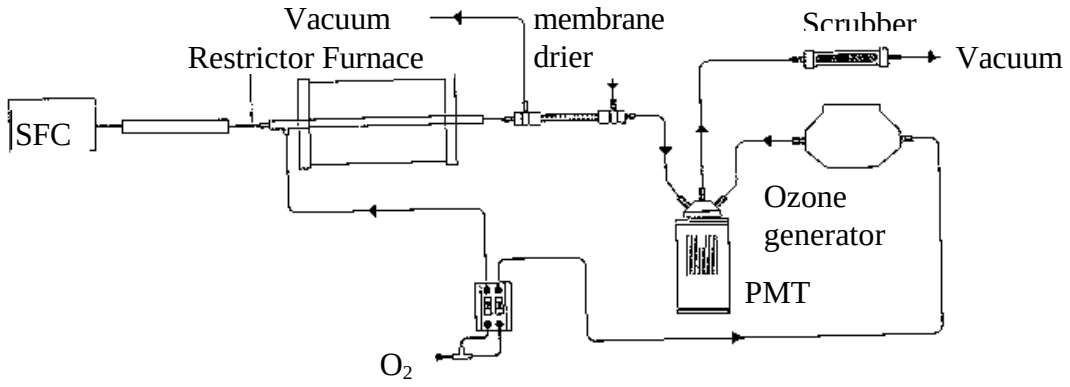


Figure 23: Flow diagram of packed column SFC-CLND system. This Figure was provided by Antek Instruments

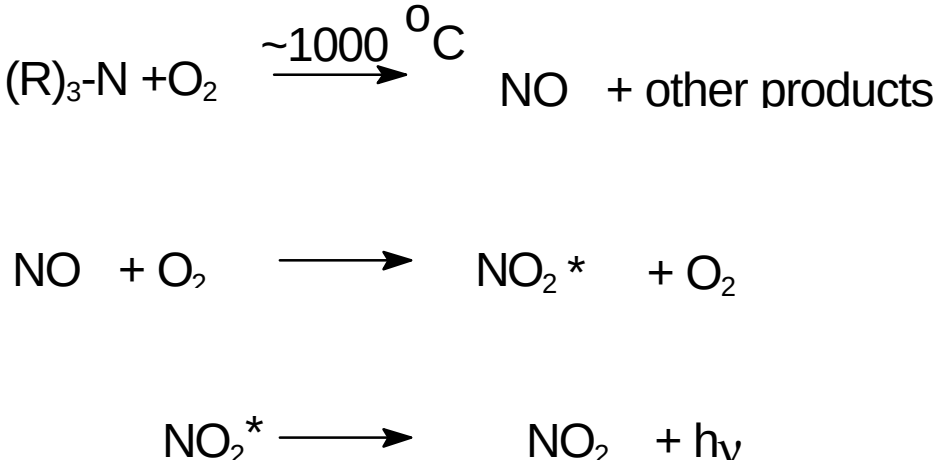


Figure 24: Proposed reaction scheme for the chemiluminescent nitrogen detector

collide with the abundant solvent ions (**Figure 25**). Solvent ions can then collide with the analyte causing ionization. Since ionization is due to molecular collisions, the APCI is considered to be a relatively soft ionization technique. Henion et al. (42) used HPLC/APCI-MS-MS for the analysis of sulfonamides. They did not report on the sensitivity of but showed the breakdown products of several sulfonamides. Analysis of racehorse urine contaminated with sulfonamides was shown to be feasible.

In this chapter, the evaluation and optimization of CLND and APCI-MS detection methods will be investigated. The first section considers the optimization of a packed column SFC-CLND system for sulfonamides with respect to ozone flow rate, decompressed CO_2 flow rate, and modifier concentration. In addition, detector design improvements and the effect of column diameter upon system sensitivity will be evaluated. The second part of this chapter evaluates HPLC/APCI MS detection for sulfonamides. System sensitivity in full scan and selected ion modes will be reported. This is only the second known report of sulfonamide analysis by HPLC/APCI-MS. Analysis of sulfonamides from chicken liver obtained using supercritical fluid extraction will also be discussed.

EXPERIMENTAL

CLND Instrumentation

A chemiluminescent nitrogen detector (CLND) Model 705D with a Model 771 pyrolysis system from Antek Instruments Inc. (Houston, TX) was interfaced to a Model G1205A supercritical fluid chromatographic (SFC) system equipped with both a variable restrictor which allows independent control of pressure and flow rate and a Model 1050 multiple wavelength UV detector (Hewlett Packard, Little Falls, DE). An auxiliary reciprocating pump in the SFC allowed methanol modifier to be added on-line to the carbon dioxide mobile phase. Samples were injected via a Model 7673 auto injector configured to an air actuated Rheodyne valve with a 5µL internal sample loop. The SFC-CLND system with the addition of the membrane drier is shown in **Figure 23**.

Simultaneous CLND and UV detection was accomplished with a post-column split of supercritical flow using a zero dead-volume tee (Chrom Tech, Inc., Apple Valley, MN). A tapered fused silica restrictor (75µm i.d.) which afforded CO_2 decompression was used to interface the SFC and CLND for all optimization procedures. An integral restrictor (75µm i.d., made in-house) was used for all subsequent analyses. The conditions for the CLND were: pyrolysis temperature 1070^{o}C, photomultiplier (PMT) voltage 740 volts, range x50, and detector output 1 mV. The pyrolysis oxygen flow rate was set to 50 mL/min. The oxygen flow rate for the ozone generator was varied to optimize detector response. At the exit end of the UV detector,

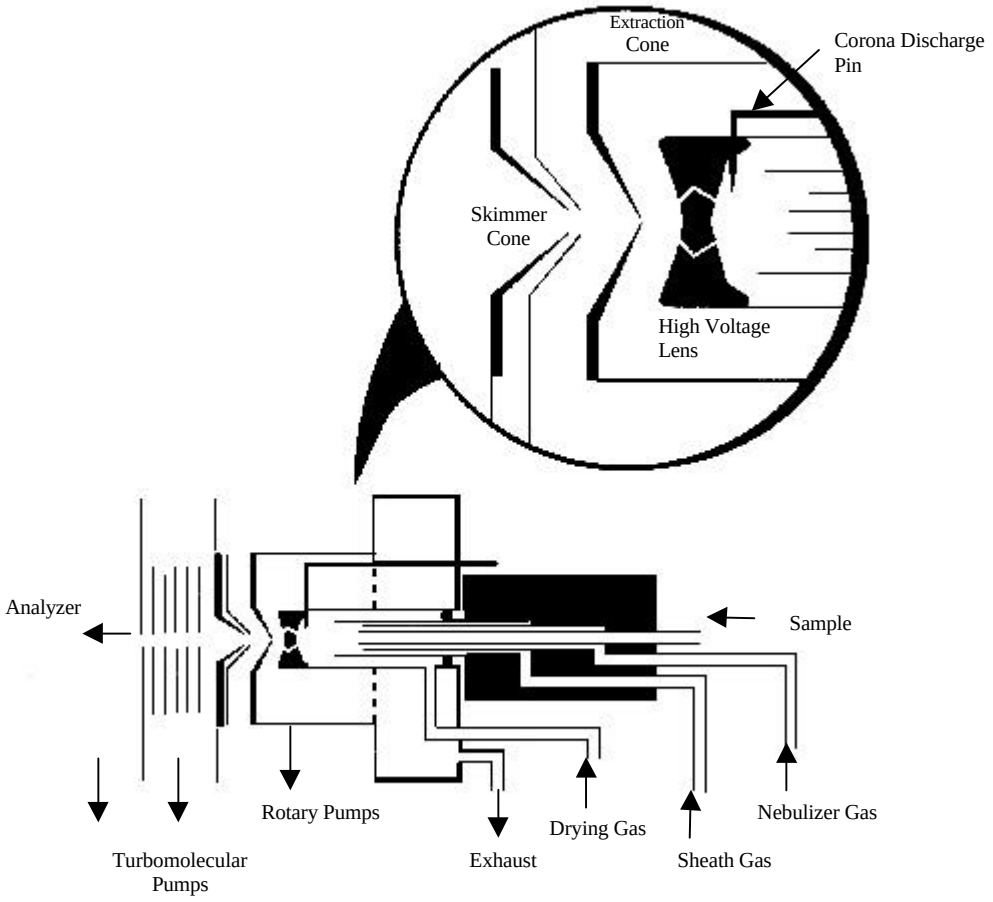


Figure 25: Atmospheric Pressure Chemical Ionization Probe. Taken from VG Platform Technical Users Manual.

a variable restrictor was fitted to maintain the SFC system pressure. The UV detector wavelength was set at 266 nm. An Alltima CN (250 x 4.6mm, 5µm d_p) column purchased from Alltech (Deerfield, IL) was used for all analyses.

A Hypersil CPS-2 (cyanopropyl) (150 x 2mm, 5µm d_p) column purchased from Keystone Scientific (Bellefonte, PA) was used for all separations that did not employ a post-column split. The entire SFC column effluent in this case was passed through the integral restrictor, used to maintain the system pressure, and was directed into the CLND furnace. In this fashion, the total sample injected was quantitated.

For the chrmoatographic method development portion of this chapter a standard HP model 1050 multi-wavelength detector (MWD) which employed a 13µL high-pressure flow cell was used. The sample wavelength was fixed at 270nm with a 4nm bandwidth. The reference wavelength was 450nm with an 80nm bandwidth. Columns were: 4.6 x 250mm, 5µm Spherisorb NH_2 (Alltech, Deerfield, IL), 4.6 x 250mm, 5µm Amino 2 (Keystone Scientific, Bellefonte, PA), 4.6 x 250mm, 5µm LiChrosorb SI-100 (Alltech), 4.6 x 250mm, 5µm Deltabond CN (Keystone Scientific), and a 4.6 x 250mm, 5µm Alltima CN (Alltech). All chromatographic conditions are noted in the figure captions.

APCI-MS Instrumentation

A VG BioTech (Altincham, UK) Platform single quadrupole mass spectrometer was interfaced to a 1050 series HPLC pump and a model 1050 variable wavelength UV detector (Hewlett Packard) via a commercially available APCI interface probe from VG shown in **Figure 25**. The APCI probe was heated to 400^{o}C to ensure complete vaporization of the column effluent. Corona pin voltage was set at 3.0kV for all analyses. Extraction cone voltage was set at 30V and the source temperature was set to 120^{o}C. A scan time of 1 second and interscan delay of 0.05 second was used for scan mode (110-300 m/z) and a 1 second dwell time was used for selected ion recording (156m/z 0-10.5 min, 265 and 250 m/z 10.5-13 min, 279 m/z 13-16 min and 156 m/z 16-23 min). Both drying gas and sheath gas were nitrogen from the gas outlet of a liquid nitrogen dewar. Optimal gas flows were adjusted daily using a simple optimization procedure described later. Samples were injected via a Rheodyne model 7125 injection valve with a 20µL sample loop.

A Prodigy C18 (250 x 4.6 mm, 5µm dp) column purchased from Phenomenex (Torrence, CA) was used for all HPLC separations. Chromatographic conditions consisted of a mobile phase of 85/15 8mM ammonium acetate/acetonitrile for the first 8 minutes. At 15.5 minutes the mobile phase was 60/40 8mM ammonium acetate/acetonitrile after a linear gradient of 100% A (85/15)

to 100% B (60/40) at 23 minutes, the mobile phase was returned to initial conditions. A flow of 1 mL/min was used throughout. The entire column flow could be added directly to the APCI probe without flow splitting.

Chemicals and Reagents

All sulfonamides used in this study were obtained from Robert Maxwell at the USDA/ARS in Philadelphia, PA. HPLC grade methanol and acetonitrile was purchased from EM Science (Gibbstown, NJ). Reagent grade ammonium acetate was purchased from Aldrich Chemical Co. (Milwaukee, WI). All chemicals were used without further purification. Grade 4.3 oxygen, used for the CLND pyrolysis furnace and ozone generator gas, and liquid nitrogen were purchased from Airco (Murray Hill, NJ). SFC-grade CO_2 (without helium headspace) was obtained from Air Products and Chemicals, Inc (Allentown, PA).

RESULTS AND DISCUSSION

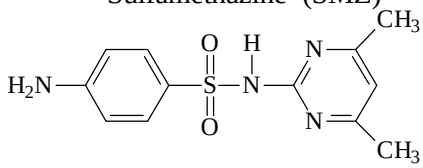
The goal of this project was two fold: (1) optimize chemiluminescene nitrogen detection (CLND) interfaced with packed column supercritical fluid chromatography (SFC) and (2) evaluate HPLC/APCI-MS for the analysis of sulfonamides. CLND parameters such as decompressed CO_2 flow rate, methanol modifier amount, and ozone flow rate were investigated. These parameters are important criteria in the optimization of detector sensitivity. No study to date has reported the effect of ozone flow rate or the effect of high methanol modifier concentrations (> 15%) on the sensitivity of the CLND coupled to SFC. In addition, the effect of detector design and column diameter changes upon system sensitivity were evaluated. HPLC/APCI-MS was investigated for sensitivity in both full scan and selected ion modes. In addition, supercritical fluid extracts of sulfonamides from chicken liver tissues were assayed by both detectors to evaluate the technique for real sample analysis.

Chemiluminescence Nitrogen Detection

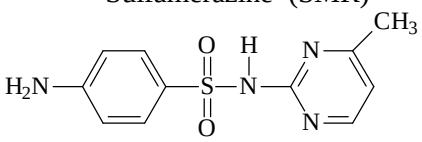
SFC Method Development

Although the first goal of this chapter was to optimize the CLND system, we felt that it was first necessary to develop a SFC separation for sulfonamides. The separation focused on the eight currently regulated sulfonamides (**Figure 26**). Choice of stationary phase/mobile phase and

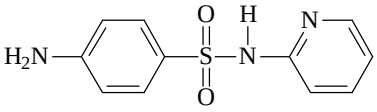
Sulfamethazine (SMZ)



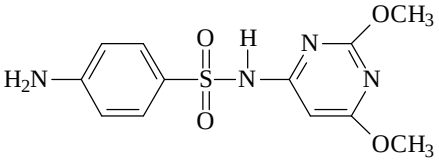
Sulfamerazine (SMR)



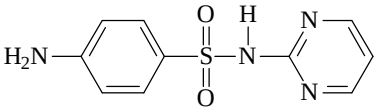
Sulfapyridine (SPD)



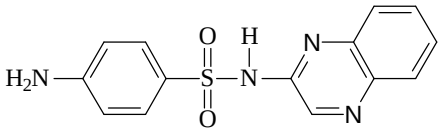
Sulfadimethoxine (SDM)



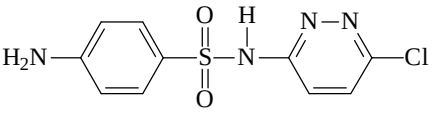
Sulfadiazine (SDZ)



Sulfaquinoxaline (SQX)



Sulfachlorpyridazine (SCP)



Sulfathiazole (STZ)

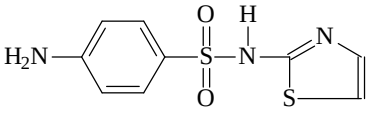


Figure 26: Structure of eight currently regulated sulfonamides

the effect of temperature upon selectivity was investigated in an attempt to provide sufficient and timely resolution of all compounds.

The first stationary phase investigated was 5µm Spherisorb NH_2. **Figure 27** shows the separation obtained with seven sulfonamides. Sufficient resolution can be obtained for all compounds (resolution (R) > 3.7 for peaks 2,3 and 3,4). However, addition of sulfapyridine (e.g. the eighth drug) caused coelution with sulfamerazine (peak 2). Ramsey et al. (49) also had reported coelution of sulfapyridine and sulfamerazine with the use of an aminopropyl silica stationary phase under similar chromatographic conditions. Due to this co-elution behavior an alternate amino column was investigated. The trace obtained using an Amino 2 column, which was said to be a highly deactivated column, decreased, for example, the retention time of sulfathiazole nearly 50%. This decreased retention also caused a loss in resolution of the early eluting peaks, therefore, precluding the use of the Amino 2 column. Since the aminopropyl silica phase did not provide an adequate separation, a Deltabond® cyanopropyl phase was investigated. Deltabond CN consists of a polymeric coated silica particle resulting in a highly deactivated phase. As with the deactivated amino phase, a poor separation was obtained for the seven sulfonamides investigated. Therefore, another cyano stationary phase was attempted. An Alltima® CN column was used. This column is deactivated by double endcapping. Seven compounds can be observed in the chromatographic trace, although baseline resolution was not obtained (R= 1.10 for peaks 3 and 4), and the sulfapyridine was not added to the mixture.

Since co-elution with the addition of sulfapyridine on the Spherisorb amino phase was observed, two columns of different selectivities were run in series (e.g. Alltima CN column followed by the Spherisorb NH_2) (**Figure 28**). This configuration was able to separate the eight compounds of interest with moderate resolution (R= 1.24 for peaks 2,3 and 2.67 for peaks 3,4). Two different stationary phases were used in hopes of separating sulfapyridine and sulfamerazine on the cyano column with their subsequent elution and separation of the other six sulfonamides on the amino column.

Earlier, Ramsey et al. (49) had achieved adequate separation of seven of the sulfonamides on either an amino or silica stationary phase. However, if sulfapyridine was added to the mixture, coelution occurred with sulfamerazine on the amino and with sulfadiazine on the silica column. In light of these events we attempted a separation on a silica column followed by an amino column in hopes of separating one pair of coeluting peaks on the silica column with the ability to maintain

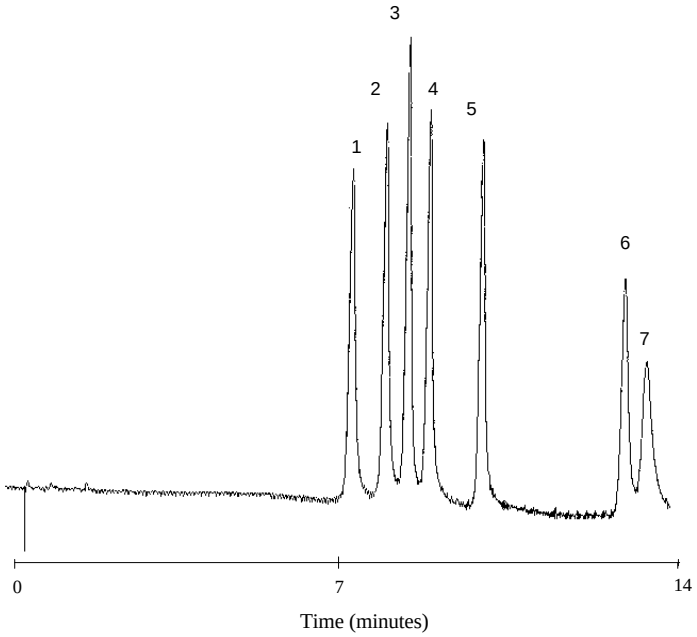


Figure 27. Supercritical fluid chromatogram of 7 sulfonamides. (1) sulfamethazine, (2) sulfamerazine, (3) sulfadimethoxine, (4) sulfadiazine, (5) sulfaquinoxaline, (6) sulfachlorpyridazine, and (7) sulfathiazole. Mobile Phase, 12% methanol for 5 minutes, 3%/min to 30%; oven temperature, 75°C; outlet pressure, 340 bar; liquid flow, 2.5mL/min; Column, 4.6 x 250mm, 5µm Spherisorb NH_2. 250 ng of each sulfonamide dissolved in methanol injected.

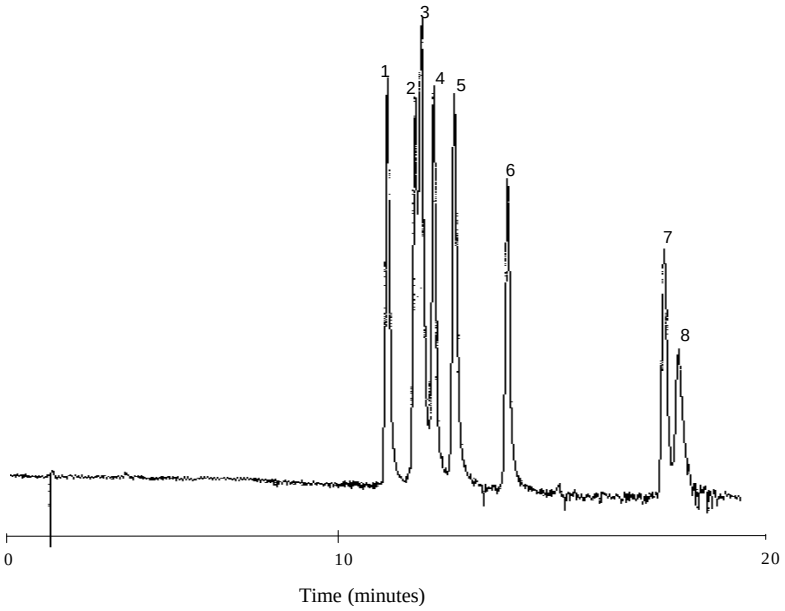


Figure 28. Supercritical fluid chromatogram of 8 sulfonamides. Tentative peak assignment. (1) sulfamethazine, (2) sulfamerazine, (3) sulfapyridine, (4) sulfadimethoxine, (5) sulfadiazine, (6) sulfaquinoxaline, (7) sulfachlorpyridazine, and (8) sulfathiazole. Mobile Phase, 10% methanol for 5 minutes, 2%/min to 30%; oven temperature, 75^{o}C; outlet pressure, 320 bar; liquid flow, 2.5mL/min; Column, 4.6 x 250mm, 5µm Alltima CN in series with 4.6 x 250mm, 5µm Spherisorb NH_2. 250 ng of each sulfonamide dissolved in methanol injected.

separation of the other components on the amino column. Separation of the eight drugs was achieved under these conditions but without baseline resolution (R= 1.31 for peaks 2,3 and 2.02 for peaks 3,4) (**Figure 29**). Injection of a smaller quantity of material under the same chromatographic conditions had little effect on the separation. Therefore, altering the temperature from 75°C was performed in hopes of improving the separation. Peaks 2,3,4 coeluted with an increase in temperature to 90°C (**Figure 30**). We next reasoned that if an increase in temperature can harm the separation, a decrease in temperature may improve the existing resolution. At a temperature of 50°C (**Figure 31**), however, more retention differences were observed. Peaks 5 and 6 now coeluted, whereas baseline separation of these peaks was obtained at both 75°C and 90°C. A more important observation was that peaks 2,3, and 4 were resolved at 50°C, although 1 and 4 coeluted and 3 coeluted with 5 and 6. An intermediate temperature was then attempted in hopes of obtaining the positive effects of lowering the temperature but maintaining separation of the later eluting peaks. **Figure 32** shows the chromatogram obtained at 65°C. By using a temperature between those previously investigated, a complete separation was achieved for all 8 compounds of interest within 20 minutes (R> 2.3 for all peaks). The separation was reproducible with the percent difference of retention times and peak areas less than 3% in most cases (n=2).

Detector Optimization

Ozone Flow Rate

The first parameter investigated was the effect of ozone flow rate on detector response with the use of supercritical methanol modified CO_2. This was accomplished at a previously optimized decompressed CO_2 flow rate of 150 mL/min and a pyrolysis oxygen flow rate of 50 mL/min (120). Ozone is one of the essential reaction components enroute to the excited state nitrogen dioxide molecule which upon relaxation produces a photon (**Figure 24**). The other necessary reaction component is nitric oxide and together with ozone they form the basis for chemiluminescent nitrogen detection. **Figure 33** shows the effect of increasing ozone flow rate on CLND response at constant modifier concentrations. At methanol modifier concentrations of 5%, 10%, and 15%, increasing ozone flow (from 1.6 to 5.8 mL/min) increased detector response. At 20% methanol however, the detector response was significantly lower and no signal was observed with 30% modifier. This observation suggests that (a) inefficient oxidation of nitrogen-containing analytes to NO in the furnace may be occurring, and/or (b) quenching of the chemiluminescence signal by uncombusted methanol is feasible (120). Low ozone flow

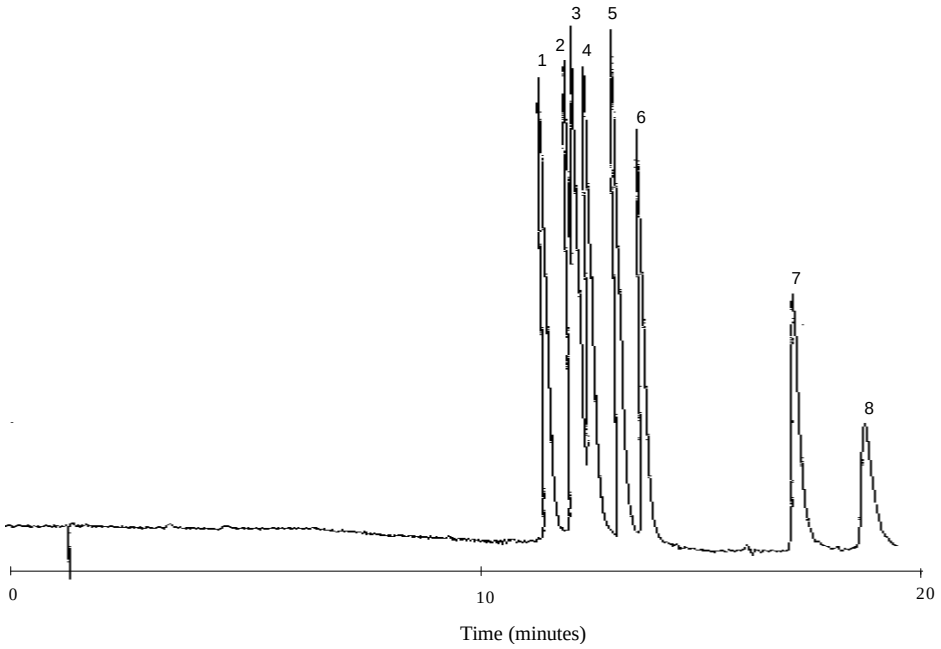


Figure 29: Supercritical fluid chromatogram of 8 sulfonamides. Tentative peak assignment. (1) sulfamethazine, (2) sulfamerazine, (3) sulfapyridine, (4) sulfadimethoxine, (5) sulfadiazine, (6) sulfaquinoxaline, (7) sulfachlorpyridazine, and (8) sulfathiazole. Mobile Phase, 10% methanol for 5 minutes, 2.5%/min to 30%; oven temperature, 75^{o}C; outlet pressure, 300 bar; liquid flow, 2.5mL/min; Column, 4.6 x 250mm, 5µm LiChrosorb SI-100 Silica in series with 4.6 x 250mm, 5µm Spherisorb NH_2. 250 ng of each sulfonamide dissolved in methanol injected.

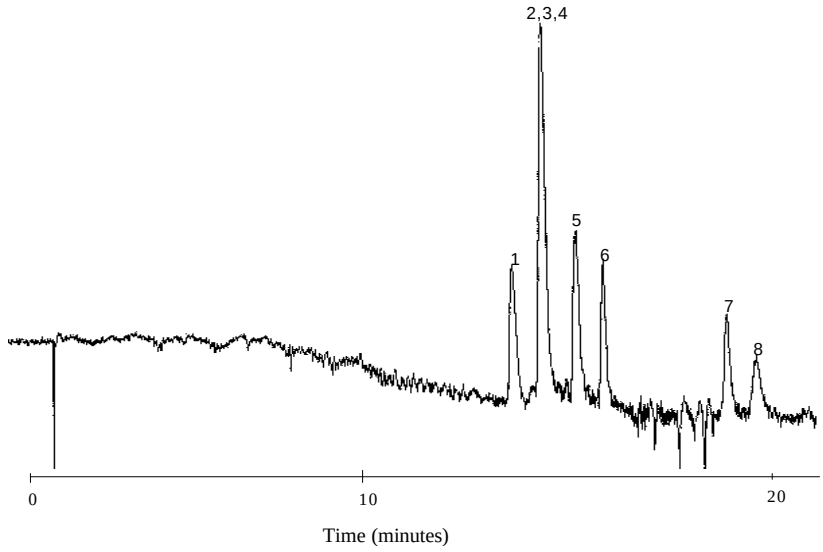


Figure 30. Supercritical fluid chromatogram of 8 sulfonamides. Tentative peak assignment. (1) sulfamethazine, (2) sulfamerazine, (3) sulfapyridine, (4) sulfadimethoxine, (5) sulfadiazine, (6) sulfaquinoxaline, (7) sulfachlorpyridazine, and (8) sulfathiazole. Mobile Phase, 10% methanol for 5 minutes, 2.5%/min to 30%; oven temperature, 90^{o}C; outlet pressure, 300 bar; liquid flow, 2.5mL/min; Column, 4.6 x 250mm, 5µm LiChrosorb SI-100 Silica in series with 4.6 x 250mm, 5µm Spherisorb NH_2. 25ng of each sulfonamide dissolved in methanol injected.

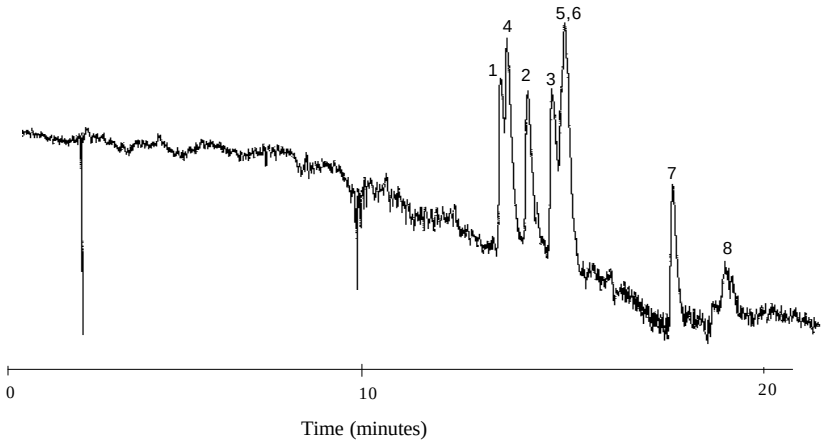


Figure 31: Supercritical fluid chromatogram of 8 sulfonamides. Tentative peak assignment. (1) sulfamethazine, (2) sulfamerazine, (3) sulfapyridine, (4) sulfadimethoxine, (5) sulfadiazine, (6) sulfaquinoxaline, (7) sulfachlorpyridazine, and (8) sulfathiazole. Mobile Phase, 10% methanol for 5 minutes, 2.5%/min to 30%; oven temperature, 50^{o}C; outlet pressure, 300 bar; liquid flow, 2.5mL/min; Column, 4.6 x 250mm, 5µm LiChrosorb SI-100 Silica in series with 4.6 x 250mm, 5µm Spherisorb NH_2. 25ng of each sulfonamide dissolved in methanol injected.

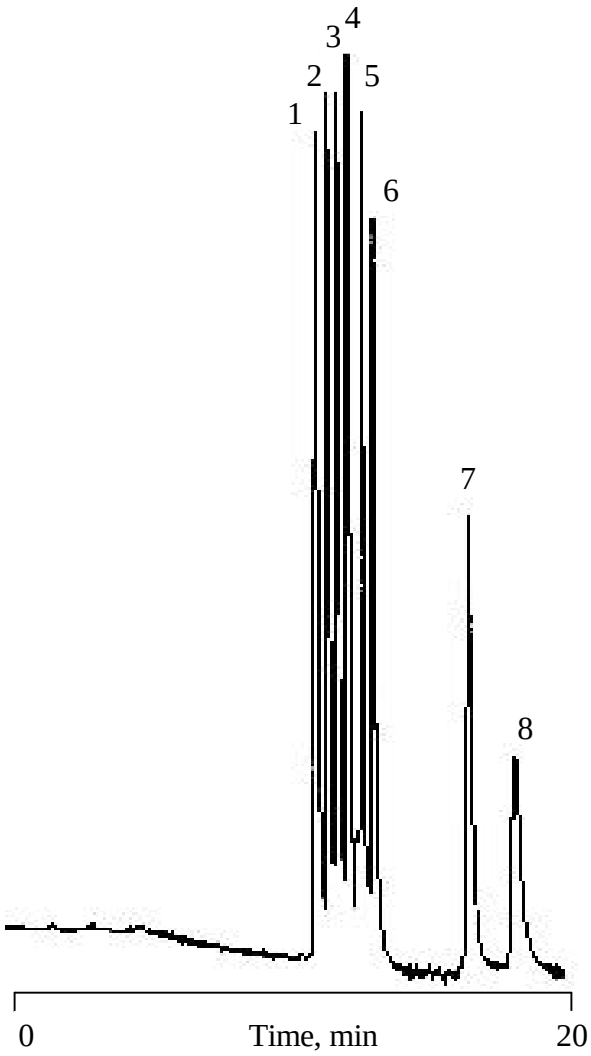


Figure 32: Supercritical fluid chromatogram of 8 sulfonamides. (1) sulfamethazine, (2) sulfamerazine, (3) sulfapyridine, (4) sulfadimethoxine, (5) sulfadiazine, (6) sulfaquinoxaline, (7) sulfachlorpyridazine, and (8) sulfathiazole. Mobile Phase, 10% methanol for 5 minutes, 2.5%/min to 30%; oven temperature, 65^{o}C; outlet pressure, 300 bar; liquid flow, 2.5mL/min; Column, 4.6 x 250mm, 5µm LiChrosorb SI-100 Silica in series with 4.6 x 250mm, 5µm Spherisorb NH_2. 250 ng of each sulfonamide dissolved in methanol injected.

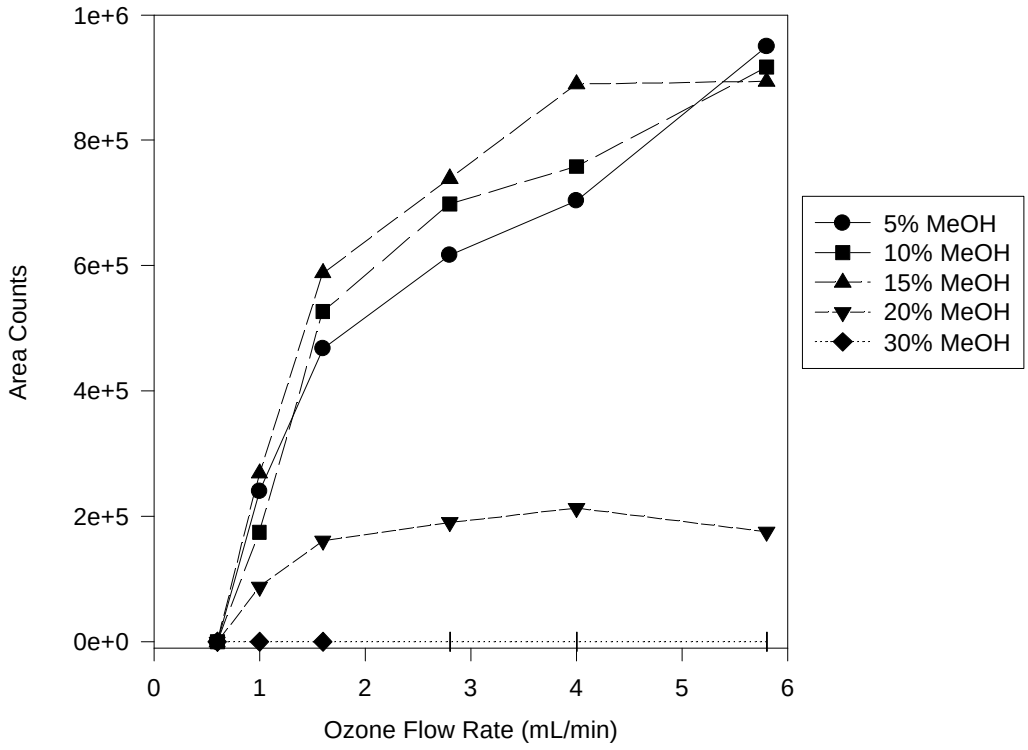


Figure 33: The effect of ozone flow rate on CLND response of sulfamethazine at 150 mL/min decompressed CO_2 flow rate using 5 to 30% methanol-modified CO_2.

(0.6 mL/min) at any modifier amount resulted in no signal due to insufficient production of excited state NO_2 molecules. From this evaluation we concluded that an ozone flow of 5.8 mL/min was sufficient for packed column SFC that employs methanol modifier content up to 15%.

Decompressed CO_2 Flow Rate

The effects of decompressed CO_2 flow rates on CLND response were further investigated in connection with variable methanol modifier concentrations. Ozone flows of 1.6 to 5.8 mL/min were used at various decompressed CO_2 flows and methanol modifier compositions. **Figure 34** shows the effects of decompressed CO_2 flow rate versus nitrogen response (1.6 mL/min ozone flow) at several methanol modifier contents. At very low decompressed flows (65 mL/min), a signal was obtained for 30% methanol, however, at flows greater than 150 mL/min no signal was observed. Similar trends were found as the decompressed CO_2 flow was increased for 10 and 15% modifier. It is important to note that even higher decompressed CO_2 flow rates can be utilized for packed column SFC when methanol modifier levels are less than 10%. In this case only a small decrease in the CLND response was observed. Similar trends in CLND response vs. decompressed CO_2 flow rate at various modifier concentrations were observed for all of the ozone flows investigated. Increasing ozone flow served to increase the signal intensity but only for methanol modifier concentrations below 10%. With 15% methanol modifier, the CLND response increased with increasing ozone flow rate, but at an ozone flow of 1.6 mL/min a decreasing trend (**Figure 34**) was observed when decompressed CO_2 flow rates were above 250 mL/min. The optimum decompressed CO_2 flow rate was thus determined to be around 250 mL/min. For 5% and 10% methanol modified CO_2, nitrogen response for the CLND could be optimized at a higher decompressed CO_2 flow rate (e.g. 310 mL/min). **Figure 35** shows a three dimensional representation of the CLND response with respect to decompressed CO_2 flow rates and ozone flow rates at 10% methanol modifier. An optimum decompressed CO_2 flow rate of 310 mL/min can be easily observed. **Figure 36** also shows a three dimensional representation of the CLND response with respect to decompressed CO_2 flow rate at methanol concentrations from 5-30% using an ozone flow of 5.8 mL/min. **Figure 36** shows local maxima in signal at different decompressed CO_2 flows as the modifier concentration changes. By setting conditions to the local maxima the best conditions for sensitivity can be used regardless of the methanol modifier concentration needed.

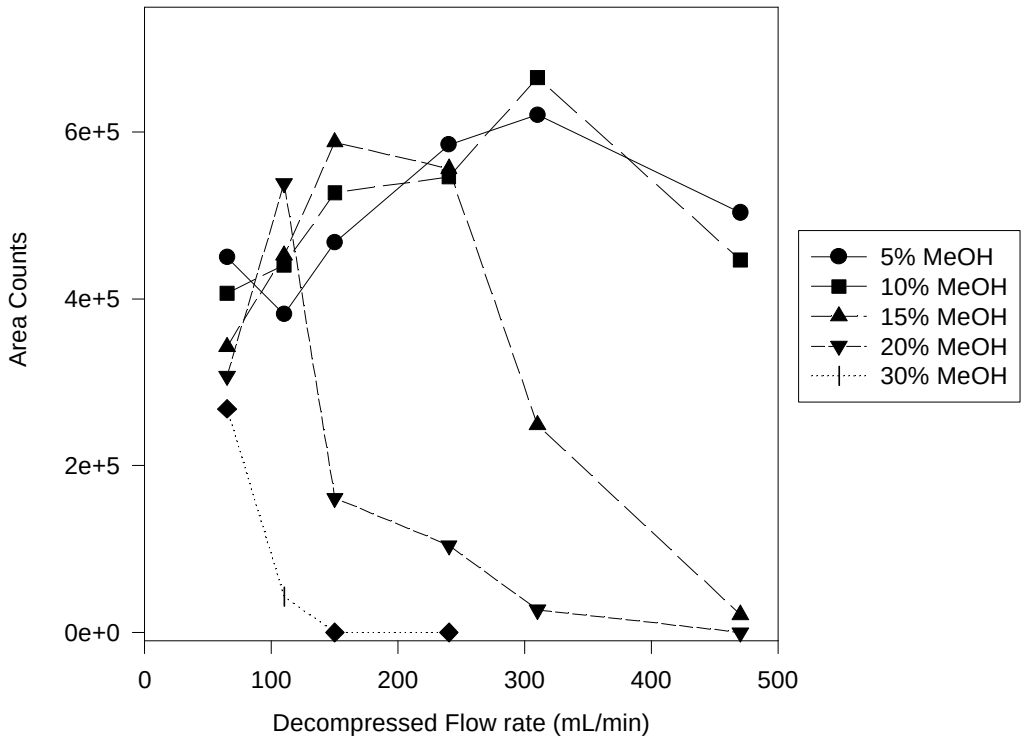


Figure 34: The effect of decompressed CO_2 flow rate on CLND response of sulfamethazine at 1.6 mL/min ozone flow rate and methanol concentrations from 5 to 30%.
even at flows as high as 470 mL/min.

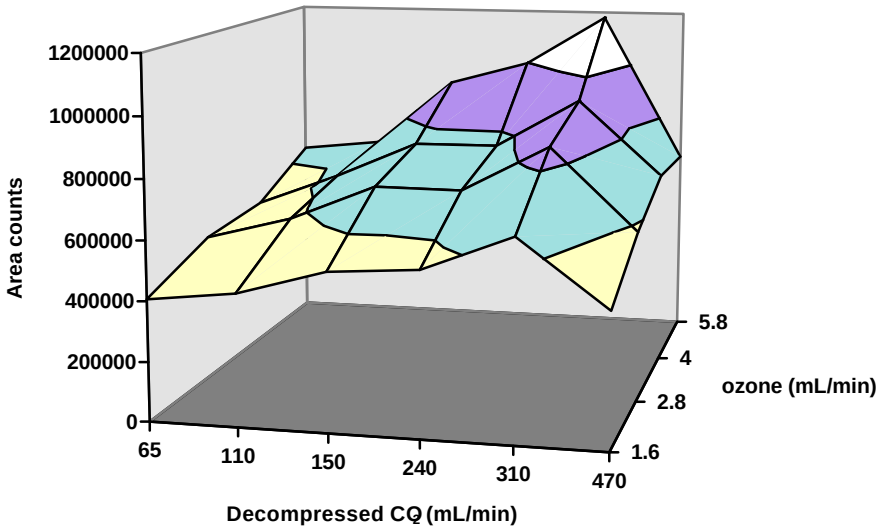


Figure 35: Three dimensional representation of CLND response of sulfamethazine versus decompressed CO_2 flow rate and ozone flow rate at 10% methanol modifer.

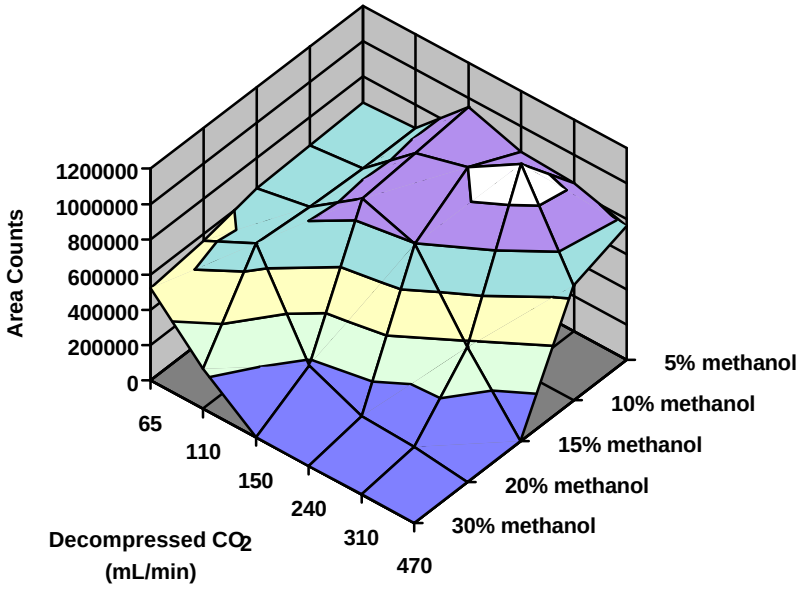


Figure 36: Three dimensional representation of CLND response of sulfamethazine versus decompressed CO_2 flow rate at methanol concentrations from 5-30% using an ozone flow of 5.8 mL/min.

Minimum Detectable Quantity Determination

Minimum detectable quantity (MDQ) with the packed column SFC-CLND system was determined in the next phase of our study. The MDQ was determined at a decompressed CO_2 flow of 310 mL/min with 10% methanol modifier. Quantities of injected sulfamethazine were decreased until a signal-to-noise ratio of 2-3 was obtained. An injection of 5 ng of sulfamethazine (1 ng N) was the lowest concentration detected at this stage of our study. Using a post-column split of the SFC flow between the UV and CLND (approximate ratio of 5:1), only 1ng of sulfamethazine (0.2 ng N) was actually present in the CLND. This limit of detection is similar to previously reported detection limits of 4.2 ng N, on column, using 5% methanol. In this study, a split ratio of 15.7:1 was assumed between UV and CLND thus resulting in only 0.3 ng N reaching the CLND (120). Strode et al. in another study reported a MDQ of 1 ng N, on-column, for packed column SFC-CLND using 8% methanol and a decompressed CO_2 flow entering the CLND of 600 mL/min (121). Their system required no post column split. Our MDQ however, is more than the 0.06 ng N previously reported for open tubular SFC-CLND using pure CO_2 (119). In summary the MDQ for all packed column systems using methanol modifier is approximately the same, but for maximum sensitivity open tubular SFC with pure CO_2 is preferred.

System Design Modification and Improvements

The second part of this section of the Chapter focuses on design modifications to the CLND made in an attempt to further reduce MDQ. The first attempt was a newly designed pyrolysis tube. This design placed the fixed (SFC) restrictor after the addition of pyrolysis O_2 to ensure better combustion in the pyro-furnace by improved mixing of effluents. This design did not offer significant improvements in detector sensitivity. Next, a membrane drier was added just after the furnace assembly (**Figure 23**). The drier selectively removes water from the combustion stream thereby 1) enriching the concentration of NO and 2) reducing the total number of colliding molecules in the reaction chamber. In this fashion, an improved quantum efficiency should be obtained for nitrogen chemiluminesce of the analyte. **Figure 37** shows a comparison of 5 ng injected both prior to and after the membrane drier was added to the CLND system. The addition of the membrane drier yielded an order of magnitude increase in sensitivity with a MDQ of 0.5 ng sulfamethazine (0.1 ng N) on column, at a S/N=2. Measurement was made using the previously determined optimum detector conditions (e.g. 15% methanol-modified CO_2 with 240 mL/min decompressed CO_2).

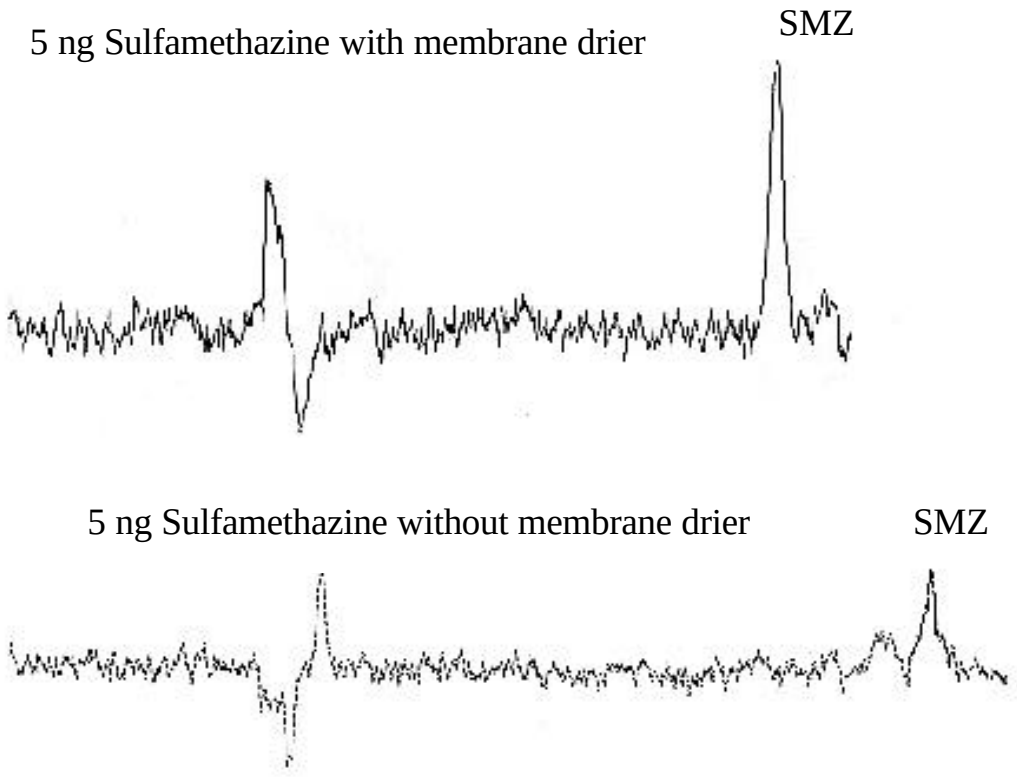


Figure 37: Supercritical fluid chromatographic comparison of 5 ng sulfamethazine (on column) prior to and after the membrane drier was added to the CLND system. (SMZ= sulfamethazine)

Once adequate CLND conditions were determined, we wanted to use the detector in a separation of the three sulfonamides we investigated in previous chapters. **Figure 38** shows all three components at a level of 2.5 ng, on column, using 15% methanol modified CO_2 and a decompressed CO_2 flow of 240 mL/min. All three peaks are baseline separated with a S/N near 2.

Reproducibility and Repeatability

A series of analyte injections was next performed to determine the reproducibility and repeatability of the SFC-CLND system. Two concentrations (0.2 ng/µL and 1 ng/µL) of sulfamethazine were used for the detector evaluation. Peak height was used for evaluation at 0.2 ng/µL due to its close proximity to the MDQ. Peak areas were used for evaluation when 1 ng/µL solutions were employed. Reproducibility studies consisted of seven injections of 0.2 ng/µL and four injections of 1 ng/µL. The relative standard deviation (% RSD) for injections of 0.2 ng/µL and 1 ng/µL was 6.9% and 4.2 %, respectively. Repeatability studies consisted of similar injections on subsequent days. Injections were performed on day 1, day 2, day 3 and day 7. The RSD for injections of 0.2 ng/µL sulfamethazine was 10.5. The higher concentration (1 ng/µL) standard over a one week period showed a lower RSD(5.6%).

Column Diameter Effect on CLND Sensitivity

Although sensitivity was greatly improved with the addition of the membrane drier, the splitting of the column effluent between the UV and CLND inherently reduces the amount of material that reaches the nitrogen detector. Therefore, a 2 mm i.d. Hypersil CPS-2 column was employed to hopefully increase the sensitivity even further. The 2 mm i.d. packed column was used with an optimum flow rate of 0.5 mL/min liquid CO_2 which corresponds to an optimum decompressed CO_2 flow rate of ~ 300 mL/min. Thus, the entire flow could be directed into the CLND, eliminating the 5:1 post-column split. The MDQ under these conditions was determined to be 0.125 ng of sulfamethazine (0.025 ng N) (**Figure 39**) using 15% methanol-modified CO_2. Considering the post-column split for the analytical scale column, the detector is responding to approximately the same amount of material from both columns (microbore: 0.125 ng) and (analytical: 0.5 ng/5= 0.1 ng). However, by eliminating the post-column split (with the packed analytical column) and by taking advantage of achieving higher peak concentration with a smaller i.d. packed microbore column, lower injected sample concentrations were detected using the

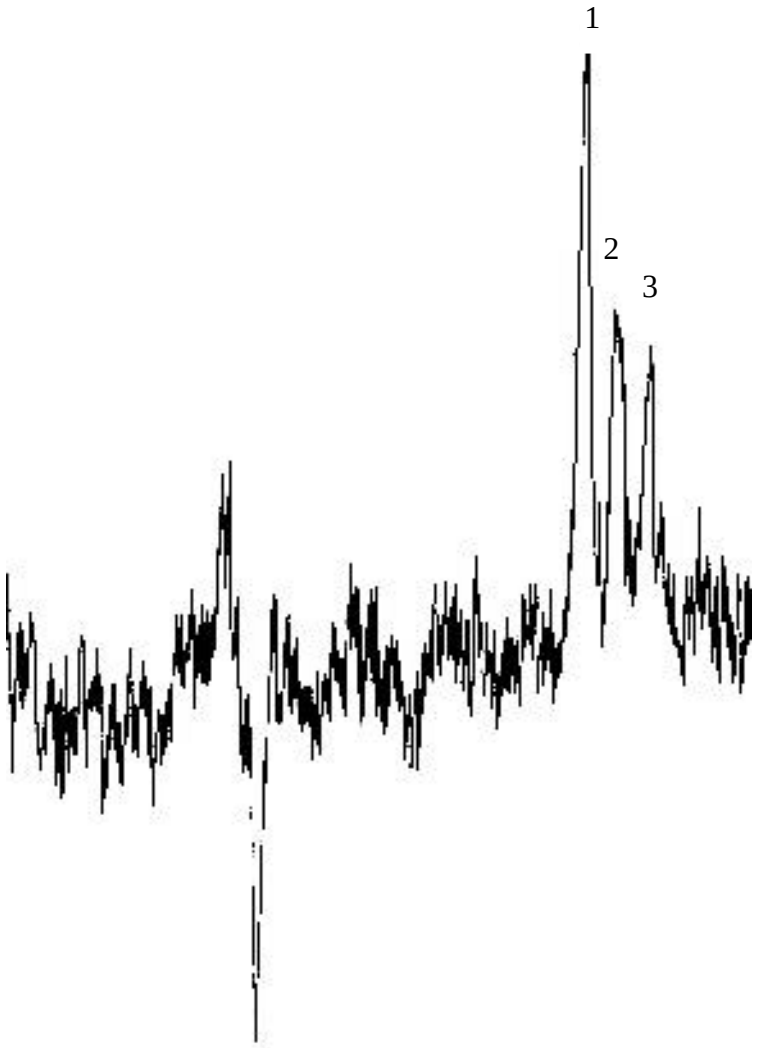


Figure 38: SFC-CLND chromatogram of three sulfonamides at 0.5 ng/µL.
15% methanol modified CO_2, 240 mL/min decompressed CO_2 entering CLND, 1.5 mL/min liquid CO_2 flow.
Column: 250 x 4.6 mm Alltima CN, 5µm d_p
Injection: 5µL in methanol
Elution order: 1) SMZ, 2) SDM and 3) SQX

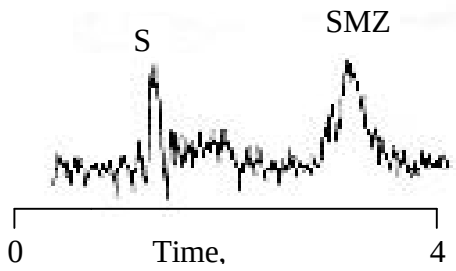


Figure 39: Supercritical fluid chromatogram of 125pg sulfamethazine on column. S = solvent, SMZ= sulfamethazine. Column Conditions: 15% methanol-modified CO_2, 0.46mL/min liquid CO_2, inlet pressure: 250 bar, 150 x 2 mm i.d Hypersil CPS-2.
Injection: 5µL in methanol

SFC-CLND system. The MDQ (0.125 ng) obtained with the 2 mm i.d. column is much better than has previously been reported for SFC-CLND. Shi et al. (120) obtained a MDQ of 0.3 ng N at the detector (4.2 ng on column) using 5% methanol modified CO_2 with packed column SFC-CLND and 0.06 ng N on column with open tubular SFC-CLND and pure CO_2 (119).Strode et al. (121) have reported a MDQ of 1 ng N, on column, using 8% methanol modified CO_2 with a decompressed CO_2 flow rate of 600 mL/min and no post column split. In this work, however, we were able to reduce the MDQ to 0.025 ng N, on column, using 15% methanol modified CO_2 packed column SFC (**Table 11**).

HPLC/Atmospheric Pressure Chemical Ionization-Mass Spectrometry

Minimum Detectable Quantity

Initially, the minimum detectable quantity for the 8 regulated sulfonamides was determined by HPLC/APCI-MS. Ion transmission of the LC interface was optimized by adjusting the sheath gas and drying gas flows (**Figure 25**), as well as, the position of the APCI probe via the positioning screws. This optimization procedure was performed each day prior to analyzing any samples. Ion abundance was monitored by initiating a blank run and viewing the total ion current (TIC). Maximum ion current signified the best sensitivity of the system. The MDQ was first determined for all eight sulfonamides (**Figure 26**) in the scan mode with a range of 110 m/z to 300 m/z (initial results showed no ions greater than 300m/z). MDQ's ranged from 100 ng to 10 ng viewed from the TIC. However, very little data manipulation is performed directly from the TIC, but instead an extracted ion chromatogram is used. The extracted ion chromatogram plots a single ion from the total scan range. In so doing, much less noise and better S/N ratios can be obtained. Mass spectra of each compound were obtained and the base peak for each compound was used for the generation of the extracted ion chromatograms. **Table 12** lists the MDQ for each of the eight compounds as well as the extracted ion used. As can be observed, extracted ion produced much lower MDQ's than total ion. **Figure 40** shows the extracted ion chromatograms at a level of 0.8 ng for each sulfonamide injected on column. SCP and STZ were not observed since they were below the MDQ. Atmospheric pressure chemical ionization is a relatively soft ionization technique producing only a few ions. The background subtracted mass spectrum of sulfamethazine at 10 ng on column is shown in **Figure 41**. The M+1 ion at 279 is clearly

Table 11: Comparison of Literature SFC/CLND minimum detectable quantity values

	Shi et al. (119)	Shi et al. (120)	Strode et al. (121)	This Study
Methanol modifier	0	5%	8%	15%
MDQ (compound)	500 pg	1.7 ng	1 ng	125 pg
MDQ (nitrogen)	60 pg	200 pg		25 pg

Table 12: Minimum Detectable Quantity (S/N ≥2) of 8 regulated sulfonamides by HPLC/APCI-MS in full scan mode from 110 m/z to 300 m/z and extracted ion mode. Sulfadiazine (SDZ), sulfachlorpyridazine (SCP), sulfathiazole (STZ), sulfamerazine (SMR), sulfapyridine (SPD), sulfamethazine (SMZ), sulfadimethoxine (SMZ) and sulfaquinoxaline (SQX).

Compound	T I C	E I C
SDZ	10 ng	0.8 ng (156)*
SCP	100 ng	10 ng (156)
STZ	20 ng	1 ng (156)
SMR	10 ng	0.8 ng (265)
SPD	10 ng	0.8 ng (250)
SMZ	10 ng	0.8 ng (279)
SDM	10 ng	0.8 ng (156)
SQX	10 ng	0.8 ng (156)

* number in parentheses is m/z used for extracted ion chromatograms

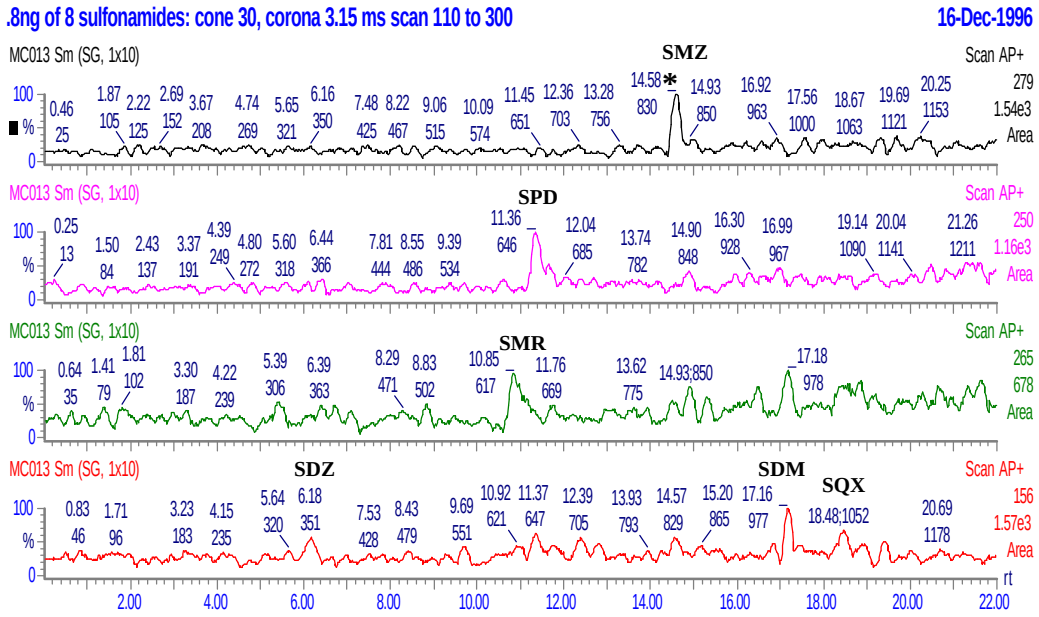
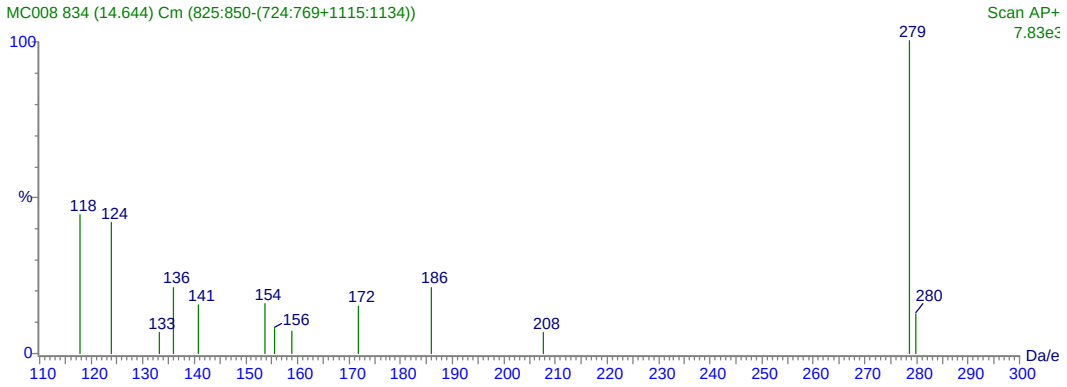


Figure 40: Extracted ion chromatogram of 8 sulfonamides using HPLC/APCI-MS in full scan mode from 110 to 300 m/z. Sulfadiazine (SDZ), sulfamerazine (SMR), sulfapyridine (SPD), sulfamethazine (SMZ), sulfadimethoxine (SMZ) and sulfaquinoxaline (SQX). Column Conditions: 0-8 min. 100% 85/15 8mM ammonium acetate/acetonitrile at 15.5 min. 100% 60/40 8mM ammonium acetate/acetonitrile, hold to 23 minutes; flow, 1.0mL/min; 250 x 4.6 mm i.d., 5µm Prodigy C18.
Injection: 20µL in methanol
MS Conditions: APCI probe 400°C; corona pin, 3.0kV; extraction cone, 30V; source, 120°C
* numbers above peak are retention time and scan number

Sulfamethazine



Sulfaquinoxaline

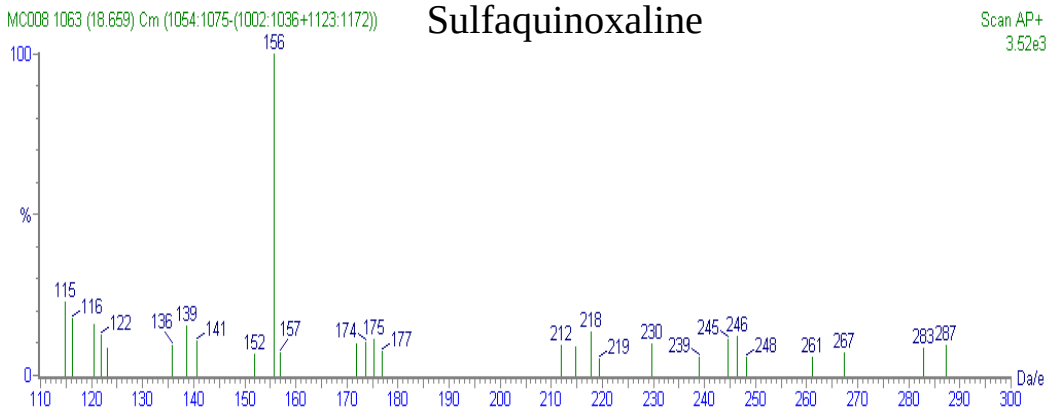


Figure 41: HPLC/APCI mass spectra of sulfamethazine and sulfaquinoxaline (10 ng of each). Full scan 110 to 300 m/z. See Fig. 40 for HPLC and MS conditions.

observed and is the most abundant peak. Other sulfonamides however did not yield such abundant molecular ions. The background subtracted spectrum of sulfaquinoxaline, also shown in **Figure 41**, has a base peak of 156 m/z and shows several other fragments up to 287 m/z. Extraction cone voltage has been shown to affect analyte fragmentation such that higher voltages cause more fragmentation. A cone voltage of 30V was used in this study since it is relatively mild and does not cause extensive fragmentation. However, it appears that some fragmentation is still occurring. The peak at m/z 156 is common for all sulfonamides. It results from the cleavage of the sulfur-nitrogen bond on the sulfonamide. This has been shown in both electron impact (EI) ionization (122) spectra and APCI-MS-MS (42) spectra of sulfonamides.

Next, the MDQ was determined using selected ion recording (SIR), where only one ion is monitored over a given period of time. SIR can further lower the MDQ since more time can be spent dwelling on an ion of the compound instead of scanning ions where the compound is not present. **Table 13** shows the results for each compound using the same chromatographic conditions and the ion used for selected ion chromatograms. SIR was able to produce a lower MDQ than extracted ion chromatograms for almost all compounds. In most cases SIR yielded 2-10 times better sensitivity than scan mode. **Figure 42** show the SIR chromatograms at 100 pg of each sulfonamide on column. The first three peaks cannot be observed since this level is below the MDQ, but the last 5 peaks can be easily seen. A previous report of HPLC/APCI-MS of sulfonamides (42) did not report the sensitivity of the system, but µg quantities of material were routinely analyzed. However, a 10% split in the column flow was necessary thus reducing the observed sensitivity. The SIR method appears to offer considerable flexibility and very sensitive detection of sulfonamides.

Analysis of Sulfonamides in Supercritical Fluid Extracts of Chicken Tissue

Since very good sensitivity was obtained for both selective detectors, SFE extracts of sulfonamides spiked onto chicken liver were assayed using each method. All extracts were obtained from the USDA/ARS in Philadelphia, PA courtesy of Robert Maxwell. Extracts were obtained at 10000 psi of pure CO_2. Following extraction the samples were collected and provided to us in 65/35 water/methanol. Published reports (53) have shown the SFE recovery of sulfamethazine and sulfadimethoxine to be greater than 90% and sulfaquinoxaline to be greater than 80% from chicken tissues using these conditions. Using a 5µL injection and non-split SFC conditions (e.g. entire decompressed CO_2 flow entering the CLND, ~300mL/min) with a 2 mm column, CLND results were much poorer for the SFE extracts than for previous MDQ studies on

Table 13: Minimum Detectable Quantity (S/N ≥2) of 8 regulated sulfonamides by HPLC/APCI-MS in selected ion recording mode. Sulfadiazine (SDZ), sulfachlorpyridazine (SCP), sulfathiazole (STZ), sulfamerazine (SMR), sulfapyridine (SPD), sulfamethazine (SMZ), sulfadimethoxine (SMZ) and sulfaquinoxaline (SQX).

Compound	Selected Ion (SIR)
SDZ	0.8 ng (156)*
SCP	6 ng (156)
STZ	0.8 ng (156)
SMR	50 pg (265,250)**
SPD	50 pg (265,250)
SMZ	50 pg (279)
SDM	100 pg (156)
SQX	100 pg (156)

* number in parentheses is m/z used for single ion monitoring.
** Both 265 and 250 m/z monitored simultaneously

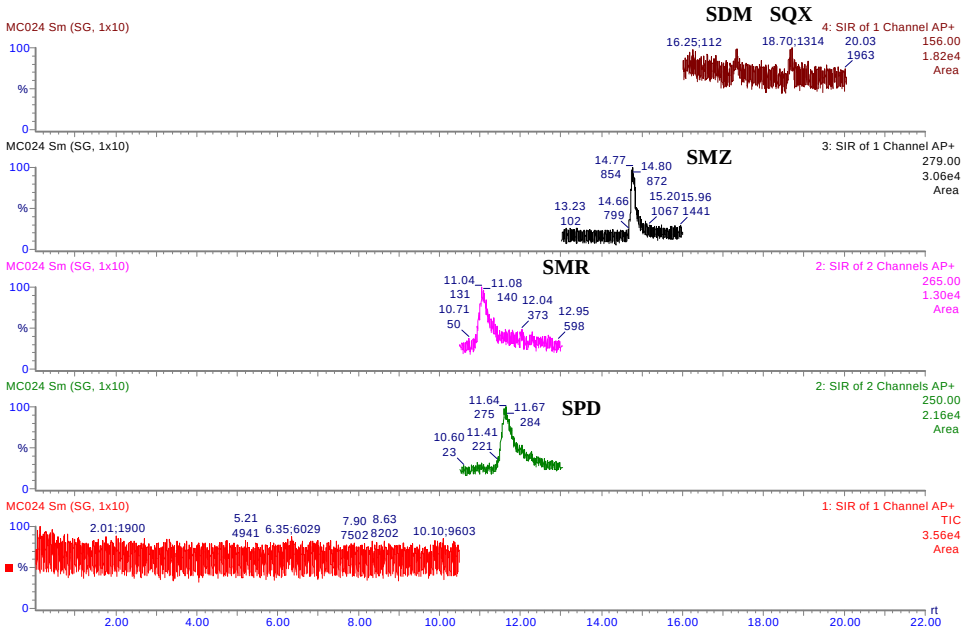


Figure 42: Selected ion recording of 5 sulfonamides at 100 pg each, on column, using HPLC/APCI-MS. Sulfamerazine (SMR), sulfapyridine (SPD), sulfamethazine (SMZ), sulfadimethoxine (SMZ) and sulfaquinoxaline (SQX). See Fig. 40 for HPLC and MS conditions.

pure compounds. Two factors may accound for the less than ideal behavior: (a) co-extracted fatty material may strongly interfere in the chemiluminescent process and (b) the large amount of water in the injection solution cannot be effectively eliminated by the membrane drier. An injection of a typical supercritical fluid (SF) extract prepared to yield a final concentration of 0.5 ng/µL of SMZ, SDM and SQX (assuming 100% recovery) is presented in **Figure 43**. The large amount of water injected possibly saturates the membrane drier causing insufficient water removal. This would result in a loss in sensitivity and may also increase band broadening effects on the small-bore column. For these chromatographic conditions (2.5 ng per drug injected) only SQX was separated from the coeluting pair of SMZ and SDM. The extracts dissolved in the methanol/water mixture showed a large water peak in the initial portion of the chromatogram which introduced much baseline instability where the sulfonamides eluted consequently decreasing the amount of sulfonamide capable of being detected with the system.

The same sample was injected onto the analytical scale (4.6 mm) column (**Figure 44**). In this case there was an approximate 1:5 split between the CLND and UV. Although less material reached the detector compared to the 2 mm column, the analytical scale column showed less band broadening and water effect, thus producing nearly the same net response for the sample. However, in both cases reduced system performance was realized for the SF extract compared to standards and was not sufficient for sample analysis.

Next, we wanted to try HPLC/APCI-MS since HPLC allows a larger sample loading than SFC due to differences in mobile phase polarity and should be less affected by the presence of water in the sample and in the HPLC mobile phase. It should be noted that HPLC conditions employed a 20µL injection, whereas, SFC only 5µL. **Figure 45** shows the HPLC/APCI-MS chromatogram (in full scan mode (110 to 300 m/z)), of the previous SF extract prepared to yield a final concentration of 0.5 ng/µL of SMZ, SDM and SQX assayed by SFC-CLND (10 ng per drug injected). Peaks for all three sulfonamides can be clearly seen, while the spectrum obtained for sulfamethazine (**Figure 46**) is quite similar to that of the standard alone (**Figure 41**). This sample shows no effect due to the presence of water and the MS exhibits sufficient sensitivity for routine sample analysis.

Another sample which had been spiked at 100 µg sulfadimethoxine /kg of tissue and extracted with SF CO_2 was assayed by HPLC/APCI-MS. Spiking at 100 µg/kg of sulfonamides in tissue is the current regulatory limit set forth by the Food Safety Inspection Service (20). **Figure 47** shows the HPLC/APCI-MS chromatogram obtained in selected ion (A) and full scan mode (B) for the low level tissue sample. The presence of sulfadimethoxine (approximately 1 ng per drug injected)can be seen at a S/N greater than 2 in both cases, but it is clearer using selected ion

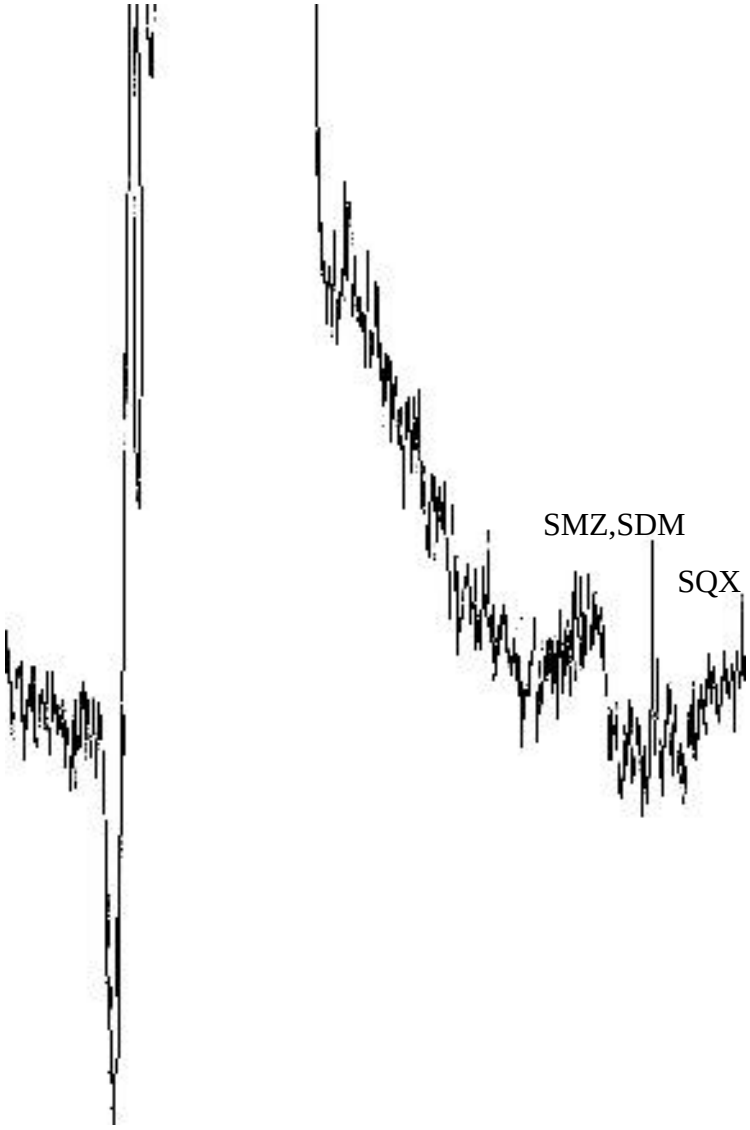


Figure 43: SFC-CLND chromatogram of a supercritical fluid extract of spiked sulfonamides (0.5 mg/kg per component) on chicken liver.
Chromatographic Conditions: 10% methanol-modified CO_2, 0.46mL/min liquid CO_2, inlet pressure: 225 bar, 250 x 2.0 mm i.d Betasil CN.
5µL injection

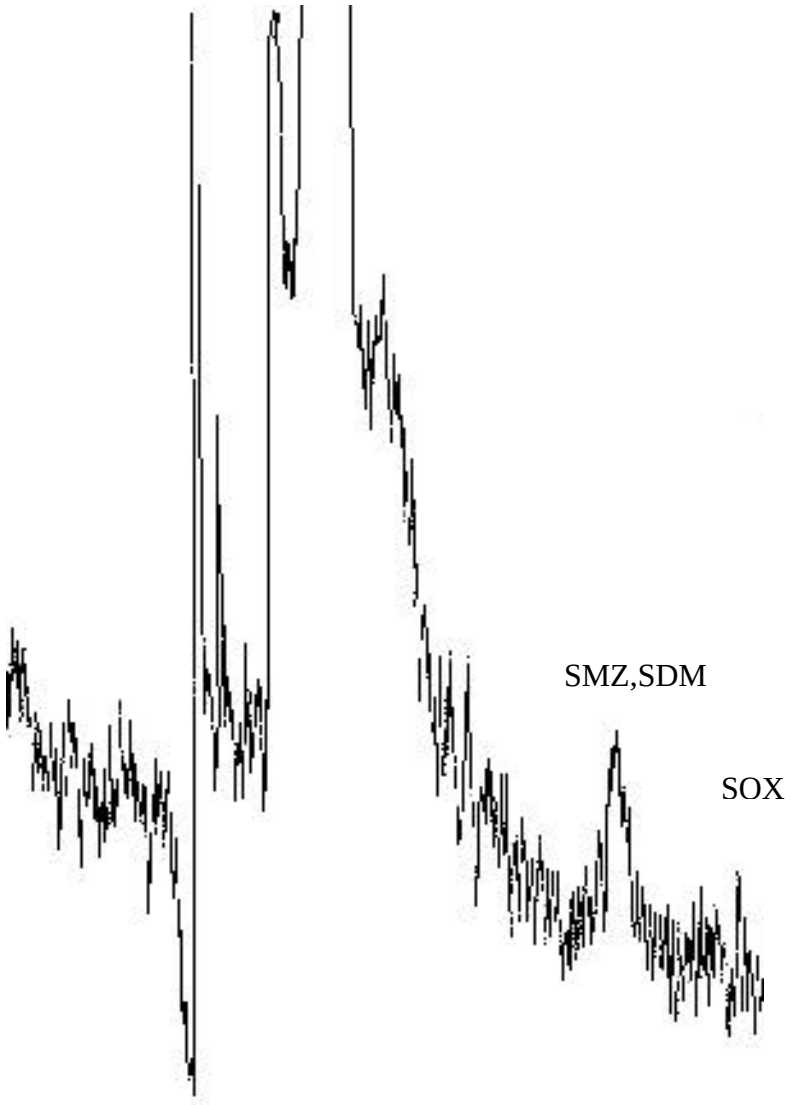


Figure 44: SFC-CLND chromatogram of a supercritical fluid extract of spiked sulfonamides (0.5 mg/kg per component) on chicken liver.
Chromatographic Conditions: 12% methanol-modified CO_2, 1.5 mL/min liquid CO_2, inlet pressure: 225 bar, 250 x 4.6 mm i.d Alltima CN. 5µL injection.

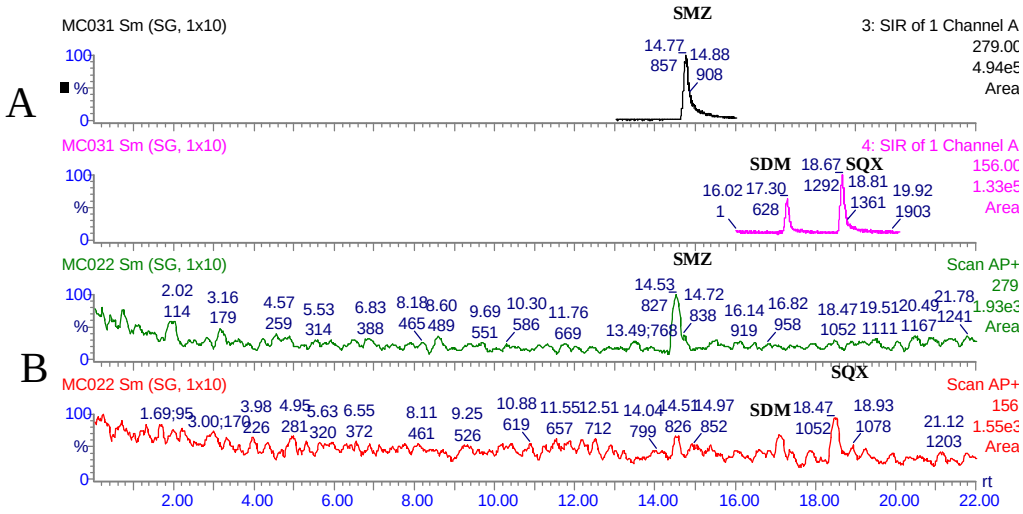


Figure 45: HPLC/APCI-MS of a supercritical fluid extract of spiked sulfamethazine (SMZ), sulfadimethoxine (SDM) and sulfaquinoxaline (SQX) (0.5 mg/kg) from chicken liver: (A) selected ion recording, (B) Scan mode. See Fig. 40 for HPLC and MS conditions.

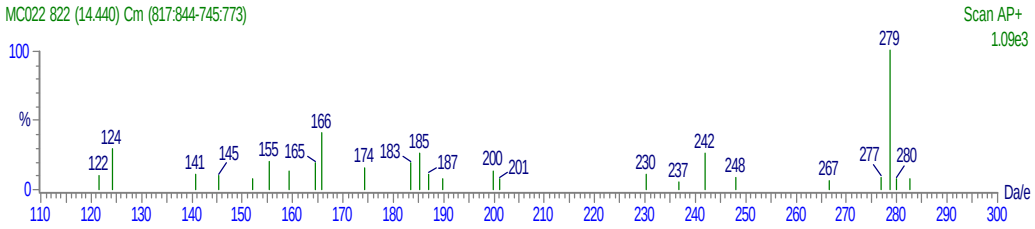


Figure 46: HPLC/APCI mass spectrum of sulfamethazine (F.W.=278) from the 0.5 mg/kg SFE chicken liver extract. See Fig. 40 for HPLC and MS conditions.

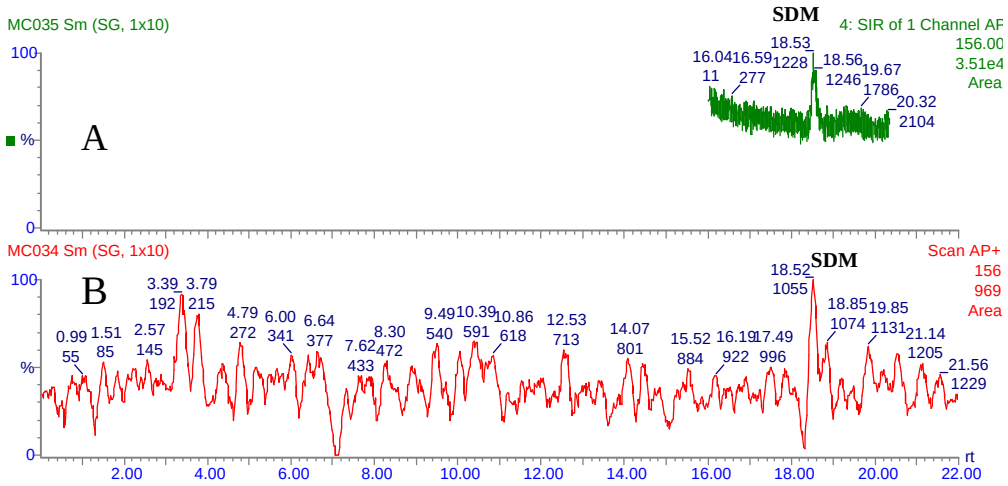


Figure 47: HPLC/APCI-MS of a supercritical fluid extract of spiked sulfonamides (100µg/kg) on chicken liver of sulfadimethoxine. (A) selected ion recording, (B) scan mode 110-300 m/z. See Fig. 40 for HPLC and MS conditions.

recording. Sulfadimethoxine was not the best compound for maximum sensitivity (i.e. sulfamerazine, sulfapyridine, and sulfamethazine) but demonstrates the usefulness of this technique for the analysis of low level sulfonamides in tissue samples.

SUMMARY

The objective of this chapter was to optimize and evaluate selective detection methods for the analysis of sulfonamides in chicken liver extracts. First, the sensitivity of packed column SFC-CLND system for sulfonamides with respect to ozone flow rate, decompressed CO_2 flow rate, and methanol modifier concentration was investigated. The detector optimization procedure found that at methanol-modifier concentrations of 15% or less, an ozone flow of 5.8mL/min, and a decompressed CO_2 flow between 240-310 mL/min exhibited maximum sensitivity of 5 ng (on column) of sulfamethazine (1ng N). The addition of a membrane drier decreased the MDQ to 0.5 ng on column (0.1 ng N). By using a microbore column (2mm i.d.) the post-column split was eliminated, and further reduced the MDQ to 0.125 ng on column of sulfamethazine (0.025 ng N).

The second method, HPLC/APCI-MS, was found to produce very low MDQ's for most of the sulfonamides investigated. In full scan mode, extracted ion chromatograms produced MDQ's of 0.8 ng on column, for six of eight compounds. Using selected ion recording of the base peak for each compound yielded a 50 pg MDQ for sulfamerazine, sulfadiazine and sulfamethazine with the other compounds presenting higher values.

Analysis of supercritical fluid tissue extracts of sulfonamides were found to be easily detected by HPLC/APCI-MS, but difficulties were encountered in the analysis of SFE tissue extracts by SFC-CLND due to the presence of water in the tissue extracts. It is believed this influence of this problem could be reduced by simply evaporating the extract to dryness and reconstituting the sample in methanol. Tissue spiked as low as 100µg/kg of sulfadimethoxine was found to be observed in both full scan and selected ion modes. HPLC/APCI-MS offers promising results for the identification and analysis of sulfonamides from biological matrices.

Chapter VI

CONCLUSIONS

The focus of this research was to develop an efficient extraction and detection method for sulfonamides, a class of frequently used antimicrobial drugs in veterinary medicine. In order to be successful, several aspects needed to be addressed.

The first goal of this work was to obtain a fundamental understanding of the parameters which effect the extraction kinetics of sulfonamides from an inert matrix. This goal was achieved by evaluating two different fluids, nonpolar carbon dioxide and polar trifluoromethane, at two different pressures and temperatures both with and without methanol modifier. Temperature effects were found to be quite complicated with increasing the fluid density not always producing increased recovery as in the case of pure CO_2 at 450 atm which increased recovery with increasing temperature. Pressure was found to improve the extraction of methanol modified CO_2 and was found to alter the kinetics of extraction at similar temperatures. Extraction behavior from sand also led to the belief that sand is not an inert matrix, but has an affinity for polar analytes. Trifluoromethane exhibited peculiar behavior with respect to extraction kinetics either with the pure fluid at 300 atm or with methanol modifier which was not able to yield quantitative recovery under any of the conditions investigated. This study demonstrated that interesting chemistry is occurring with SFE and that the highest solvating strength does not always correlate with highest recovery.

The second portion of this work applied the extraction of sulfonamides to biological matrices in which they may be found including 1) milk powder, 2) egg yolk, and 3) beef liver. CHF_3 and CO_2 were compared for both extractability and selectivity. Further increasing the pressure to near the instrument limit of 490 atm was found to further increase the extraction efficiency of the sulfonamides using pure CO_2, but was found to decrease recovery with methanol modified CHF_3. A pressure of 400 atm was required to obtain quantitative recovery of all three sulfonamides since at higher pressure there is more fluid-fluid interaction which decreases the ability of the fluid to interact with the analyte. Non-fat milk powder was found to be easily extracted with quantitative recovery obtained using either CO_2 or CHF_3 with methanol modifier. However, due to differential matrix interaction both egg yolk and beef liver yielded very poor recoveries. The matrix effect is believed to arise since different proteins are present in each matrix and sulfonamides are known to reversibly bind to proteins that more chemisorption is occurring

with the egg yolk and beef liver. Trifluoromethane in each case was found to greatly improve the extraction efficiency of SMZ and SDM relative to CO_2 since CHF_3 with a dipole moment of 1.6 debye is able to solvate the polar sulfonamides better than nonpolar CO_2. CHF_3 was also found to be more selective such that less fatty coextractives were obtained while improving recovery of the polar analytes of interest.

The third portion of this work focused on further improving the extraction of sulfonamides from difficult biological matrices by studying the effect of modifier identity and concentration. This was achieved using 1) methanol, 2) ethanol, 3) acetone and 4) acetonitrile as modifiers at 5, 10, and 20% by volume in CO_2. It was discovered that either 20% acetone or 20% acetonitrile was able to produce quantitative recovery of SMZ, SQX and SDM from chicken liver for the first time. This method also demonstrated its functionality at levels below 1 ppm. Matrix was found to effect the optimal modifier needed for extraction with acetonitrile being better for chicken liver while acetone was required for beef liver. For the first time quantitative recovery of each sulfonamide was obtained from beef liver with 20% acetone modifier. Acetone, however, caused significant interferences in the HPLC method. This was alleviated by evaporating the sample extract under a stream of nitrogen to remove the acetone and reconstituting the sample with the HPLC mobile phase. The egg yolk matrix was found to be quantitatively extracted with either 20% acetone or 20% acetonitrile. In addition to complete and efficient extraction from each matrix being obtained for the first time, the method required only minimal sample cleanup following the extraction. The entire sample cleanup step consisted of passing the sample extract through a 0.2µm Teflon filter.

The final portion of this work focused on evaluating and optimizing selective detection methods for the analysis of sulfonamide residues using either chemiluminescence nitrogen detector (CLND) interfaced to supercritical fluid chromatography or HPLC/atmospheric pressure chemical ionization-mass spectrometry.

Evaluation of CLND detector parameters was found to greatly effect overall system sensitivity. Ozone, a primary reactant for detection, was found to produce the best sensitivity when increased to a flow of 5.6 mL/min. In order to obtain optimal sensitivity the amount of column effluent entering the detector was found to decrease as the amount of methanol modifier in the system increased. The greatest sensitivity using 10% methanol modifier and 310 mL/min of decompressed CO_2 entering the CLND allowed a 5 ng injection of sulfamethazine (1 ng nitrogen) to be detected. The SFC method found to produce baseline resolution required 30% methanol and 30% methanol produced very poor sensitivity thus eliminating the use of this method for CLND detection. With novel detector design improvements this minimum detectable quantity

was reduced by an order of magnitude to 0.5 ng of sulfamethazine (0.1 ng nitrogen). The discovery of column effluent effect on sensitivity led to using a 2.1 mm column for the SFC system since the required flow parameters correlate directly with the optimal flow rate needed. In so doing it was discovered a further reduction in the minimum detectable quantity to 125 pg of sulfamethazine (25 pg nitrogen) could be obtained with the optimized system. This reduces the best previous detection limits of other nitrogen containing compounds in packed column SFC by over a factor of 10 and bettered open tubular SFC by over a factor of 2. HPLC/APCI-MS was found to produce low minimum detectable quantities for most of the sulfonamides investigated. In full scan mode, extracted ion chromatograms produced MDQ's of 0.8 ng on column, for six of eight sulfonamides. Using selected ion recording of the base peak for each compound yielded a 50 pg MDQ for sulfamerazine, sulfadiazine and sulfamethazine with the other compounds presenting higher values.

Analysis of supercritical fluid tissue extracts were found to be easily detected by HPLC/APCI-MS, but difficulties were encountered in the analysis of SFE tissue extracts by SFC-CLND. Tissue spiked as low as 100µg/kg of sulfadimethoxine was found to be observed in both full scan and selected ion modes. HPLC/APCI-MS offers promising results for the analysis of sulfonamide drug residues from biological matrices.

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Presentations:

- **Combs, M.T.**; Ashraf-Khorassani M.; Taylor, L.T. "Optimal SFE of Sulfonamides from Various Biological Matrices" Tri-State Supercritical Fluid Discussion Group, November 1996.
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