SUPERCRITICAL FLUID EXTRACTION AS A SAMPLE PREPARATION TECHNIQUE FOR THE ISOLATION OF POLAR PHARMACEUTICAL COMPOUNDS

by

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(ABSTRACT)

The low polarity of the supercritical fluid extraction (SFE) solvent, CO_2, causes problems when applying this technique to polar compounds. The objective of this research was to expand the applicability of SFE to include polar compounds.

The first phase of this research investigated "inverse SFE", which is the selective extraction of the matrix while leaving behind the target analytes, as a means of isolating four polar polymyxin sulfate compounds from several challenging matrices due to the liquid-like consistency and the low concentrations (ppm) of the target analytes. Higher recoveries (>100%) were achieved with low RSD's (5.0 and 1.9% for two separate matrices), compared to the conventional method of isolation, solid phase extraction.

The second portion of this research investigated the effect of high levels of polar modifier on trapping efficiency. The mode of modifier introduction was found to have a considerable effect on trapping efficiency, as in-line modifier addition rather than matrix

spiking was determined to be best when introducing high levels of liquid modifier. Also, three separate mechanisms, aerosol formation, blow-by, and modifier elution, were identified which caused analyte loss at the solid phase trap, and the extraction and trapping conditions for which each mechanism was most favorable were identified.

The next phase of this research developed two separate SFE methods which were used to isolate two pharmaceuticals from leaves. Each SFE method reduced the time, sample handling, costs, and liquid solvents needed as compared to the conventional liquid extraction technique. While achieving comparable recoveries, reproducibilities were much improved for the two SFE methods with RSD's of 3.8 and 5.2%, respectively, while the liquid extraction technique yielded RSD's of 14%.

The last phase of this research investigated several strategies for improving selectivity in SFE. Three separate strategies for improving selectivity were studied: 1) alternative fluids (pure and modified fluoroform and tetrafluoroethane), 2) pre-extraction, and 3) selective rinsing of the solid phase trap. Advantages were realized with each technique, and it was concluded that they be used in conjunction with one another in order to maximize selectivity.

This dissertation is dedicated to my wife, Angela Taylor Moore, and my parents,

Mr. and Mrs. William E. Moore, Jr.

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Burroughs-Wellcome, Co. for their financial support and pharmaceutical samples; Air Products and Chemicals, Inc. for supplying the supercritical fluids; Suprex Corporation and Hewlett Packard Company for the loan of their supercritical fluid extractors; and the physics machine shop staff for their help in building accessories for the experiments.

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List of Abbreviations

Words	Abbreviations
Supercritical Fluid	SF
Supercritical Fluid Extraction	SFE
High Performance Liquid Chromatography	HPLC
Ultraviolet	UV
Environmental Protection Agency	EPA
Carbon Dioxide	CO_2
Fluoroform	CHF_3
Tetrafluoroethane	CH_2FCF_3
Solid Phase Extraction	SPE
Relative Standard Deviation	RSD
3.22Digitalis lanata	*D. lanata*

Chapter I

INTRODUCTION

Supercritical fluids (SFs) have been known to exist since the early 1800's when Cagnaird de la Tour observed the disappearance of the meniscus of the two distinct phases, liquid and gas, as the temperature and pressure of the system was increased above the critical parameters (1). However, only recently has this "superstate" received increased attention both in the scientific and engineering communities. Recent concerns about the toxic properties of many conventional organic solvents have fueled a significant growth in the field of supercritical fluid technology, both in the area of sample preparation/extraction and separation science.

A supercritical fluid can be defined simply as any substance which is taken above its critical pressure and temperature. The physical properties of these dense gases deviate substantially from those of normal liquids or gases and offer substantial advantages when used for the extraction of analytes from complex matrices.

SFs typically possess physical properties which are intermediate between those of a conventional liquid and a gas. For example, SFs possess densities which can be varied from 0.1 g/mL to approximately 1 g/mL, as a function of temperature and pressure. This unique ability to vary density stems from the high degree of compressibility of gases. Consequently, this phenomenon allows one to not only emulate a variety of organic

solvents by varying the solvating strength of the fluid, but specifically tailor the fluid's density so as to achieve maximum selectivity for a particular application. SFs also possess enhanced mass transport properties when compared with liquids, as their coefficient of diffusion and viscosity are close to that of a gas, around 10^{-3} cm^2/sec and 10^{-4}g/cm sec, respectively. These gas-like properties ensure fast mass transfer when dealing with complex matrices. Moreover, SFs exhibit near zero surface tension, which further facilitates their rapid penetration into and out of porous matrices when compared with methylene chloride or other organic solvents typically employed for sample preparation techniques such as Soxhlet or liquid extractions.

SFs possess many other desirable properties which are conducive to sample preparation. The most common SF, CO_2, is not only inert, non-toxic, non-flammable, and currently unregulated by the Environmental Protection Agency (EPA), but it is also a gas at ambient conditions which eliminates the need for a solvent concentration step following the extraction and prior to analysis. SF CO_2 also has easily accessible critical parameters, 31.3°C and 72.9 atm, and such a low critical temperature allows extractions which provide assurance against chemical reactions taking place.

Perhaps the greatest disadvantage of SF CO_2 is its low polarity which makes it incompatible with high polarity analytes. As a result, the technique has been limited to nonpolar and moderately polar compounds. While a variety of polar modifiers, additives, and coanalytes can be added to increase the polarity of the SF and hence, solubility of the

analyte, problems with analyte solubility still exist which limit the scope of SFE for polar compounds.

This problem is especially true in the pharmaceutical industry in which many metabolites and water soluble compounds, which contain numerous polar functional groups, render themselves insoluble in pure SF CO_2. Consequently, few applications of SFE to pharmaceutical compounds can be found in the literature. In addition to analyte/fluid incompatibility of polar compounds, other obstacles also exist which prevent successful SFE of polar compounds and contribute to the slow advancement of SFE in the pharmaceutical field. Some of these obstacles which will be discussed in detail shortly, include increased analyte/matrix interaction, decreased trapping efficiency, and decreased selectivity.

Even if the analyte of interest is soluble in the modified SF, analyte extractability is still not ensured. For example, the location of the analyte within the matrix is critical. Whether the analyte is found on the matrix surface or within the matrix network can contribute to the success of the extraction. The type and degree of analyte interaction with the active sites of the matrix is also crucial and must be disrupted. This is especially true when extracting polar analytes from complex natural product matrices such as leaves, as strong analyte/matrix interactions are prevalent which often impede a successful quantitative extraction.

Analyte insolubility in the SF and the strong interaction of the analyte with the matrix can often times be overcome through the addition of a polar modifier or additive as

stated previously. However, as the modifier concentration in SF is increased, problems trapping the decompressed analytes are encountered due to the propensity of the gas/liquid mixture to form an aerosol. Therefore, inefficient trapping remains a contributing factor to the poor success which SFE has for polar pharmaceutical compounds.

Yet another problem related to the large amounts of modifier which are often employed to achieve dissolution of the target analytes is a loss in the selectivity of the extraction fluid. As the solubility of the target analyte increases, the extraction experiences a corresponding decrease in selectivity. As the solvating power of the fluid is increased, so is the solubility of the coextractives which not only can interfere with the quantitation of the target compounds, but also will result in a "dirty" extract which must undergo some additional type of sample cleanup such as solid phase extraction (SPE) which utilizes additional liquid solvents and time.

The focus of this research is therefore to expand the applications of SFE in the pharmaceutical field by attempting to reduce, and if possible eliminate, the contributing factors responsible for the slow progress of this new and powerful extraction technique. The goal is to overcome the aforementioned limitations encountered in the extraction of polar compounds and help facilitate the progress of this technique so that the pharmaceutical industry can take full advantage of the assets which SFs have to offer.

Chapter II involves the SFE method development of a highly polar amino acid complex, present at low concentrations (0.16% and 0.08%), from both a liquid-like cream

and solid-like ointment matrix via a novel extraction method which is referred to as inverse-SFE (2,3,4). This technique is based on the idea that the matrix is extracted, while the analytes of interest are isolated within the extraction vessel. This work not only focuses on new matrices, but also on overcoming some of the current limitations of the technique proposed by Messer et al. (2). The inverse SFE methods are then compared with the currently employed solid phase extraction (SPE) technique.

Chapter III is a fundamental study designed to identify the different parameters which contribute to the loss of polar analytes from the solid phase trap during the collection stage of SFE when high levels of modifier are employed. The objective of this research is not only to understand what factors are responsible for sample loss from the trap following decompression of a modified SF, but also to determine if the causes can be eliminated thereby ensuring efficient trapping of extracted analytes even when high modifier levels are employed. Three polar cardiac glycosides were employed as the probe analytes for this study, and the ability to trap these components was investigated as a function of the four primary SFE parameters thought to be crucial in the trapping process: 1) modifier amount, 2) mode of modifier introduction, 3) extraction flow rate, and 4) trap temperature.

Chapter IV describes novel SFE procedures used for the isolation of two polar cardiac glycosides, digoxin and acetyldigoxin, both from an inert matrix as well as from their natural product leaf matrix. The potential of SFE as a sample preparation technique for these highly polar and water soluble compounds is investigated and the results are

compared with a current liquid extraction method which employs hazardous chlorinated solvents.

As stated previously, increasing the solvating strength of the SF decreases the selectivity. Chapter V investigates several cleanup strategies which can be employed in order to regain the selectivity advantage of SFE. Several of the techniques, which are inherent to the SFE process, show definite promise for cleaner extracts. For example, some of the cleanup techniques explored involve fluids other than CO_2 which may perhaps exhibit a greater degree of selectivity, selective pre-extraction of interfering compounds prior to extraction of the analyte of interest, and selective rinsing of the solid phase trap following the extraction step.

Chapter II

Analytical Inverse Supercritical Fluid Extraction of Polar Pharmaceutical Compounds from Cream and Ointment Matrices

INTRODUCTION

There are relatively few applications of SFE for the extraction of polar drugs from pharmaceutical matrices. Published applications include the extraction of antihistamines from transdermal patches, polar vitamins and drugs from animal feeds, and metabolites from plasma (5-10). Other applications deal with the extraction of polar drugs from natural product matrices, and these will be discussed in Chapter 4.

Even with the addition of a polar modifier, there still are a large number of compounds which remain insoluble and are thereby not extractable when CO_2 is employed as the primary fluid.

A recent study involved the use of SFE to isolate a polar drug, acyclovir, by extracting the drug carrier matrix which was a hydrocarbon-based ointment and leaving behind the analyte of interest. This process has been referred to as "inverse SFE" (2-4). To the author's knowledge, this is the only application of its type in the literature. Although still in its early stages of development, the technique has shown promise in the pharmaceutical industry. Messer and Taylor have identified five parameters which appear to play important roles in the outcome of "inverse SFE" (2).

First, the analyte must be totally insoluble in the supercritical fluid. Second, the matrix must be soluble in the SF. Third, a highly efficient washing method must be used to transfer the analyte from the extraction vessel for analysis. Fourth, the analyte concentration in the matrix should be >2%. Fifth, an assay method with low detection limits for the analyte is advantageous. Messer reported quantitative recoveries with good reproducibility for the inverse extraction of acyclovir, the water soluble, active ingredient found in Zovirax® Ointment 5%.

This chapter assesses the feasibility of achieving two primary objectives. One objective was to try and overcome the concentration limitation as defined by Messer. The second objective was to assess the feasibility of using inverse SFE as a way of isolating a polar drug from a cream matrix, which has near liquid-like properties, compared to the hydrocarbon based ointment which has physical properties closer to that of a solid. It was believed that this liquid-like consistency would pose problems not encountered in the extraction of analytes from the ointment carrier.

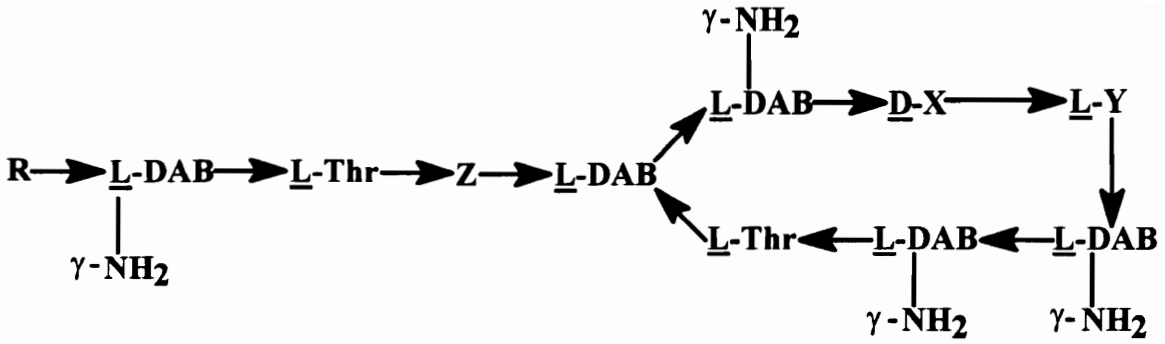
While it is important for quantitative reasons to work with concentrated drug formulations, concentrations of this magnitude are typically not encountered in the pharmaceutical industry. The work reported here focuses on the isolation of an active ingredient polymyxin B sulfate from its cream carrier (Neosporin®Cream) in which the drug is present at 0.17%, and also from its ointment carrier (Neosporin® Ointment) in which the drug is present at 0.08%. While Polymyxin B sulfate represents a class of about eight compounds with the general structure shown in **Figure 1,** Burroughs-

Wellcome, Co. is interested in only four of these compounds which constitute the majority of the antibiotic complexes present in the drug formulations. Each polymyxin B sulfate compound has a molecular weight of approximately 1200 amu and consists of many polar amino acid functionalities which make the compounds insoluble in both pure and modified supercritical carbon dioxide. In addition to the problem of its liquid-like consistency, the cream matrix is also more complex than that of the ointment carrier extracted by Messer et al. (2). This cream matrix consists of methyl paraben, emulsifying wax, mineral oil, polyoxyethylene polyoxypropylene compound, propylene glycol, purified water and white petrolatum, while the ointment matrix consisted simply of a white petrolatum base.

EXPERIMENTAL

Supercritical Fluid Extraction and Quantitation

All extractions were performed on the Suprex Prepmaster (Suprex, Pittsburgh, PA) along with a reciprocating modifier pump (SSI, State College, PA). The extractor employs a dual head reciprocating pump which delivers the CO_2 as a liquid and is capable of achieving 500 atm (7,348 psi). Extraction vessels (Keystone



R	=	(+)-6-methyloctanoyl or 6-methylheptanoyl
X	=	leucine or phenylalanine
Y	=	threonine or leucine
Z	=	D-serine or L-DAB
DAB	=	α,γ-diaminobutyric acid
Thr	=	threonine

Figure 1. General structure of polymyxin B sulfate, an antibiotic polypeptide complex (top). Structures vary only by the R, X, Y, or Z constituent.

Scientific Inc., Bellefonte, PA) with a 3 mL volume were employed for all extractions. An empty polyethylene solid phase extraction (SPE) tube which was placed within the vessel served as an insert and was used to contain the sample to make easier sonication of the non-CO_2 extractables following inverse SFE. The vessel configuration employed is shown in **Figure 2**. The supercritical fluid Prepmaster was modified slightly by disconnecting the solid phase trap from the flow path of the SF so that the extracted material and compressed fluid were decompressed at the restrictor into the trunk of a hood. The modified restrictor consisted of a plugged zero dead volume union which allowed the SF to be decompressed at a controlled rate. This setup was found to minimize plugging of the restrictor by the two types of matrices. Moreover, the restrictor employed was externally heated to 300°C via two high temperature heating cartridges which further discouraged any plugging of the restrictor. SFE/SFC grade helium headspace CO_2 (Air Products and Chemicals Inc., Allentown, PA) was used for all extractions. HPLC grade methanol (EM Science, Gibbstown, NJ) was employed as the modifier.

Polymyxin B sulfate extracts were assayed by an HPLC method supplied by Burroughs-Wellcome Company (Research Triangle Park, NC). Analysis of the extracts was performed with isocratic elution on a 4.6 X 250 mm i.d. Synchropak SCD reversed-phase proprietary column (5 μm particle size and 100 angstrom pore size) received from Burroughs-Wellcome Company. The mobile phase consisted of

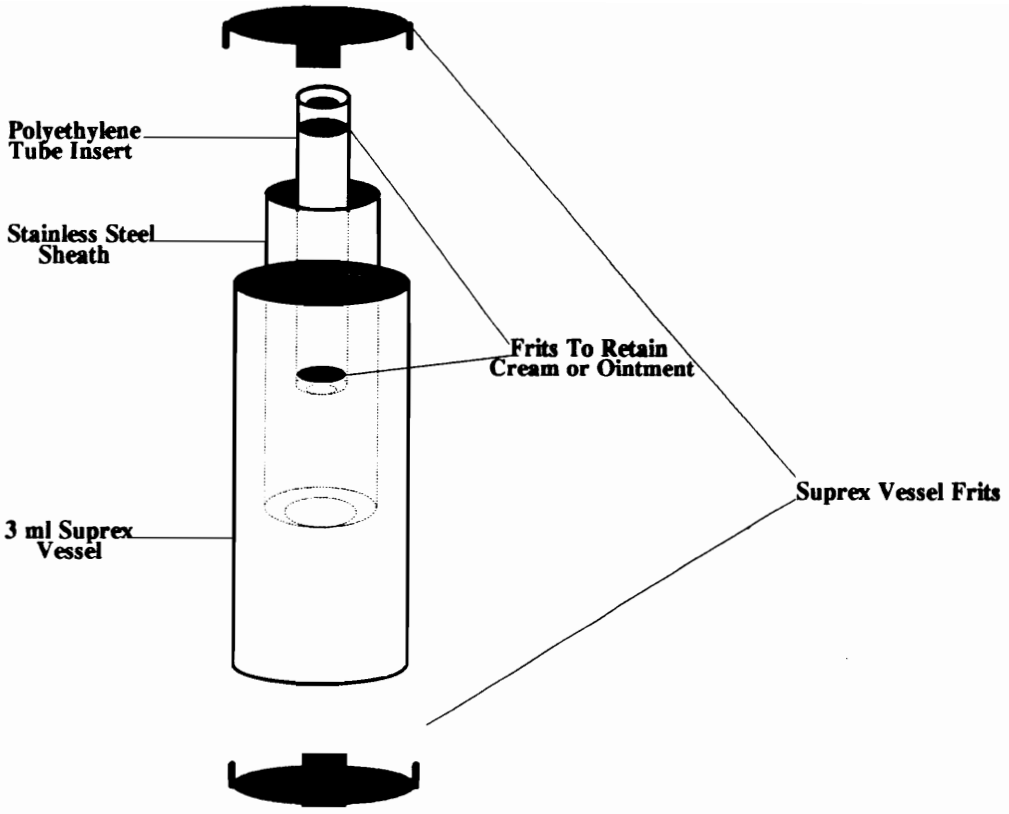


Figure 2. Initial vessel design employed which placed a polyethylene solid phase extraction tube within the supercritical fluid extraction vessel. Two frits at either end of the SPE tube were used to contain the sample to be extracted.

0.1 M potassium phosphate monobasic with 0.1% trifuoroacetic acid:acetonitrile (78.5:21.5, v/v). The mobile phase was filtered through a 0.45 μm filter (Millipore, Bedford, MA) prior to use. The flow rate was 1.5 mL min^{-1}. A Hewlett-Packard (Avondale, PA) 1050 series isocratic pump was used and connected to a Valco (Austin, TX) model EQ-60 LC injector using a 50 μL loop. A Hewlett-Packard (Avondale, PA) 1050 series ultraviolet detector was employed with detection set at 215 nm. A Hewlett-Packard model 3394A integrator was used. All Neosporin® samples and standards were provided by Burroughs-Wellcome Company (Research Triangle Park, NC). HPLC quantitation was by an external calibration curve of the cumulative areas of the four peaks of interest relative to solid phase extraction results achieved in house. A typical chromatogram can be seen in **Figure 3**. The same tube of cream or ointment was used in comparative extractions, both SPE and SFE, as it was stated by personnel at Burroughs-Wellcome Company that the tubes could vary in concentration from lot to lot. All samples were filtered prior to HPLC analysis through 0.2 μm filter unit.

Solid Phase Extraction

The current sample preparation method for polymyxin B sulfate in Neosporin® Cream and Ointment is solid phase extraction on a silica gel SPE column which uses 1 gram of sample and roughly 100mL of total solvent, hexane and ethylacetate, per

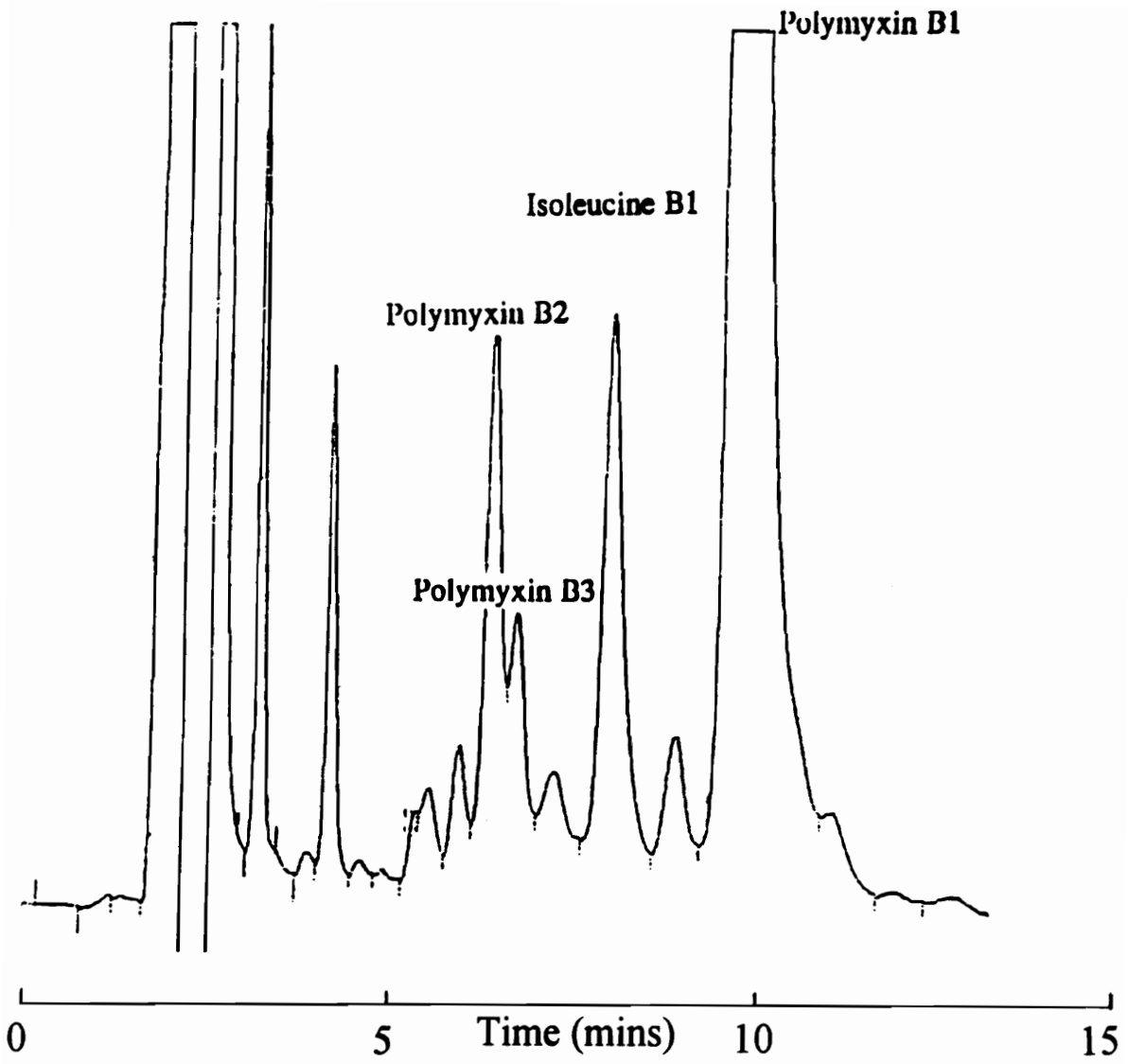


Figure 3. Typical HPLC chromatogram of the polymyxin compounds extracted from either the cream or ointment formulation. The four peaks of interest have been labeled for clarity.

extraction to load the cartridge. In order to elute the polymyxin compounds from the silica stationary phase, 2 mL of 0.1N HCl are added in a dropwise fashion followed by 2 mL of a 50/50 mixture of 0.1N HCl/methanol. The extract is collected in a 5 mL volumetric flask and diluted to the mark. Typical extraction times are approximately 2 hours for the solid phase extraction procedure. Following SPE, the compounds were then separated on the proprietary column given above and quantitation performed via the external calibration curve of the four cumulative peak areas.

RESULTS AND DISCUSSION

SFE of Cream Samples

This project began with an attempt to inversely extract and quantitate the amount of polymyxin B sulfate present in Neosporin®Cream. The average concentration reported by Burroughs-Wellcome Company for the cream was 1.66mg of polymyxin B sulfate in 1 gram of cream (0.17%). Sample sizes of approximately 200mgs were weighed into an empty SPE tube, frits were placed in either end to retain the sample of interest, and placed within the stainless steel extraction vessel. Following supercritical fluid extraction (SFE), the SPE tube was removed from the vessel, and the SPE frits within the extraction tube were removed and placed with the SPE tube into a 3.7 mL vial and sonicated with 1 mL of the solvent

which dissolved the polymyxin B sulfate compounds most successfully, 75:25: of 0.1N HCl:methanol with 0.1% Tween 80 (polyoxyethylene (20) sorbitan monooleate) (J.T. Baker, Phillipsburg, NJ). Sonication times were varied from 15 to 45 minutes, and it was found that 15 minutes was sufficient for an exhaustive washing of the SPE tube. All extractions were attempted under a variety of 100% CO_2 pressures and temperatures, however the results seemed to follow the same trend. In each case, the percent recoveries of polymyxin B1, B2, and B3 were well below those found with solid phase extraction assay (≈10%), while the percent recovery of the third peak of interest, isoleucine B1, was found to be well above that found with SPE (≈300%) (**Figure 4**). These initial results were puzzling.

After placing a sample of the cream within a GC oven and slowly ramping the temperature, it was discovered that the cream, which consisted of numerous components, became a free flowing liquid between 65 and 70°C. While all extractions were performed below 65°C (45-60°C), it seemed reasonable that the polymyxin B sulfate compounds which were known to be completely insoluble in supercritical-CO_2 could be undergoing physical entrainment or mechanical transfer through or around the SPE frits (50 μm pore size) during extraction of the matrix. This theory would explain the loss of the three polymyxin compounds, but the question as to how 300% recovery of a compound very similar in nature to the other three compounds could be achieved remained a mystery.

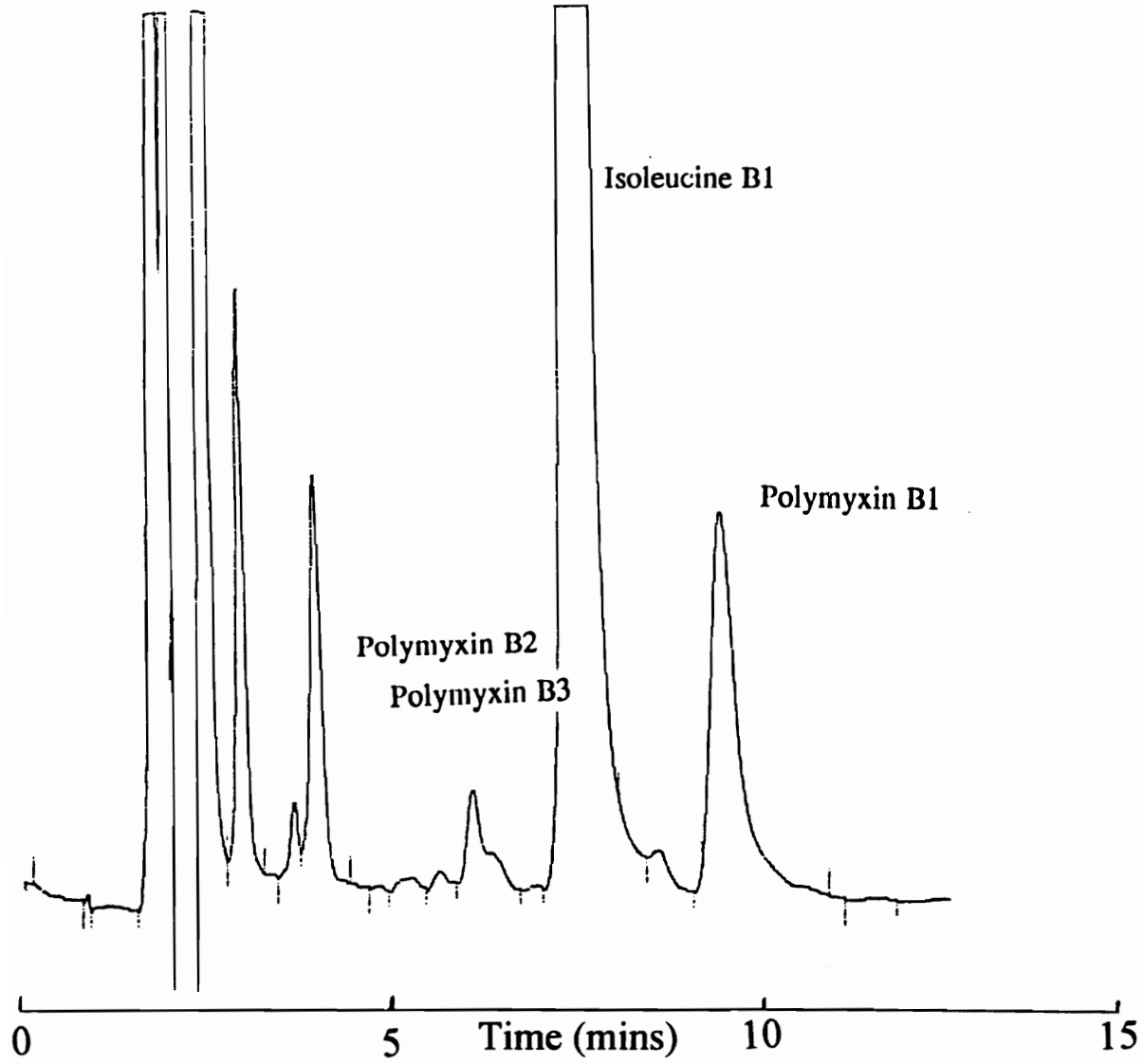


Figure 4. HPLC chromatogram showing the decrease in peaks 1,2 and 4, and the large increase in peak 3, relative to the HPLC chromatogram of the conventional solid phase extraction procedure shown in Figure 3.

Further investigation led to the discovery that methyl paraben seen in **Figure 5**, a preservative found in the cream, coeluted with the isoleucine B1 in the HPLC assay. This fact was proven as some of the methyl paraben standard purchased from Sigma, was injected under the employed HPLC conditions and found to elute at the same retention times as isoleucine B1. The 100% CO_2 was then discovered to be unsuccessful at exhaustively extracting the methyl paraben within the employed extraction time as the standard was extracted under the SFE conditions, collected in a liquid trap, and assayed. While some the methyl paraben was in fact extracted, a significant amount was still found within the extraction vessel as is confirmed by an assay of the contents of the extraction vessel following SFE. However, an extraction profile (**Figure 6**) produced from data obtained at 300 atm and 60°C showed that methyl paraben could be removed from the extraction vessel in approximately 30 minutes with 5% methanol modified CO_2 at a flow of 2 mL min^{-1}. Further extractions of the Neosporin® cream at these conditions proved to remove all of the methyl paraben from the cream, as well as the other cream components. However, recoveries of the four polymyxin B sulfate components continued to range from 5 to 15% when compared with the corresponding SPE results.

At this point there were several possible reasons which could explain the loss in recovery of the polymyxins. First, the analytes could still be undergoing some type of physical transfer out of the vessel. Second, the analytes could be reacting with the

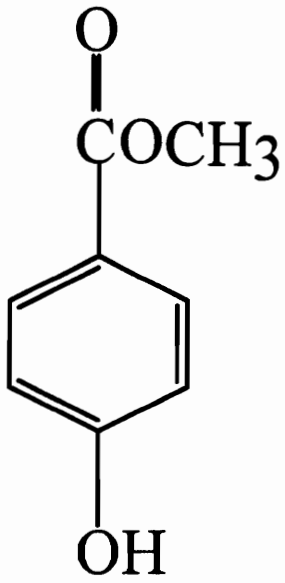


Figure 5. Molecular structure of methyl paraben, a preservative found within the cream matrix.

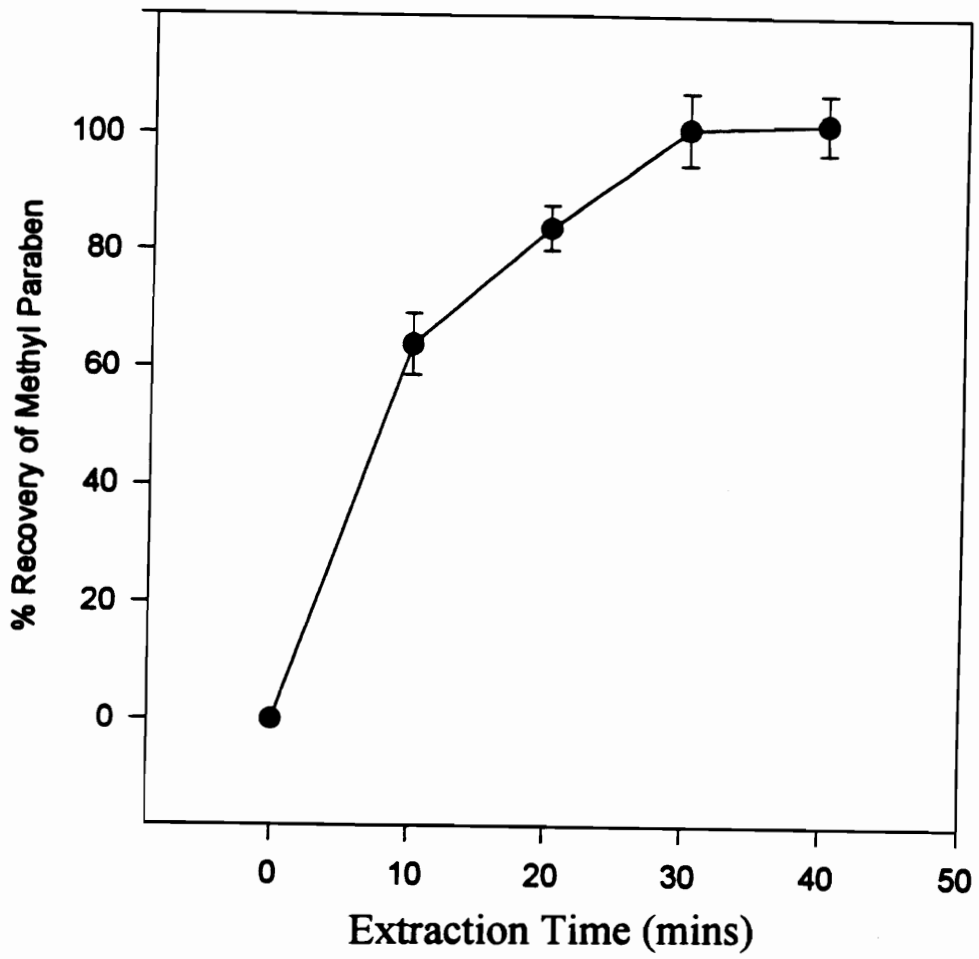


Figure 6. An extraction profile of methyl paraben at 300 atmospheres and 60°C with 5% methanol modifier. The extracted methyl paraben was collected in a liquid trap of 5 mLs of methanol.

SF. This premise was considered highly unlikely due to the relative inertness of the CO_2. Third, the analytes could be degraded as a result of the heat the compounds are subjected to during the sonication process. It seemed reasonable to assume that possibilities two and three were not responsible for the low recoveries, but in order to confirm reason one, experiments were carried out to eliminate the remaining possibilities. First, in order to eliminate the second possibility, known amounts of the polymyxin B sulfate standard were assayed with HPLC before and after interaction with the SF. This experiment ruled out the possibility that any of the compound was reacting with the SF as the chromatograms were identical. In order to rule out the third possibility, a solution of polymyxin B sulfate standard in the solvent was assayed by HPLC before and after sonication. Again, both chromatograms were identical. It was subsequently discovered that the analyte was undergoing transfer out of the vessel resulting in low recoveries.

Several extractions were carried out at relatively low temperatures (40-45°C) in order to prevent any melting of the cream. However, recoveries were still unsatisfactory and RSDs approached 100%. Various lower pressures, lower temperatures, and lower flow rates were tested in hopes of preventing mechanical transfer, but to no avail.

It seemed clear at this point that some other vessel design was needed in order to keep the analyte of interest within the vessel while allowing the exhaustive

extraction of the matrix components. A new design was tested in which the cream sample was sandwiched between two layers of 200 mesh silica gel (approximately 200mg on either end). (**Figure 7**) The goal of this design was to promote the selective trapping of the analyte of interest prior to supercritical fluid decompression, while allowing the cream to be completely removed from the system. This configuration was tested and found to result in an extremely efficient extraction. For the cream, at a pressure of 300 atm with an extraction temperature of 55°C with 5% methanol, and a flow rate of 2 mL min^{-1}, average recoveries of the analytes of interest were 108% (n = 6) when compared to SPE, and RSDs of 5.0% were generated. The sample size of Neosporin® Cream was approximately 200 mg, and 345μg of polymyxin B sulfate were recovered with the SFE method. The extraction time required for exhaustive extraction of each of the matrix components was 75 minutes with the 5% methanol-modified CO_2. The restrictor was heated to approximately 300°C, and roughly 140 grams of CO_2 was used (≈150mL), and roughly 7 mL of methanol. Following the extraction, the frits, silica gel, and SPE tube were placed into the 3.7 mL vial and sonicated for 15 minutes in 1 mL of the diluent. The sample was filtered and assayed by HPLC.

SFE of Ointment Samples

It seemed feasible that if 345μg of polymyxin B sulfate could be

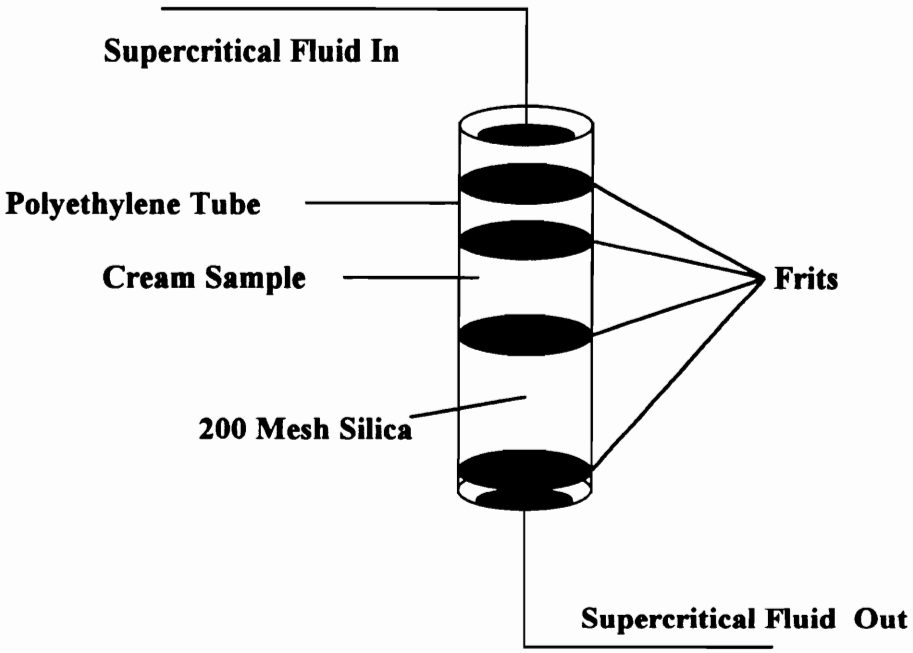


Figure 7. Diagram of the SPE tube insert modified with additional frits, along with silica to eliminate mechanical transfer.

quantitated, then approximately half as much (160μg), by SPE, found within the ointment could also possibly be quantitated. Other than the low concentration obstacle, this extraction should have been relatively simple as the only component of the ointment matrix was a white petrolatum base. This scenario was in fact the case. The extractions were carried out at a pressure of 450 atm and an extraction temperature of 60°C with a flow rate was 2 mL min^{-1}. It was discovered in the extraction of the cream sample, that not only did the methanol aid in the extraction of the methyl paraben preservative, but it also helped prevent any clogging of the restrictor as it helped solvate the plug upon decompression. Therefore, the extraction fluid of 5% methanol-modified CO_2 was again employed. The time required for an exhaustive extraction of the ointment matrix was 45 minutes. The restrictor was again kept at 300°C to assist in the prevention of plugging. Roughly 83 grams of SF-CO_2 (90 mL) were used along with 5 mL of liquid methanol. These conditions resulted in an average recovery of 137% (n = 6) for the ointment relative to the SPE method with great RSDs of 1.9%.

These high recoveries could be attributed to a coelution of another ointment ingredient causing inaccurate quantitation. However, while this is feasible, it seems unlikely. Perhaps this observation is best explained by an inaccurately low determination of the antibiotic via solid phase extraction, which would results in a high recoveries of the SFE method relative to the conventional method.

Summary

This study confirms that not only can inverse SFE be successfully employed for cream matrices, as well as ointment matrices, but this technique can also be a viable sample preparation technique for drugs present in carriers at concentrations lower than those proposed by Messer et al. (2). In addition, it was discovered that some type of stationary phase is needed in the extraction of the cream matrix in order to prevent any physical loss out of the vessel. Inverse SFE is an alternative which is not only mostly automated and results in a ten-fold decrease in the amount of liquid solvents employed, but also yield comparable recoveries.

Chapter III

Mechanisms Responsible for Sample Loss at a Solid Phase Trap in Supercritical Fluid Extractions Using Carbon Dioxide Modified with High Levels of Methanol

INTRODUCTION

The success of Supercritical Fluid Extraction (SFE) with liquid trapping is limited by the ability to (1) remove the analytes of interest from the matrix, (2) quantitatively trap the extracted components, and (3) accurately assay the resulting solution. For successful SFE with solid phase trapping the additional step of thoroughly rinsing the analytes from the trap prior to analysis is required. Failure to optimize each of these steps will limit the success of SFE. Typically, the removal of the analytes from the matrix is viewed as the most complex and complicated of the steps presented, and is achieved by optimizing extraction parameters such as fluid density, static and dynamic extraction time periods, flow rate, and fluid composition. If the fluid has insufficient solvating power for the analytes in question, and if one is restricted to a particular fluid, modification with a polar liquid will be required.

Trapping Techniques in SFE

Liquid Trapping

At first glance, the collection of the analytes in SFE seems fairly simple and straightforward. The extracted analytes can be collected via two basic trapping schemes. The first and most popular trapping technique to date is liquid trapping in which the analyte-containing CO_2 is decompressed into a compatible organic solvent such as methanol or hexane. Its popularity owes largely to the fact that it is mechanically simple to carry out.

Solid Phase Trapping

The second type of SFE collection technique which has become very popular is known as solid phase trapping. In this case the analyte-containing CO_2 is decompressed onto either a relatively inert solid phase such as stainless steel or glass beads, or onto a solid sorbent which is often a chromatographic stationary phase such as octadecylsilica or silica itself. The analyte must then be rinsed off the solid phase surface with a suitable solvent. For 100% CO_2 systems, Mulcahey et al. have provided a detailed study of solid phase trapping (11). The trap temperature was found to be critical for certain analytes

(e.g. acetophenone and N,N-dimethylaniline), but for others it did not seem to have an effect (e.g. tetracosane, decanoic acid, and 2-naphthol).

Mulcahey and Taylor have also shown that both volatile and nonvolatile components of a test mixture are lost from a C-18 sorbent trap when modifier in excess of 4% is utilized (12). It is believed that this analyte loss stems from aerosol formation with the expanding SF mixture of CO_2 and condensed organic solvent (13,14,15). Yoo and Taylor reported that an increased trap temperature aids in the recovery of an array of compounds at higher (4-16%) modifier percentages, but percent recoveries plateau when the trap can no longer vaporize the condensed modifier (16). Currently, it is believed that the ability to trap compounds on a solid phase at high (>4%) modifier concentrations is directly related to the trap temperature during extraction and the supercritical fluid flow rate.

Tandem Trapping

For some applications a single trap may be insufficient for quantitative purposes, in which case a less common but rapidly growing form of trapping in SFE known as tandem trapping could be employed. This type of trapping system employs sequential solid and liquid trapping schemes in order to maximize the collection of analytes. There are several ways in which this tandem trapping system can be configured. Dionex (Sunnyvale, CA) manufactures a supercritical fluid extractor which employs in tandem a glass surface plus

liquid trapping system. (**Figure 8**) This device employs a fully heated restrictor housed within a glass tube that extends into the liquid. The glass tube serves as the solid phase trap while a variety of different organic solvents can be utilized in the liquid trap. Work has been performed on this instrument which shows that tandem trapping is more efficient in trapping some compounds than conventional liquid trapping alone (13,17). In cases where the fluid employed was 100% CO_2, several applications and studies have revealed recoveries of PAHs from marine sediment that are comparable to that of Soxhlet extraction, while employing this type of tandem trapping system (17). Also, Thompson and Taylor reported on the tandem solid/liquid collection of an array of nonpolar and polar compounds using the aforementioned Dionex SFE system (18). For analytes of varying polarity a solvent mix composed of hexane, chloroform, and methanol (1:1:1) proved best for 100% CO_2 extractions.

Detailed studies involving tandem solid/liquid trapping when modified CO_2 was employed have recently been reported by these same workers (19). When modifier was employed, recoveries were generally poor regardless of the modifier amount and identity when employing the 100% CO_2 optimum collection solvent mixture. Meyer et al., however, have reported high recoveries of polynulcear aromatic hydrocarbons (PAHs) from soils with modified fluid extractions using a tandem solid/liquid trap in which an octadecylsilica plug is placed within the glass tube, indicating that perhaps a tandem trapping system may be beneficial when modifiers are employed (13).

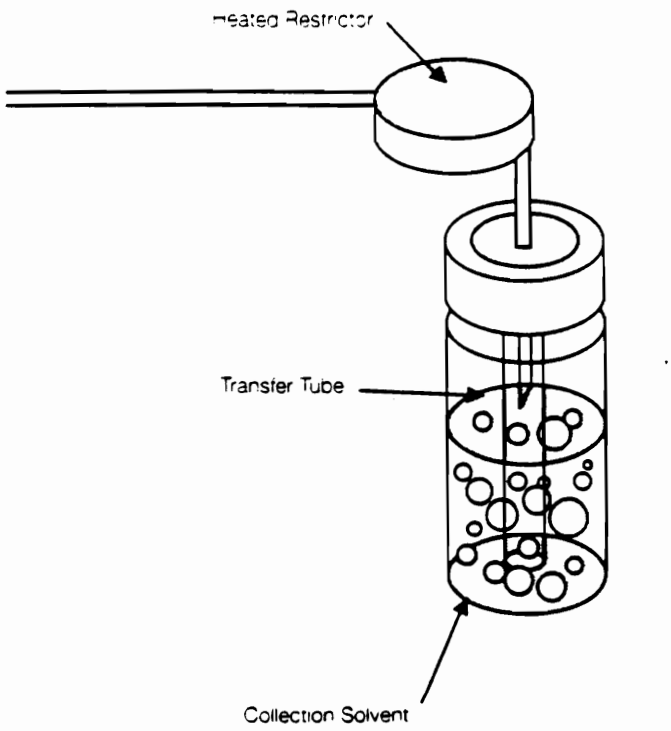


Figure 8. Diagram of the tandem trapping setup utilized by the Dionex supercritical fluid extractor.

Work has also been performed on extractors other than the Dionex instrument with tandem trapping in which a sorbent solid phase is used in conjunction with liquid trapping. This setup has been employed extensively in our laboratory including the extraction of sulfonamides from various food matrices while using both modified and nonmodified SF CO_2 (20).

Modifier Introduction and Its Effect on Trapping

As was previously stated, to increase the polar nature of the fluid for solvating more polar compounds and to overcome any analyte-matrix interaction which may be present, a polar liquid modifier is employed. There has been an ongoing debate concerning the various ways in which modifier can be introduced into the SFE experiment. Modifier introduction may be of three types: (1) premixed modified fluid, (2) modifier addition to CO_2 with the aid of a micro-HPLC pump (in-line), and (3) direct modifier addition to the matrix via spiking (off-line).

While premixed tanks are still in use, they possess several disadvantages. One disadvantage of this type of introduction is that in order to obtain different percentages of modifier, different cylinders containing a range of modifier amounts are necessary. Also, it has been observed experimentally that as the fluid is drawn from the cylinder, the concentration of modifier in the CO_2 increases. This occurs because the vapor pressure of

CO_2 is greater than that of the modifier, and as a result more CO_2 evaporates into the headspace volume of the cylinder relative to the amount of modifier, thereby resulting in a greater concentration of modifier in the fluid to be used for extraction. These changes in modifier concentration may cause reproducibility problems in the extraction recoveries (21). Consequently, the later two techniques have surpassed premixed tanks in popularity.

It is known but not widely studied that the mode of modifier introduction into SFE can have a profound effect on the extraction efficiency. Knipe et al. have suggested that matrix modification rather than fluid modification is more effective for the extraction of atrazine, cyanazine, and diuron from celite and soil (22). Also, Ashraf-Khorassani et al. have also reported that perhaps spiking the matrix may be better than in-line modifier addition, especially when attempting to overcome severe-analyte matrix interactions (23). Ashraf-Khorassani reports high recoveries of polychlorinated biphenyls from river sediment when employing the spiking technique. We believe that the mode of modifier introduction not only affects analyte/matrix interactions, but may also significantly affect trapping in SFE as well.

Understanding and optimizing the variables involved in the collection of the extracted analytes are as important as understanding and optimizing the extraction variables themselves. The focus of the work reported here is three-fold. First, the effect of the mode of modifier introduction on solid phase trapping in SFE is investigated. Second, the various types of mechanisms which can lead to analyte loss at the solid phase trap when high levels of modifier are used is considered. Third, whether or not a tandem

trapping system employing a liquid trap helps in trapping the extracted analytes which pass through the solid phase trap is of interest.

EXPERIMENTAL

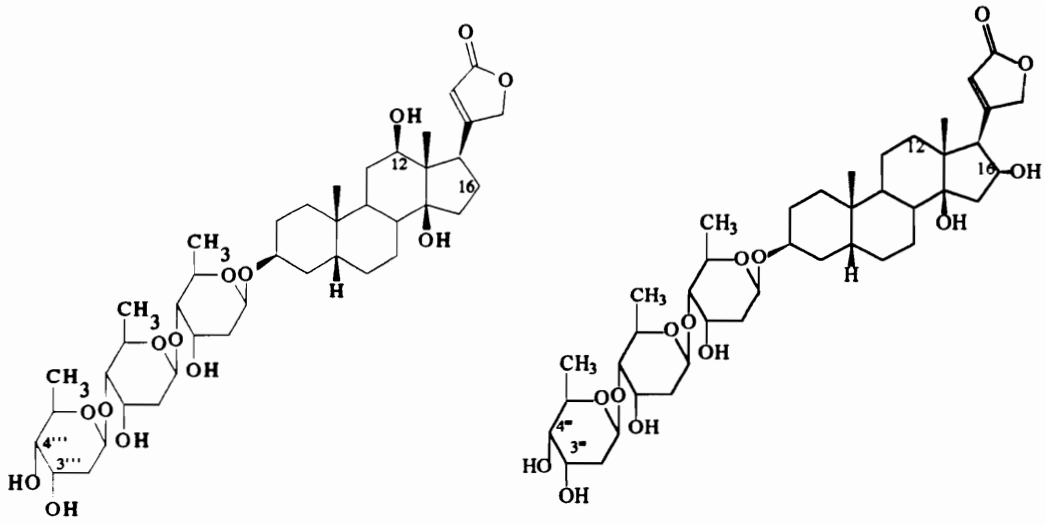
Supercritical Fluid Extraction and Quantitation

All supercritical fluid extractions were performed on the Suprex Prepmaster (Suprex Corp., Pittsburgh, PA) which employs an automated variable restrictor. The Accutrap (Suprex Corp., Pittsburgh, PA) which is a module used for off-line collection of the analytes via either solid, liquid, or tandem trapping was employed. This component can achieve solid trap temperatures from -50°C to +100°C, and its automated restrictor can also achieve a temperature of 100°C which further helps to reduce restrictor plugging. The extractor employs a dual-head reciprocating pump which can deliver up to 7 mLs/min liquid flow while achieving a maximum pressure of 500 atm (7,348 psi). The oven can achieve temperatures up to 150°C. The extraction fluid was SFE/SFC grade CO_2 with helium headspace and was obtained from Air Products and Chemicals, Inc. (Allentown, PA). Modifier was introduced in-line with an HPLC pump obtained from SSI (State College, PA), or it was added directly to the vessel. All solvents were HPLC grade and purchased from EM Science (Gibbstown, NJ). All samples for extraction were prepared by spiking 0.5 mg (1 mL of a 0.5 mg/mL solution in methanol) of each of the three

analytes digoxin, gitoxin, and digitoxin onto Celite (Supelco, Bellefonte, PA) in a 3.7 mL vessel obtained from Dionex (Sunnyvale, CA). The methanol was completely evaporated prior to each extraction by placing the vessel under a stream of nitrogen overnight. The glycoside standards were purchased from Sigma Chemical Company (St. Louis, MO) (**Figure 9**).

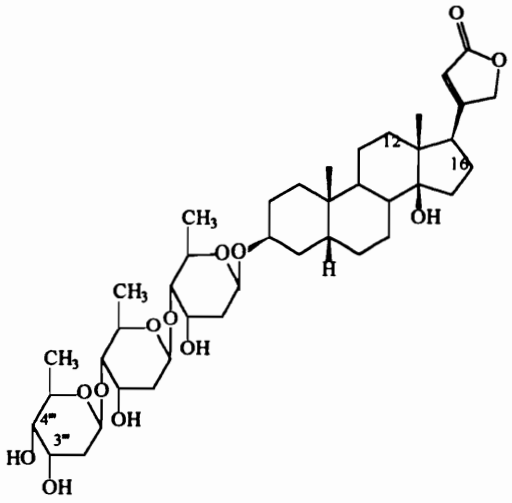
All extractions were performed at a pressure of 450 atm and an oven temperature of 50°C. The solid phase trap was packed with stainless steel beads obtained from Suprex and a liquid trap of 5 mLs of methanol in a 10 mL capped microcollection flask (Supelco, Bellefonte, PA) was employed in tandem (**Figure 10**). Notice that this trapping setup varies quite substantially from the Dionex trapping system eluded to earlier. An 18 gauge needle was used to vent the liquid trap by piecing the septum allowing the decompressed gas to escape. Following each extraction the liquid trap was assayed for percent recovery, followed by an assay of the solid trap rinse which was 5 mLs of methanol at 25°C. The restrictor during extraction was heated to either 50°C or 100°C, and the solid phase trap during rinsing was held at a temperature of 25°C. Flow rates and percent modifier were varied throughout the experimental process.

An HPLC-UV assay of the glycoside standards was performed on a 1050 Series HPLC System (Hewlett-Packard, Avondale, PA) which incorporates a quaternary gradient pump and a variable wavelength UV detector. A gradient 75/25-water/acetonitrile- 100% acetonitrile program was employed. A model EQ-60 LC six port external loop injection valve (Valco, Houston, TX) was used with a 10 μL sample loop. A 4.6 x 250 mm



Digoxin

Gitoxin



Digitoxin

Figure 9. Chemical structures of the three glycoside compounds used for the trapping study. Molecular weights are ≈ 800 amu, while the temperatures of decomposition are approximately 225°C for digoxin and gitoxin and the melting temperature is 285°C for digitoxin.

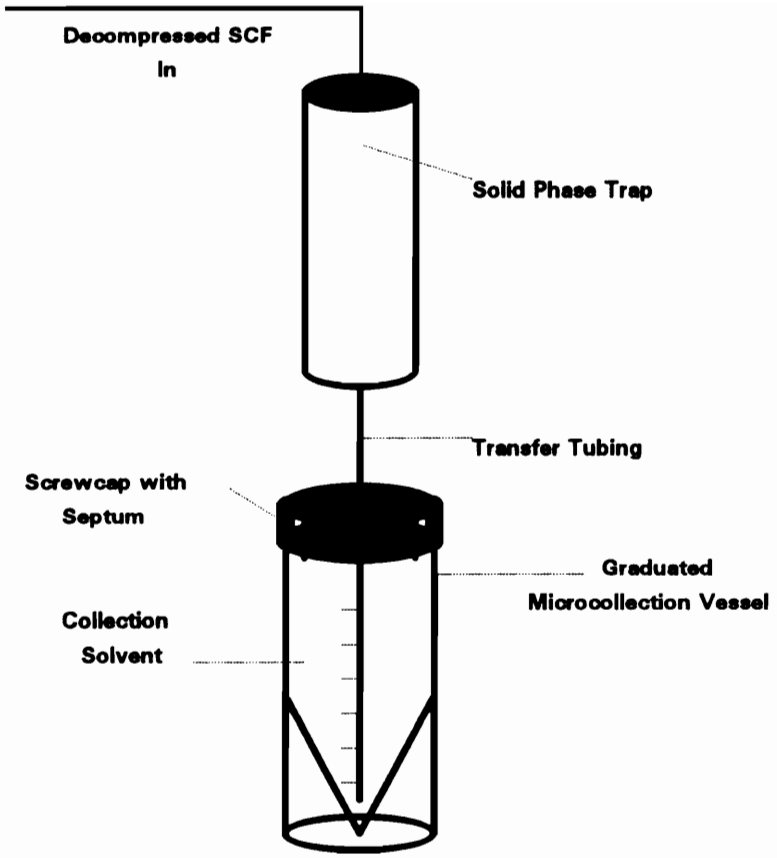


Figure 10. Diagram of the tandem trapping system employed throughout the study.

Hypersil ODS (5 μm) (Keystone Scientific Inc., Bellefonte, PA) liquid chromatographic column was employed. The wavelength of detection was 215 nm, and the flow rate employed was 1.5 mLs/min. All samples were filtered through a 0.45 μm filter (Millipore, Bedford, MA) prior to injection. A typical chromatogram is shown in **Figure 11**.

Extraction profiles were obtained by carrying out a step-wise extraction in which both the liquid and solid phase traps were periodically assayed and their cumulative recoveries plotted as a function of extraction time. During each of the trap rinsing steps, the extraction vessel was maintained under supercritical conditions with the aid of a six port static/dynamic valve.

Analyte recoveries relative to an external standard were calculated based on a 5 point calibration curve of each glycoside compound in which the correlation coefficient for each curve was ≥0.9900. (**Figure 12**) Relative standard deviations of the chromatographic method were found to be ≤1% for n=3 for each of the three compounds when employing the separation method discussed above.

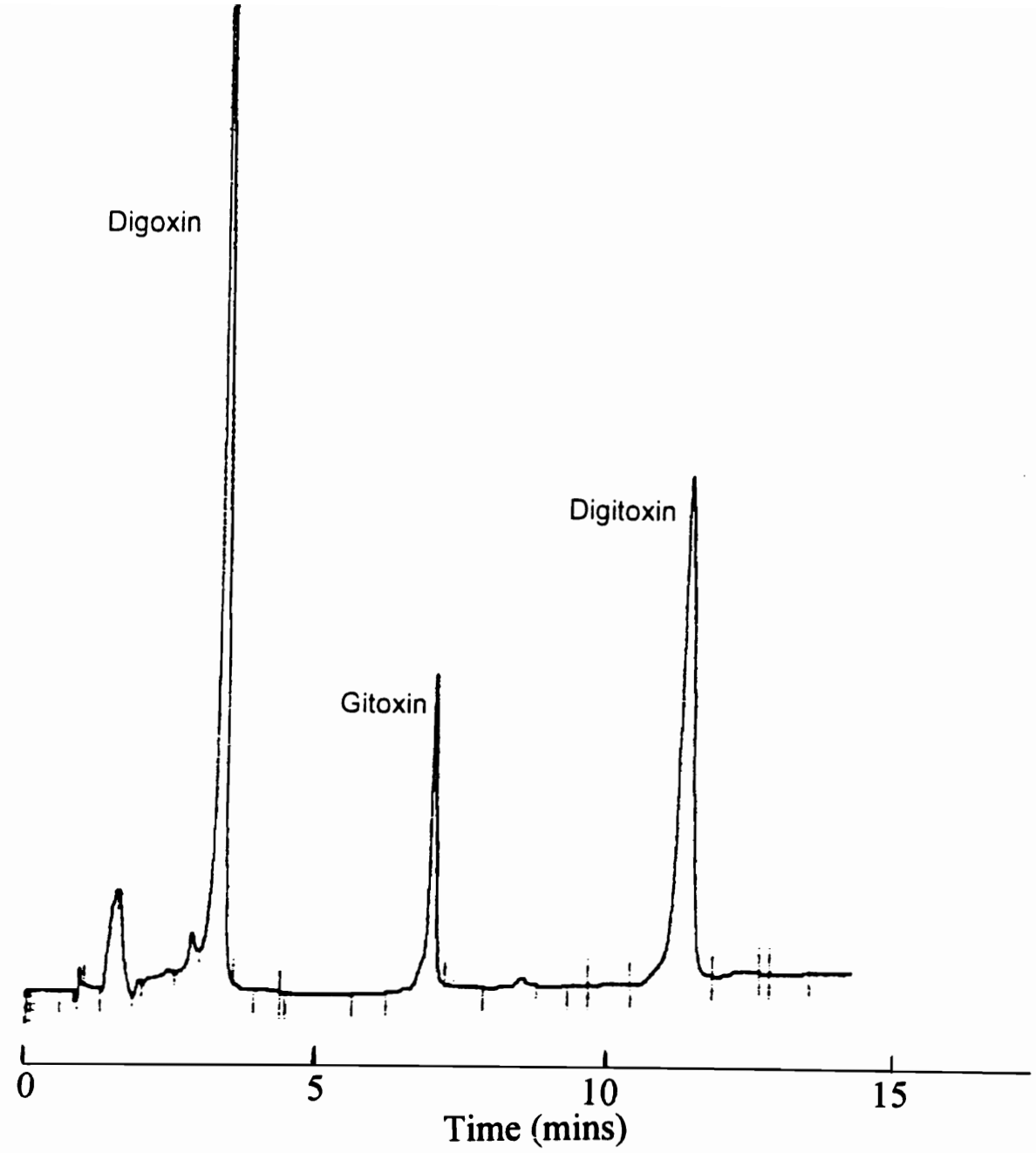


Figure 11. A typical HPLC chromatogram showing the elution order of the three glycosides employed for the trapping study.

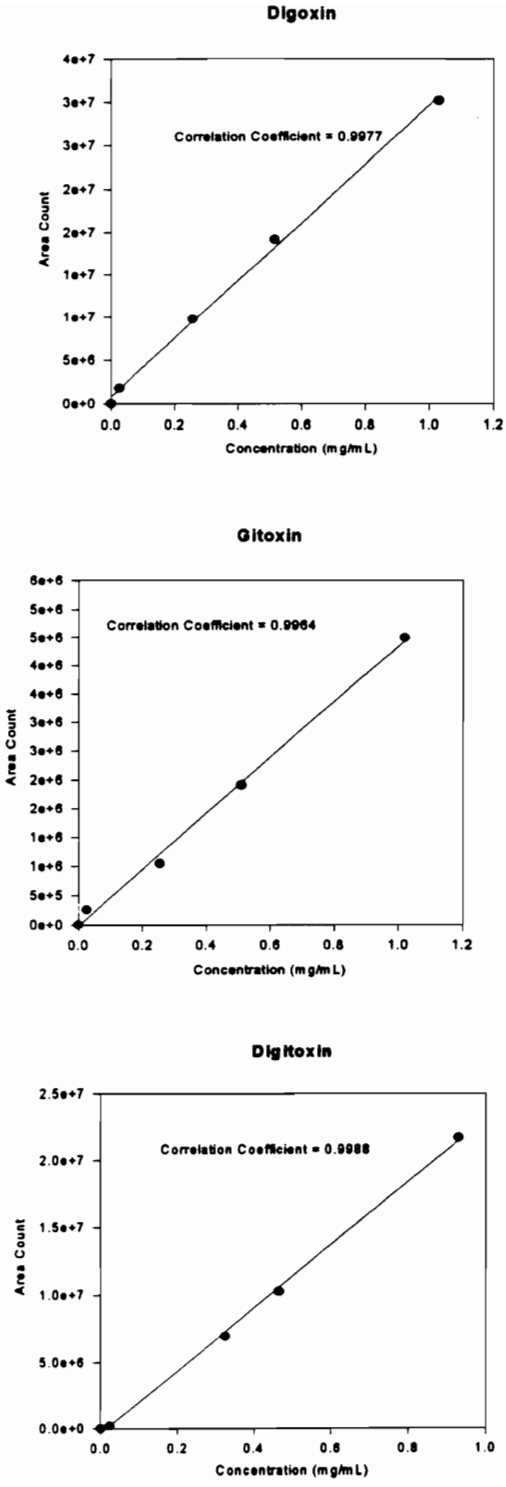


Figure 12. Calibration curves for the three analytes employed in the trapping study.

RESULTS AND DISCUSSION

Modifier Concentration and Introduction

This portion of our trapping study focuses on the effect of modifier concentration and the manner in which the modifier is introduced. The benefit of employing a tandem liquid trap was also examined. A liquid CO_2 flow rate of 1 mL/min was used. The solid trap and restrictor temperatures were maintained at 100°C, and the liquid phase of the tandem trap was at 25°C. It should be noted that a solid phase trap temperature of 100°C is unusually high, but since our analytes are thermally stable at this temperature, no decomposition of analytes was anticipated. Initially, methanol modifier was introduced in-line to the fortified (spiked) matrix. Increasing percentages (0 to 30%) of methanol-modified CO_2 were used in each dynamic extraction which lasted 30 minutes (**Figure 13**). Recovery increased for all three analytes as the percent methanol increased, to a maximum of approximately 80%. This was surprising because in the past just the opposite had been observed because of decreased trap efficiency with higher modifier levels, but in these previous cases the solid phase trap temperature was below the boiling point of the modifier. (24) Since the solid phase trap was at 100°C, no liquid methanol should have

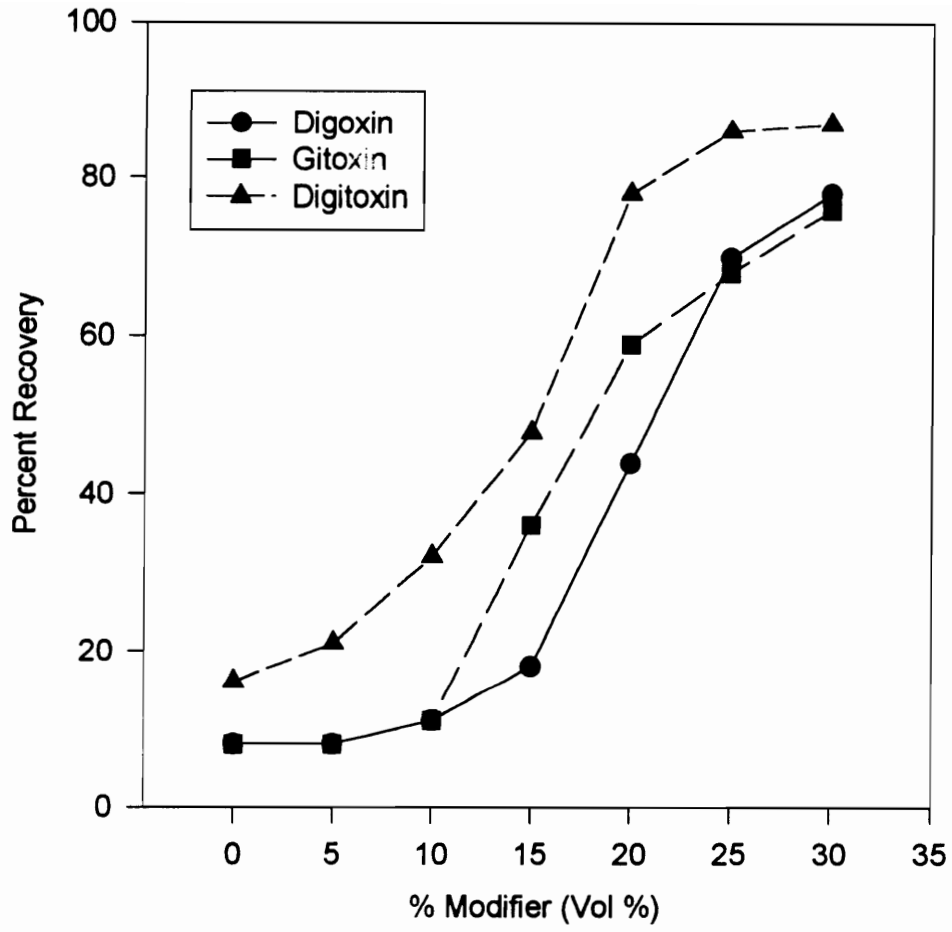


Figure 13. Plot of in-line modifier addition showing the combined solid and liquid trap recoveries as a function of modifier amount added in-line.

accumulated on the stainless steel trap. The large percentage (30%) of methanol was deemed to be needed to solubilize these high molecular weight analytes. In a second extraction, the modifier was introduced into the system by spiking the fortified matrix with methanol just prior to extracting with 100% CO_2 for 10 minutes in the static mode followed by 20 minutes in the dynamic mode (**Figure 14**). A comparison of both Figures 11 and 12 naively suggest that approximately the same (but less than quantitative) recovery is obtained with either a total of 9 mL methanol (in-line/30 minutes dynamic) in the modified fluid case or 2 mL methanol (spike/10 minutes static-20 minutes dynamic) in the modified matrix case.

Extraction profiles were next constructed under optimum conditions for both "in-line" and "spiked" extractions (20% methanol by volume and 2 mL methanol, respectively) (**Figures 15 and 16**) in order to gain some insight as to why a larger amount of modifier for the in-line extraction was required for a comparable recovery of the three glycosides. Both extraction profiles appeared somewhat similar in that initial slopes were rather steep but each profile quickly reached a plateau. This type of extraction profile is indicative of minimal analyte-matrix interaction which was expected since the matrix was inert. Surprisingly, the in-line modifier (30%) profile (**Figure 15**) showed a greater recovery in 5 minutes than was seen after 30 minutes of 30% in-line modifier (**Figure 13**) under identical dynamic extraction conditions. The only difference in the two experiments was that the stainless steel trap was rinsed after every 2.5 minutes for the in-line extraction profile data, whereas, the trap was only rinsed once after 30

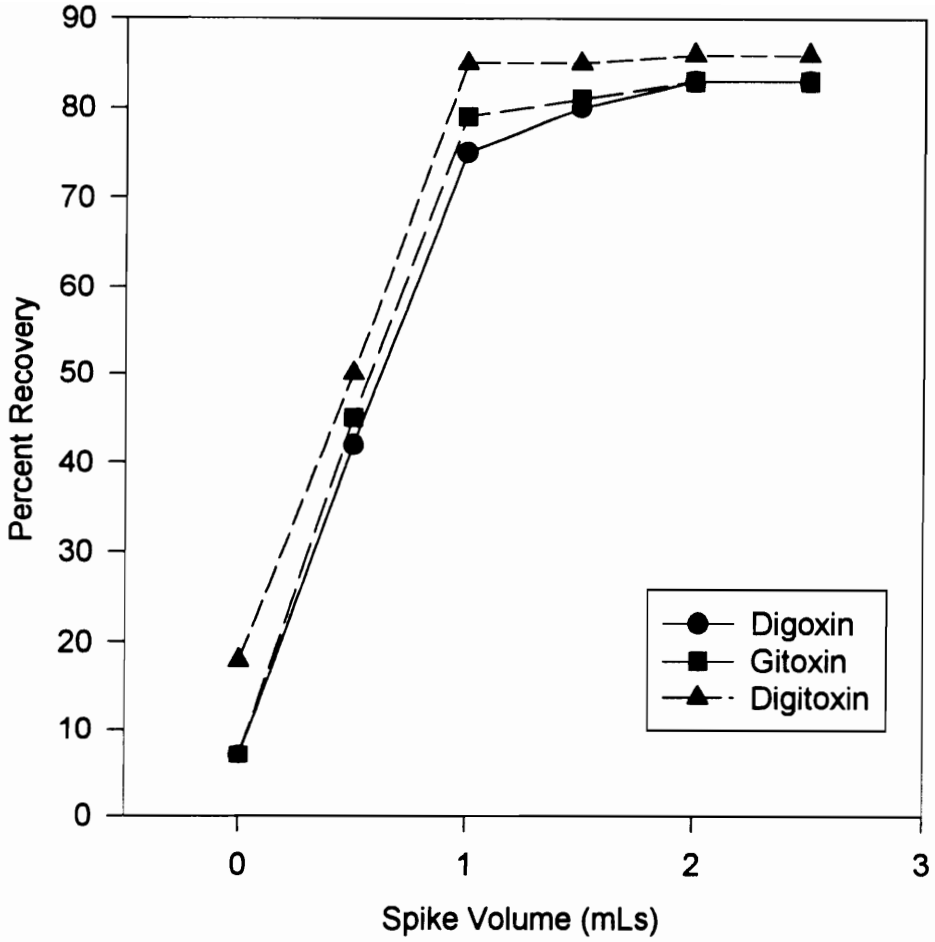


Figure 14. Plot showing the combined solid and liquid trap recoveries as a function of modifier amount added by spiking directly into the vessel.

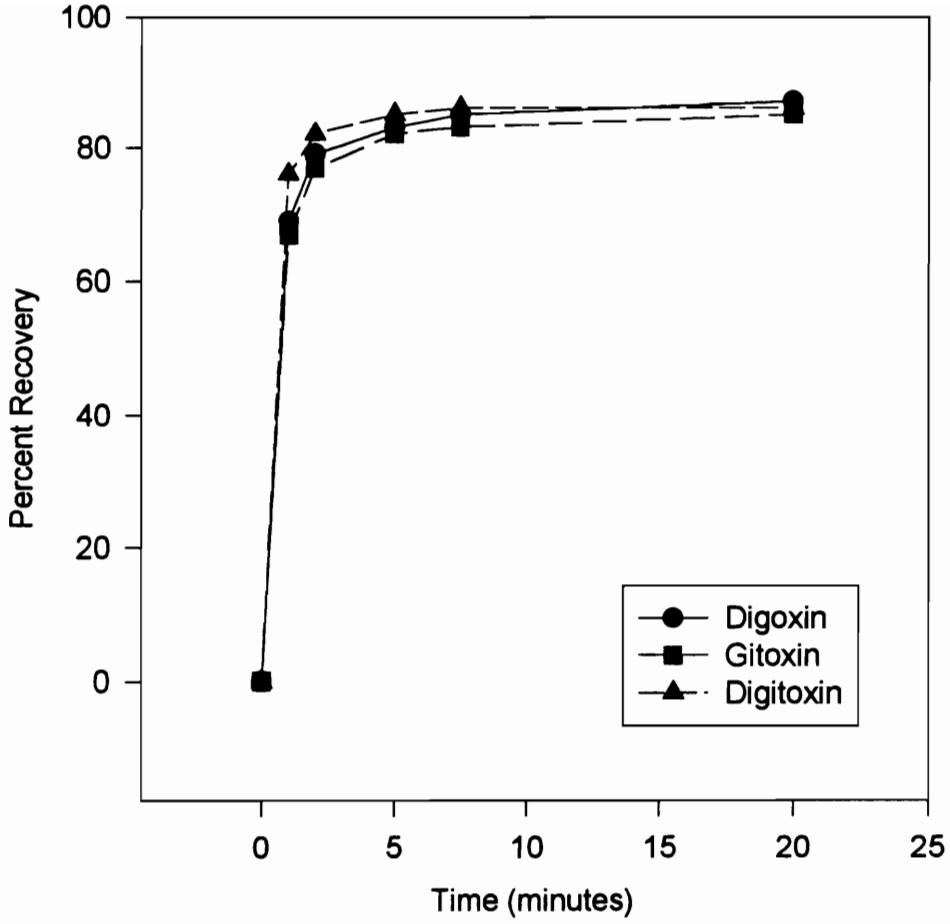


Figure 15. Extraction profile for in-line modifier addition under optimum conditions with combined recoveries from both solid and liquid trapping.

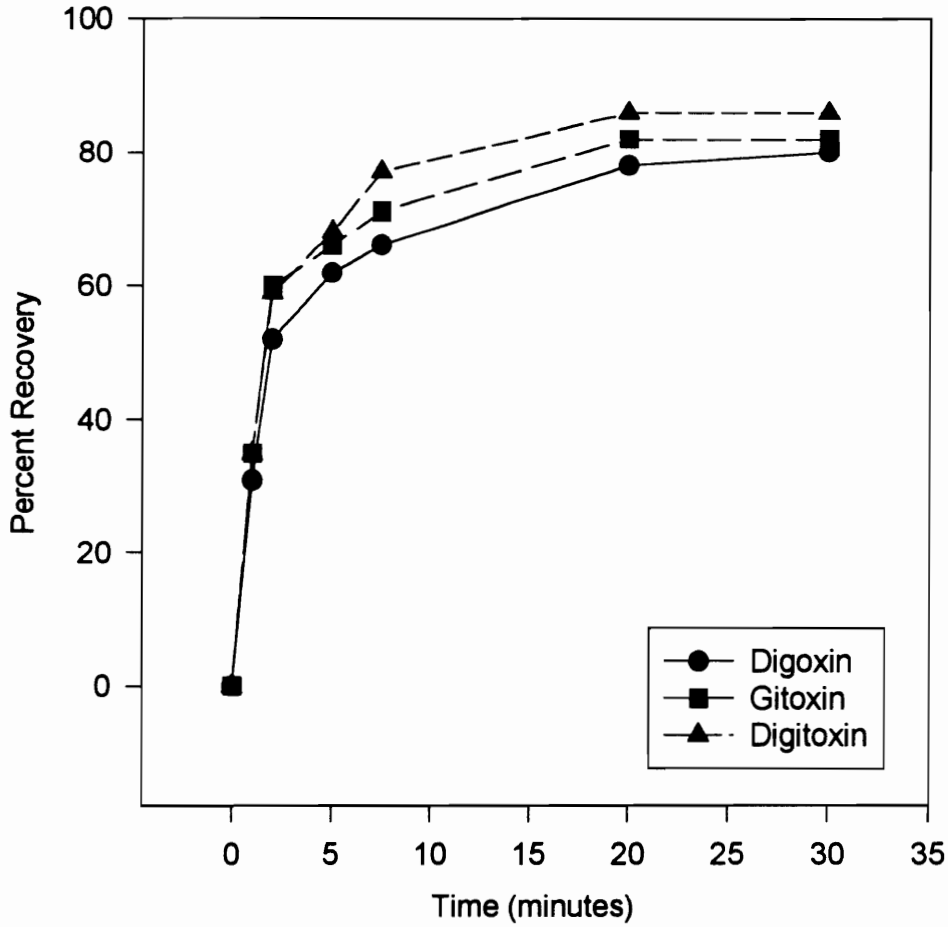


Figure 16. Extraction profile showing the combined recoveries from solid and liquid trapping as a function of optimum amount of modifier spiked directly into the vessel.

minutes dynamic extraction in the other (**Figure 13**). The in-line 30% modifier extraction profile (**Figure 15**) also showed a greater initial recovery of the analytes after 5 minutes when compared to the extraction profile results employing an optimum methanol matrix spike (2mL) (**Figure 14**). This finding can be explained by the idea that solvent breaks through the trap taking analyte with it during the initial stages of the spiked matrix extraction when modifier levels are high. This large amount of methanol which condenses on the trap is believed to form an aerosol in the initial stages of the extraction resulting in lower recoveries for the spike extraction throughout the extraction profile. This aerosol formation was subsequently proven to increase when the CO_2 flow rate increased (**Figures 17-19**). It appears that the solid trap when heated to 100°C is able to vaporize the 0.3 mL/min of methanol introduced by in-line modifier addition, but it is unable to vaporize the larger initial cascade of 2 mLs of methanol introduced through matrix spiking.

The lower recoveries witnessed initially with the in-line 30% modifier 30 minute extraction compared with a 5 minute in-line 30% modifier extraction under identical conditions can be attributed to analyte loss from the solid phase trap by physical removal of the analytes from the stainless steel trap over the 30 minute extraction period as decompressed supercritical fluid continued to enter the solid trap. It was thought originally that the tandem liquid trap under these conditions (i.e. continuous 30 minute extraction) would have trapped any compounds which were not trapped on the solid phase, but this proved not to be the case as <5% of the total recovery could be attributed

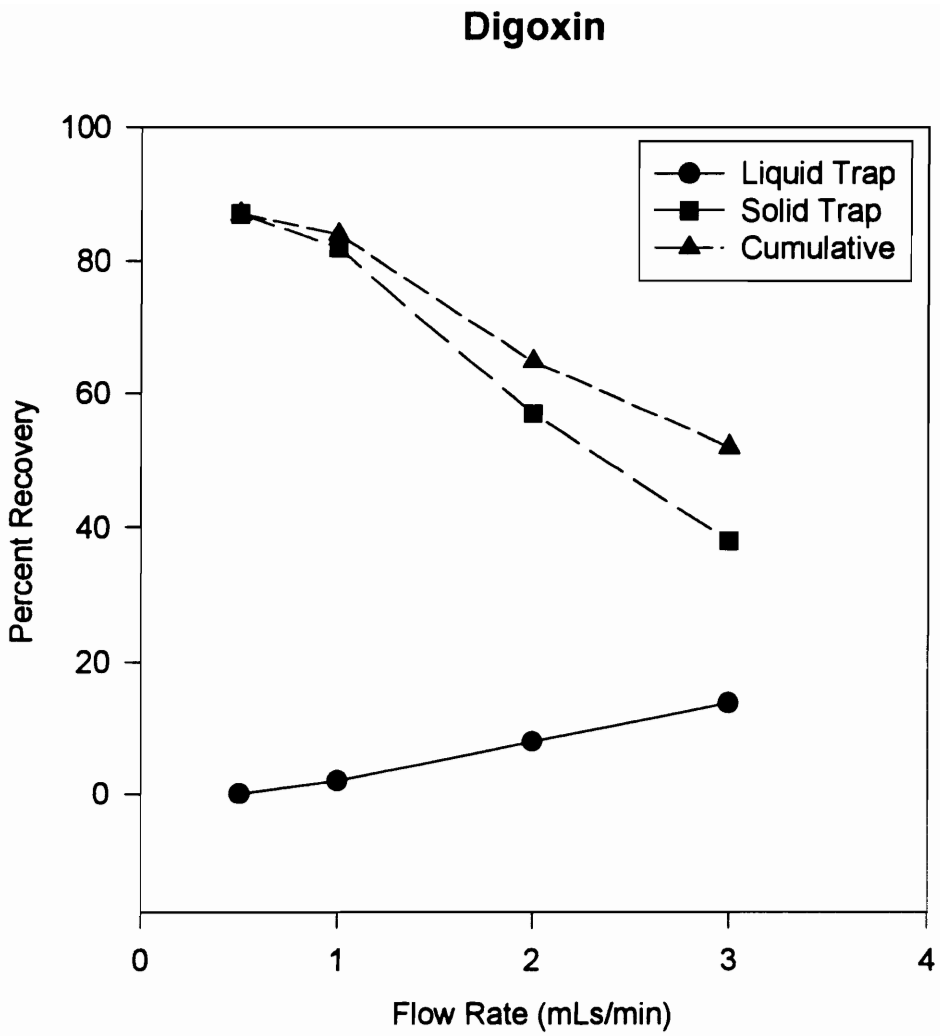


Figure 17. Plot showing the effect of increasing flow rate on the trapping efficiency after matrix modification of the solid and liquid phase traps for digoxin.

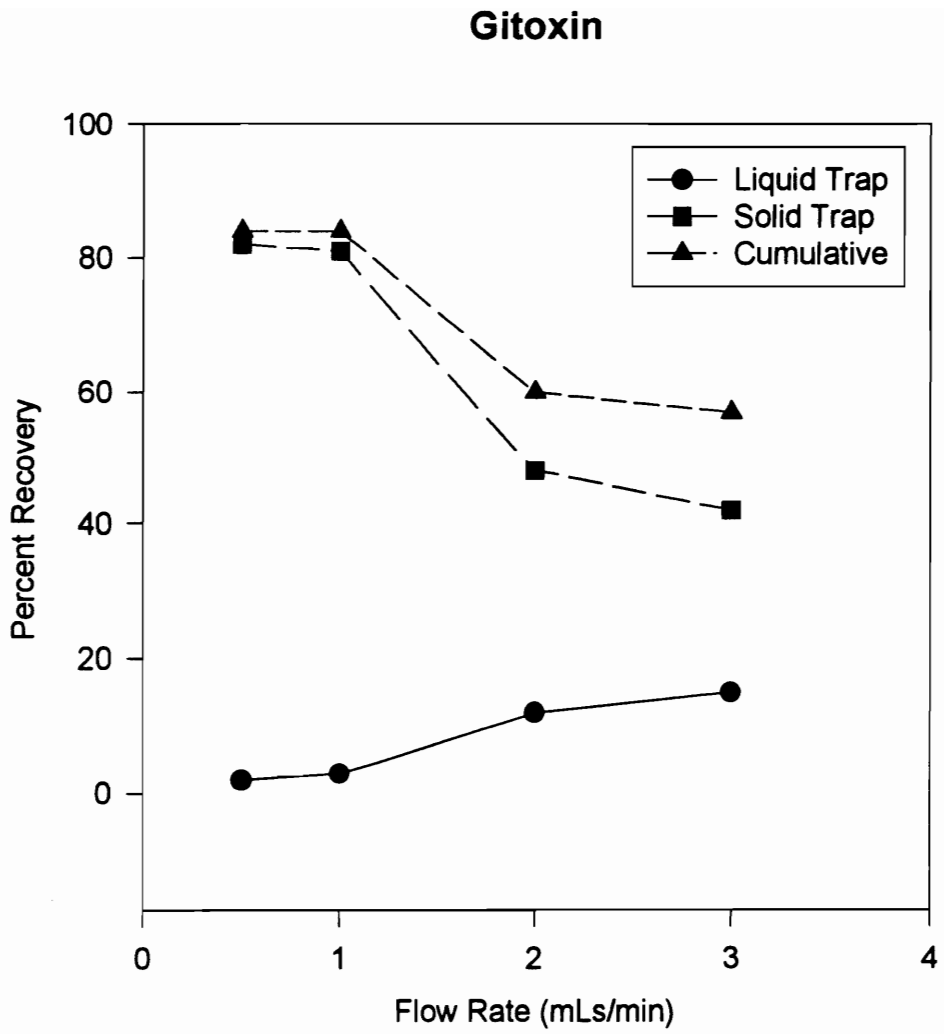


Figure 18. Plot showing the effect of increasing flow rate on the trapping efficiency after matrix modification of the solid and liquid phase traps for gitoxin.

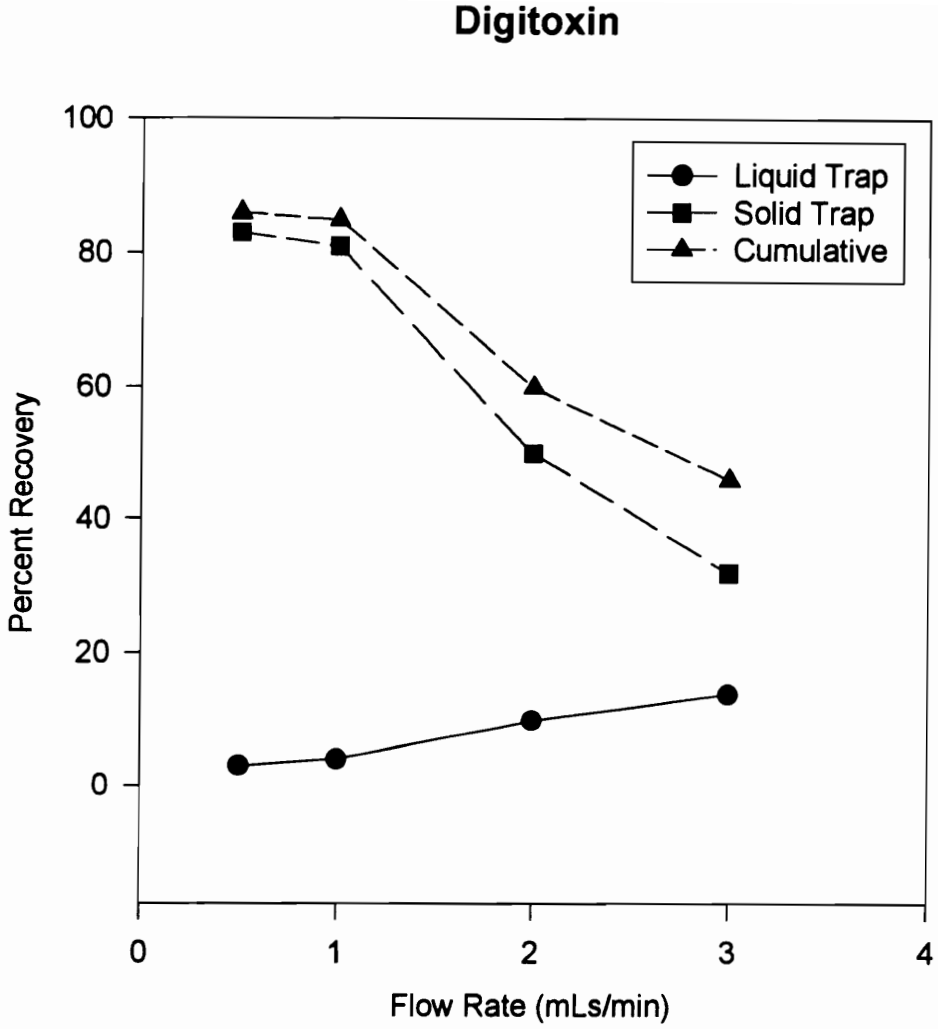


Figure 19. Plot showing the effect of increasing flow rate on the trapping efficiency after matrix modification of the solid and liquid phase traps for digitoxin.

to the liquid trap. For the spiked matrix study the liquid trap in tandem with the solid phase trap did accumulate a portion of the analytes with increasing CO_2 flow, but the overall result was lower cumulative recoveries with increasing flow. This observation once again points out the ineffectiveness of the tandem liquid trap, especially at higher flow rates.

Trapping Loss Mechanisms

There appear to be three possible mechanisms responsible for analyte loss from a solid phase trap: 1) blowing off of the solid phase trap due to the force generated by the decompressed gas (i.e. blow-by), 2) blowing by the trap in the form of an aerosol generated by the decompression of the mixture of CO_2 and modifier (i.e. aerosol formation), and 3) analyte being mechanically rinsed from the solid phase trap by condensed modifier which is produced during the SF decompression (i.e. modifier elution). Three experiments are described in which one of the mechanisms appears to be in effect. In each case, it is believed that each of the three mechanisms contribute to analyte loss from the trap; however, for each scenario given, one mechanism is predominant.

Blow-By

This particular experiment was described in detail in the previous section, *Modifier Concentration and Introduction*. An extraction profile was constructed with the "in-line modifier addition" extraction (**Figure 15**) and a greater overall recovery in 5 minutes was seen compared with a 30 minutes extraction under identical extraction (**Figure 13**) conditions with the only difference being the frequency of washing the solid phase trap. The lower recoveries witnessed initially with the in-line modifier extraction for 30 minutes (≈80%) compared with a 5 minute (≈90%) extraction under identical conditions can be attributed to analyte loss from the solid phase trap by physical blow-by of the analytes from the stainless steel trap over the 30 minute extraction period. Analytes which are lost from the solid trap as a result of being mechanically forced off due to SF decompression appear to be surprisingly unretained by even the liquid trap (e.g. <5% of the glycosides were found in the in-line liquid trap).

Aerosol Formation

Methanol (2 mL) was spiked onto the matrix and extracted as in the previous experiment. The solid phase trap during extraction was kept at 30°C, restrictor at 50°C, and the flow rate was increased from 0.5 mL/min to 3 mLs/min. Under these conditions,

extracted methanol would condense onto the trap. Decompressed carbon dioxide thus flowing past the stainless steel surface on which the methanol had condensed would no doubt blow off the methanol which contained the dissolved analytes. Proof for this comes from the fact that the lowest recoveries could be attributed to solid phase trapping at the highest flow rate as was expected. In addition, only a slight increase in the amount of analyte trapped within the liquid trap was observed (not accounting for the greater amounts lost from the solid trap) indicating an increased amount of blow-by of both traps due to aerosol formation at increased flow rates. Recoveries of combined solid and liquid traps were below 60% for each of the three analytes. It is believed that higher flow rates propel the aerosol further than at lower flow rates thereby resulting in less analyte being trapped in the liquid trap.

Modifier Elution

Varying amounts of methanol were spiked into the vessel and the fortified matrix was extracted statically 10 minutes followed by a 20 minute dynamic extraction wherein 15 mLs of extraction fluid were used. The conditions of the extraction were kept the same as above with the exception that the solid phase trap temperature during extraction was lowered from 100°C to 30°C, restrictor temperature was set at 50°C, and the flow rate was set at 0.5 mL/min. The ability of the solid phase trap to retain the extracted analyte was very poor at low trap temperatures and all spiked modifier levels (**Figures 20-22**). For example, a 2.5 mL modifier spike revealed very little (5%) had been trapped on

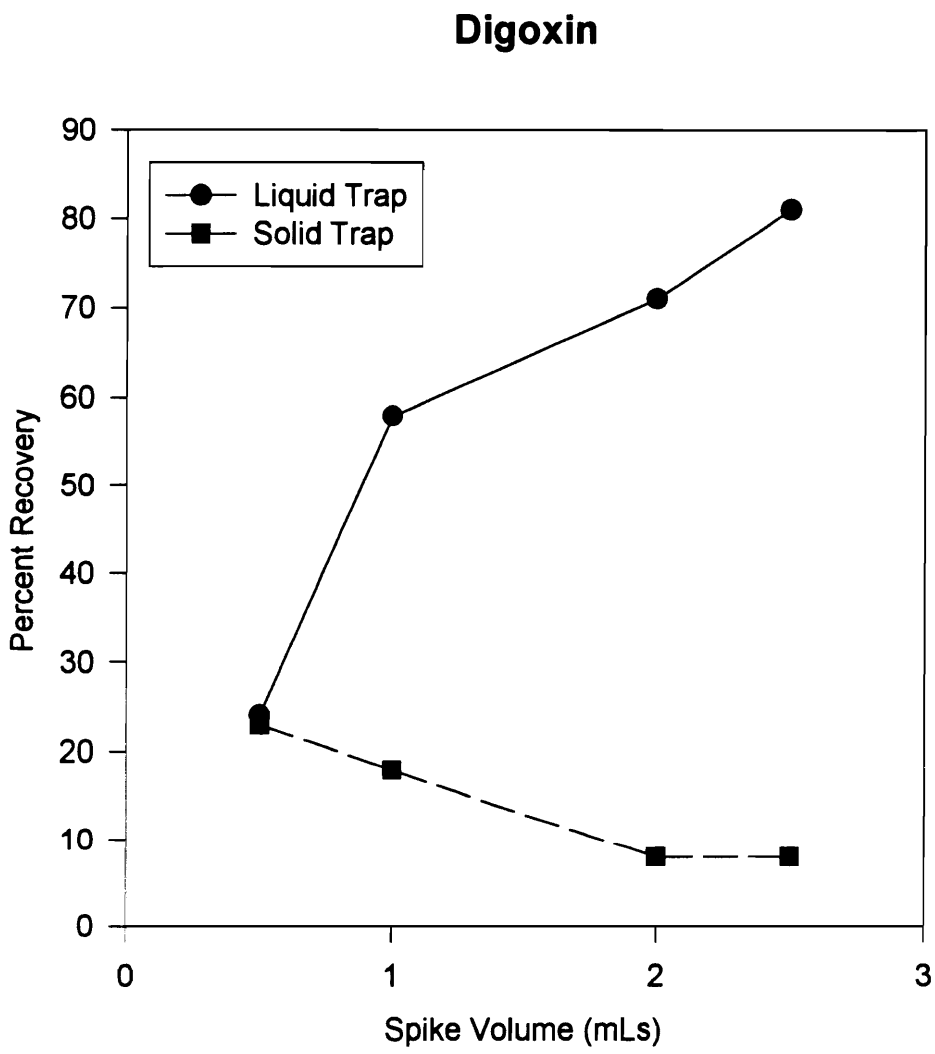


Figure 20. Plot showing how matrix spike volume affects where digoxin is trapped within the tandem trapping system.

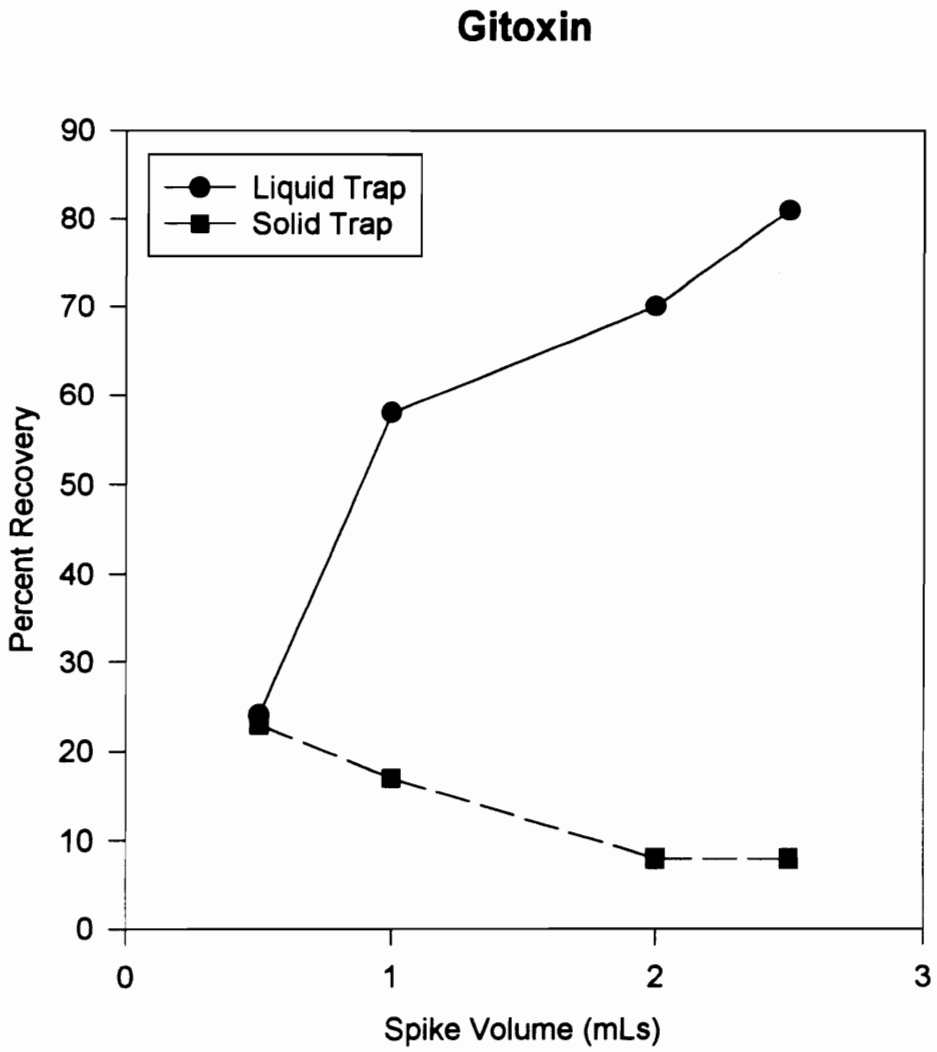


Figure 21. Plot showing how matrix spike volume affects where gitoxin is trapped within the tandem trapping system.

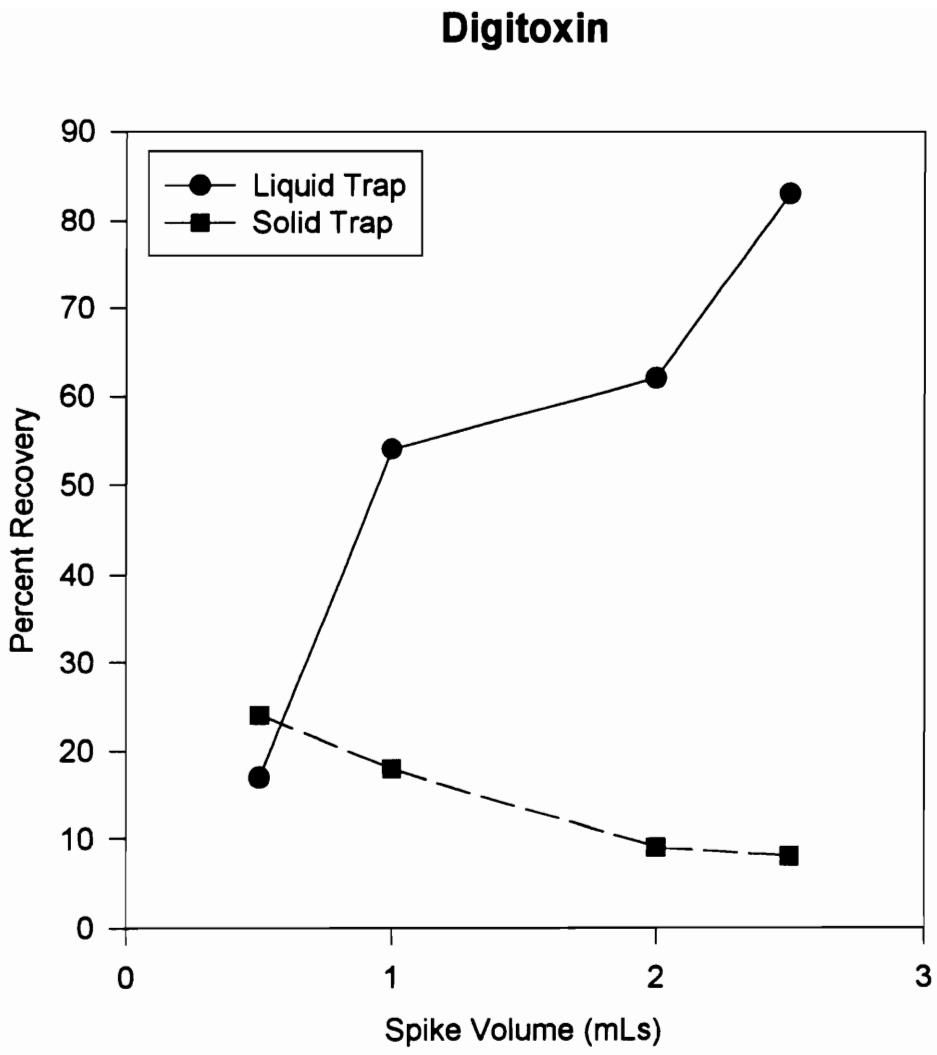


Figure 22. Plot showing how matrix spike volume affects where digitoxin is trapped within the tandem trapping system.

the solid phase. While the liquid trap appeared to exhibit increasing efficiency with increasing level of spiked modifier, less than a quantitative cumulative recovery was found. At low spike levels the combined solid and liquid traps are probably efficient but the level of modifier is too low to achieve sufficient solubility. For example, approximately 45% recovery was found for a 0.5 mL modifier spike. At high modifier levels a relatively large amount of modifier condenses on the solid phase such that one can observe liquid modifier dripping from the solid trap. Under these conditions, it is clear that the presence of the tandem liquid trap is very important as it trapped components which were ineffectively trapped on the solid sorbent.

A final experiment was performed to prove the hypothesis that high trap temperature and low flow rate would provide the highest recoveries thus far with the stainless steel trap. Methanol (2 mL) was spiked onto the matrix and extracted 10 minutes in the static mode followed by 20 minutes in the dynamic mode. The liquid flow rate was 0.5 mL/min and the trap temperature was held at 100°C throughout the extraction. These extraction parameters represent optimum high temperature/low flow conditions, and quantitative recoveries were obtained on each analyte as shown in **Figure 23**. The recoveries along with the RSDs of the three compounds were: Digoxin (93.8%-3.2%); Gitoxin (95.9%-5.1%); Digitoxin (93.5%-2.9%).

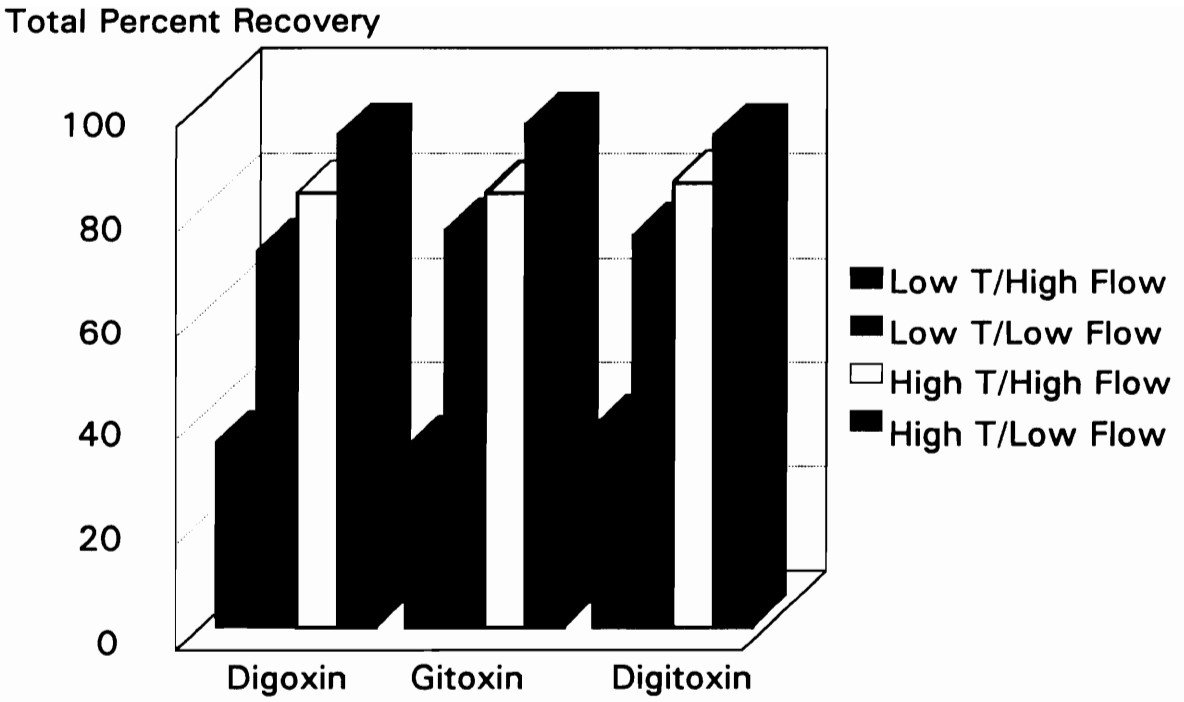


Figure 23. Bar graph showing the cumulative recoveries, using the tandem trapping system of stainless steel beads and methanol, after matrix modification as a function of the four combinations of high/low trap temperature and high/low liquid flow rate.

Summary

The use of modified-CO_2 adds another dimension to SFE in the trapping process as well as in the extraction step. When employing modifier, the user should be aware that low recoveries may not be due to the fluid's inability to solvate and remove the analyte of interest from the matrix, but rather a problem exists in the ability to trap the extracted components effectively. It is known that the method by which modifier is introduced into the SFE system affects the extraction efficiency of different matrices, and it can also have an effect on the ability to trap the components effectively. While larger initial amounts of modifier may help to solvate more of the analytes within the vessel, problems with trapping are observed at these higher spiked modifier percentages which cannot be eliminated totally even at high trap temperatures.

For extractions which exhibit matrix effects a modified matrix may be preferred over a modified fluid depending on the amount of modifier employed. However, a large volume of modifier added to the vessel may actually lead to lower net recoveries than in-line modifier addition even at high trap temperatures because of aerosol formation at the trap. Extractions that use high flows and that place lots of decompressed CO_2 through the stainless steel trap (between trap rinses) run the risk of analytes blowing off the trap. For this reason, it may prove beneficial to use frequent rinse steps. For the best recoveries when modifier concentration is high regardless of the mode of modifier

introduction, it is believed high trap temperatures, low flow rates and periodic trap rinsing are preferred.

The ability of the solid phase to quantitatively trap extracted components at high modifier percentages as well as the type of mechanism that gives rise to sample loss from the tandem trapping system has been shown to be dependent on flow rate and trapping temperature. Tandem trapping proved to be beneficial in instances where modifier elution was occurring (low trap temperature/low flow rate/modifier condensation), however in instances where the analyte was being lost due to aerosol formation (low trap temperature/high flow rate/modifier condensation) and/or physical blow-by of the analyte (high trap temperature/high flow rate/no modifier condensation) due to the force of the decompressed gas, the liquid trap in tandem provided very little benefit, even for the nonvolatile, higher molecular weight glycosides. It was shown that the optimum trap temperature and flow rate combination for extractions where high modifier levels are employed exists at high trap temperature to boil away any modifier and low flow rates to maximize contact time with the tandem liquid trap. Under these high modifier conditions a sorbent trap should be investigated, but one should verify with a tandem trap (at high/low trap temperature and high/low decompressed flow rate) as was performed here, the sufficiency of a single solid phase trap.

Chapter IV

Extraction of Digoxin and Acetyldigoxin from the *Digitalis lanata* Leaf via Supercritical Fluid Extraction

INTRODUCTION

The appealing properties of supercritical fluids have resulted in their use for the extraction of many classes of compounds within a variety of areas, including environmental, food, and polymer. Another area of chemistry which has benefited particularly well from the advantageous properties of SFs is natural products. While this segment is not intended to be an exhaustive literature review of the many applications of SFE to natural products, it will cover some of the classes of compounds to which SFE has been applied.

SFE has been used extensively on both the analytical scale and the larger industrial processing scale for the isolation of naturally occurring compounds from a variety of matrices. One of the most well known applications is the decaffeination of coffee with SF carbon dioxide, a technique which has allowed the replacement of the hazardous extraction solvent, methylene chloride (25). Other well-known alkaloids which have been extracted using SFs include nicotine from tobacco, as well as the compounds thebaine, codeine, and morphine from poppy straw (26,27). Lipids, oils, and pigments have been

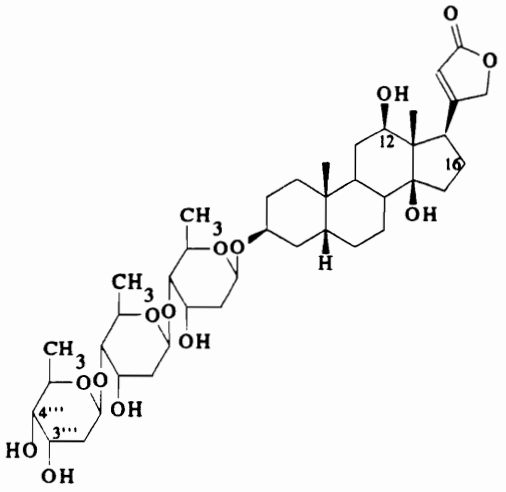
extracted from matrices which include sunflower seeds, peanuts, potato chips, fungi, algae, fruits, medicinal herbs, and numerous types of leaves (28-30). Triglycerides and fatty acids have been the focus of many extractions from matrices which include vegetable, olive, tusk and fish oils (31-33). Cholesterol has been extracted from egg yolk in an effort to yield a diet-friendly product, and other steroids such as stigmasterol have been extracted from plant materials (34,35). Numerous classes of terpenes have been isolated using SFE in the flavor and perfume industry (36-38). Other significant applications of SFE in natural products include the isolation of Taxol, an anti-tumor compound, from the bark of Taxus Brevifolia (39).

While the positive extractive properties of SFs have been and continue to be exploited extensively in the natural products area, the complexity of most natural product matrices presents the analytical chemist with a variety of problems. The strongly bound nature of the incurred analytes as well as numerous coextractable materials which result in complex chromatograms are two examples.

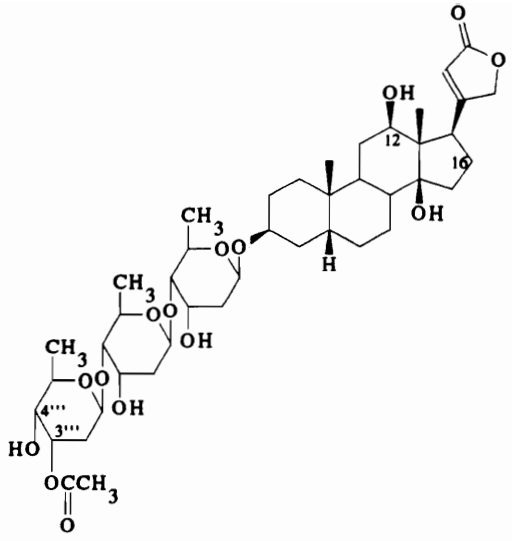
Project Overview

The primary objective of this project was to develop the quickest, safest, cheapest, and easiest supercritical fluid extraction method possible which will yield comparable recoveries of digoxin from the *Digitalis lanata Ehrh.* (Scrophulariaceae) leaf relative to the conventional liquid extraction method, while achieving comparably clean extracts. The

current method of extraction involves a solid-liquid extraction (soak step) followed by a liquid-liquid extraction with chloroform (40). As can be seen in **Figure 24**, digoxin is a moderately polar compound consisting of a steroid-like aglycone bonded to numerous sugar units which contain multiple polar hydroxyl functionalities. It is a cardioactive drug used universally in the treatment of atrial fibrillation and congestive heart failure. The multiple hydroxyl groups which are present on the sugars render the compound soluble within the body may present problems of solubility for nonpolar carbon dioxide. Therefore, before addressing the SFE of the natural product leaf matrix, preliminary studies involving the extraction of standards from an inert matrix were carried out in order to address their solubility in the supercritical fluid (Chapter 3). This preliminary work also revealed the ideal conditions for trapping the extracted analytes. This project involved the isolation of digoxin and acetyldigoxin from the actual leaf matrix using SFE. The method development process is covered in detail for two separate SFE processes, along with a comparison of the two novel extraction procedures and the conventional liquid extraction method.



Digoxin



Acetyldigoxin

Figure 24. Structures of the two cardiac glycosides of interest, acetyldigoxin and digoxin. Acetyldigoxin is converted to digoxin via base hydrolysis.

Leaf Chemistry

In addition to the digoxin compound of the *Digitalis lanata* leaf, we were also interested in extracting acetyldigoxin (**Figure 24**) which can be readily converted to its nonacetylated form, digoxin, through base hydrolysis. This involves raising the pH of the extract solution to 12.0 for 15 minutes with dilute NaOH and then lowering the pH back down to 6.0. Care should be taken not to overshoot pH 12.0 as this results in the opening of the butenolide ring. The acetylated form of the compound differs from its nonacetylated form by the presence of one acetyl group substituted in place of the glucose hydroxyl group located on the 3''' position of the terminal sugar unit. The molecular weight of these two compounds are approximately 800 amu with a temperature of decomposition above 200°C (41). Lanatoside C (**Figure 25**) is the naturally occurring precursor to digoxin which undergoes enzymatic hydrolysis under proper conditions and is converted into acetyldigoxin. This hydrolysis reaction is initiated when the leaf is soaked in the presence of an ethyl alcohol/water mixture which is known to activate the enzyme digilanidase (40). Subsequent liquid extraction with chloroform is used to further clean up the extract liquor following base hydrolysis of the acetyldigoxin. The relationship of lanatoside C, acetyldigoxin, and digoxin is shown clearly in **Figure 26**.

A literature search revealed only one paper which deals with the extractability of glycosides via supercritical fluids. Shibuta et al. reported the cumulative glycoside

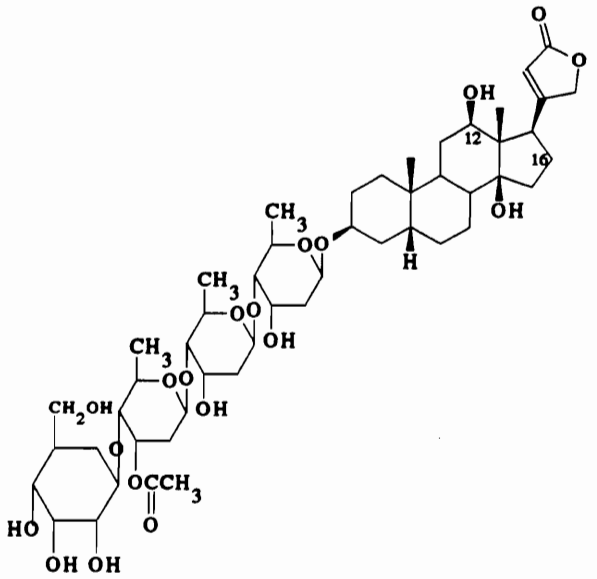


Figure 25. Structure of the naturally occurring precursor to digoxin, lanatoside C, which is converted to acetyldigoxin through enzymatic hydrolysis.

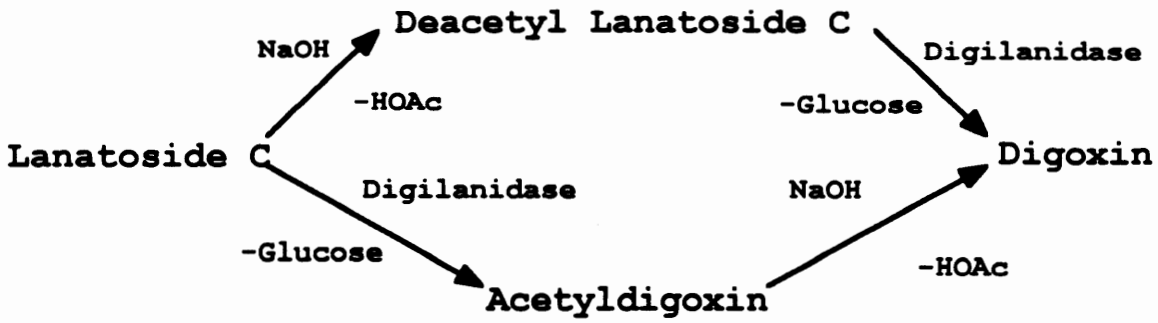


Figure 26. Chemical relationship of the three glycosides within the *Digitalis lanata* leaf.

amounts which could be recovered from various leaf types, of which the foxglove leaf was one example (42). They achieved 0.4% total glycosides from the *D. lanata* leaf with 10 mol% alcohol modifier in SF carbon dioxide which was introduced in-line through a modifier pump. A breakdown of the individual glycoside amounts was not given. However, it was discovered that water and acetone modifiers yielded fairly poor recoveries relative to the ethanol-modified carbon dioxide extraction.

EXPERIMENTAL

Supercritical Fluid Extraction and Quantitation

Extractions were performed on the Hewlett-Packard (HP) supercritical fluid extractor model 7680T (Wilmington, DE). This extractor delivers the solvent as a liquid through the pump heads which are cooled. Modifier is introduced into the system using a HP 1050 HPLC isocratic pump. This instrument employs an automated variable restrictor which decouples extraction pressure and flow rate. Vessels with a 7 mL vessel volume were employed for all extractions, and Celite® (Supelco, Fairlawn, NJ) was used both to fill any dead volume remaining in the vessel and also to prevent leaf particulates from plugging the exit frits and the system tubing. SFE/SFC grade carbon dioxide without helium headspace (Air Products and Chemicals Inc., Allentown, PA) was used for all extractions. All spike solvents and modifiers were HPLC grade and were obtained from

EM Science (Gibbstown, NJ). Sample sizes for all extractions in phase II were 200 mgs as it was discovered early in the leaf extraction work that the larger sample sizes resulted in quicker system and trap plugging. Also, due to the high toxicity of digoxin, smaller sample sizes were desired. As a result of the trapping study (Chapter 3), flow rates of 1.0 mL/min liquid flow were used. These low flow rates help to prevent aerosol formation and blow-by of the solid phase trap as was shown in Chapter 3. All extractions used a solid phase trap of stainless steel beads, with the exception of a final study which compared the trapping efficiency of stainless steel beads with octadecylsilica trapping material. Both restrictor and trap temperatures during the extractions were held above the boiling point of methanol to discourage any liquid modifier condensation on the trap thus causing aerosol formation. Methanol was used as the rinse solvent in all cases to remove the extracted materials from the solid phase trap. The temperature at which the trap was rinsed was 25°C for all extractions. Following SFE, all samples were pH adjusted to deacetylate any acetyldigoxin in the extract solution.

The SFE extracts were assayed by HPLC with ultraviolet detection at 218nm. A calibration curve for the compound digoxin which exhibited a correlation coefficient of 0.9999 is shown in **Figure 27**. The relative standard deviation of the chromatography method was calculated experimentally to be less than 1%. The method used a linear mobile phase gradient of 100% water to 100% acetonitrile in 25 minutes, 100% acetonitrile for 10 minutes, gradient back to 100% water in 5 minutes. The total run time was 40 minutes which allowed for the elution of all coextracted materials from the

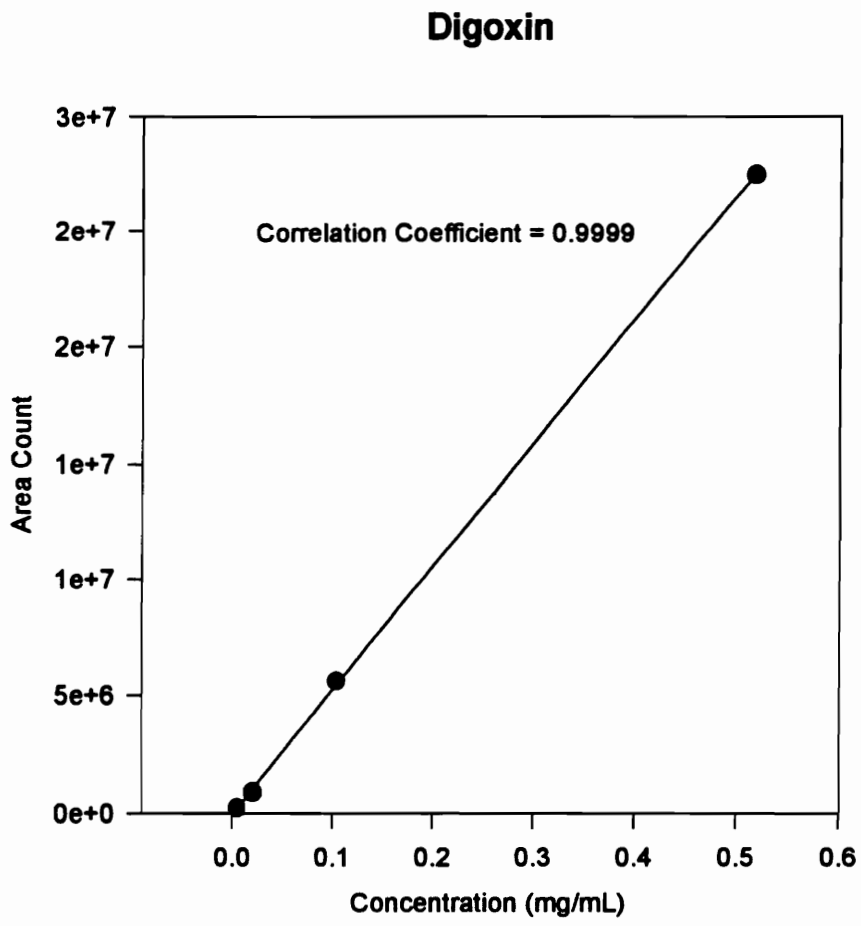


Figure 27. External calibration curve employed for the quantitation of digoxin using HPLC with UV detection at 218nm.

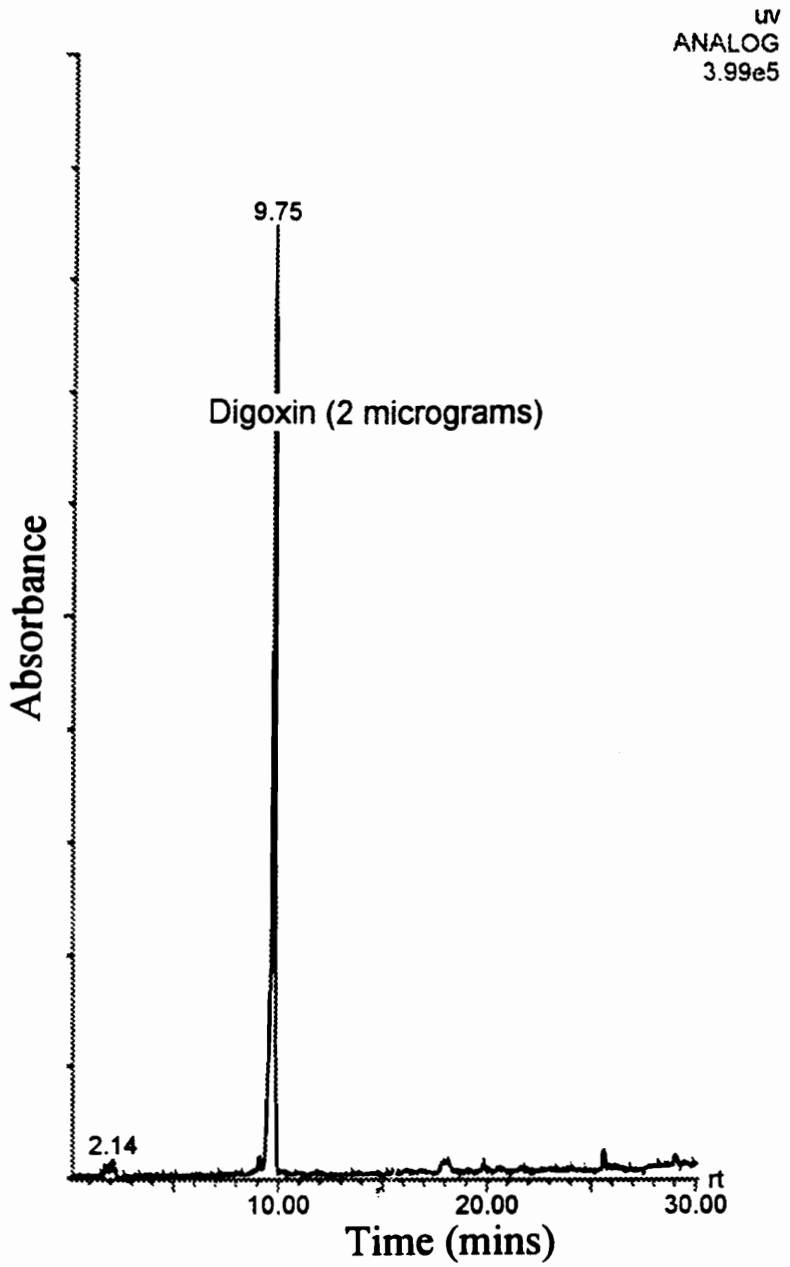


Figure 28. HPLC chromatographic trace of digoxin at 218 nm.

RESULTS AND DISCUSSION

Conventional Liquid Extraction

Prior to SFE of the leaf matrix, the conventional liquid extraction method which involves soaking the *D. lanata* leaf in a mixture of water/ethanol (80/20) for 24 hours, followed by a liquid extraction of the supernatant with chloroform, was performed in order to establish a 100% value to which subsequent SFE recoveries can be compared. The method used was developed by Paul Cobb in 1976 (40). Using this conventional liquid extraction procedure, digoxin was found experimentally to be present in the leaf at 0.25% by weight with a 14% relative standard deviation. A typical HPLC chromatographic trace of the conventional liquid extract is shown in **Figure 29.**

The SFE work to follow is divided into two sections for clarity. The first section, entitled *SFE of the Leaf* describes attempts to extract the as-received leaf which has not been soaked to promote the conversion of lanatoside C to acetyldigoxin, and leaf which has been soaked. The second section entitled *SFE of the Liquid Supernatant from the Soak Step* involves the extraction of the liquid portion of the maceration step in which the leaf has been soaked. **Figure 30** shows more clearly the matrices which are extracted for each of the methods covered in this chapter.

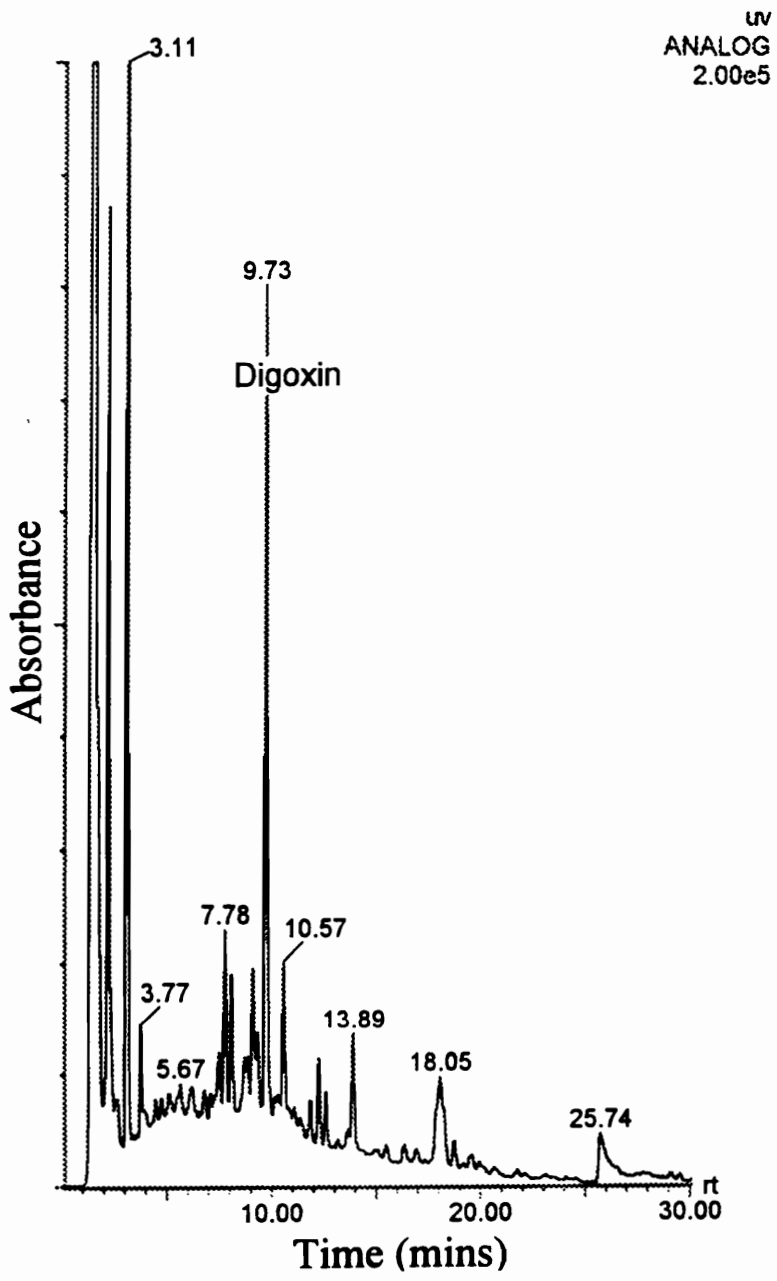


Figure 29. HPLC-UV (218nm) chromatogram of the conventional liquid extract of the *D. lanata* supernatant from the soak step.

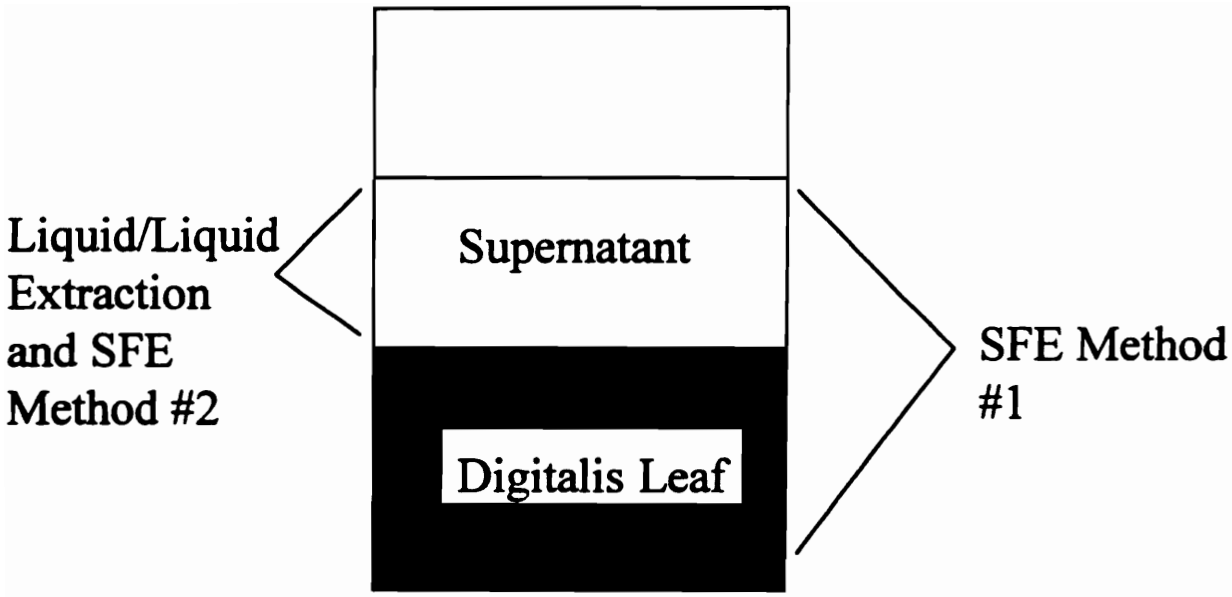


Figure 30. Representation showing what is extracted for the conventional liquid extraction, as well as the two SFE methods discussed.

SFE of the Leaf

All efforts to extract the as-received, dried *D. lanata* leaf yielded only trace quantities of the desired compounds. This result was expected as it was known that the soak step is required to activate the enzyme, digilanidase, which converts the natural precursor to acetyldigoxin and digoxin.

Several preliminary extractions of the wet leaf matrix (after the soaking step) were performed. It was discovered that any residual water left within the leaf from the soaking step resulted in low extraction recoveries. The water in this case is believed to hinder the extraction of the digoxin as the moderately polar digoxin partitions into the liquid H_2O medium to a greater extent as opposed to the extracting CO_2/methanol medium. In addition, large amounts of liquid solvent in the vessel were believed to contribute to trapping problems. Therefore, following the soak step in which 10 grams of the leaf was allowed to sit in approximately 300 mL of water/ethyl alcohol (80/20, v/v) for 24 hours, the leaf was lyophilized at -60°C and 128 mtorr, in order to remove any water and alcohol prior to supercritical fluid extraction. This freeze-drying step of the wet leaf resulted in improved extraction recoveries, therefore each extraction from this point involved freeze-drying the previously soaked leaf.

A high density was chosen as a starting point for the extraction of the freeze-dried (soaked) leaf since this gives the fluid its greatest solvating power, and the optimized trapping conditions found in the trapping study were used. A modifier amount of 20% by

volume was chosen as an initial starting amount to test for feasibility. The freeze-dried leaf (200mgs) was loaded into a 7 mL extraction vessel and Celite was placed at the exit end of the vessel to prevent frit plugging and system contamination. The leaf was then extracted for a 15 minute static period followed by a 45 minute dynamic period with 20% methanol-modified (mixed in-line) carbon dioxide at 0.95 g/ml (383 bar, 40°C) at a liquid flow of 1.0 mL/min. The extracted materials were trapped on stainless steel beads at a temperature of 80°C to prevent liquid methanol condensation. The restrictor temperature was set at 60°C. The trapped materials were then rinsed from the stainless steel with 4.5 mL of methanol with the restrictor set at 45°C and the trap at 25°C. Digoxin was recovered at 0.11% relative to the conventional extraction method eluded to earlier. A typical LC chromatogram of the SF extract of the leaf both before and after pH adjustment is shown in **Figure 31** showing the conversion of acetyldigoxin to digoxin.

This same extraction method was repeated immediately on the raffeinate (previously extracted leaf) and a cumulative recovery of 0.20% of digoxin was achieved. The extraction was immediately repeated a third time on the extracted leaf with very little additional recovery. The extracted raffeinate was then allowed to sit at room temperature for approximately 3 days while still wet with methanol from the third extraction. It was then extracted for a fourth time with the same extraction conditions listed above. Surprisingly, another 0.06% of digoxin was recovered. This resulted in cumulative recoveries for the four extractions of 0.26%, approximately the same amount of digoxin recoverable with the liquid extraction method.

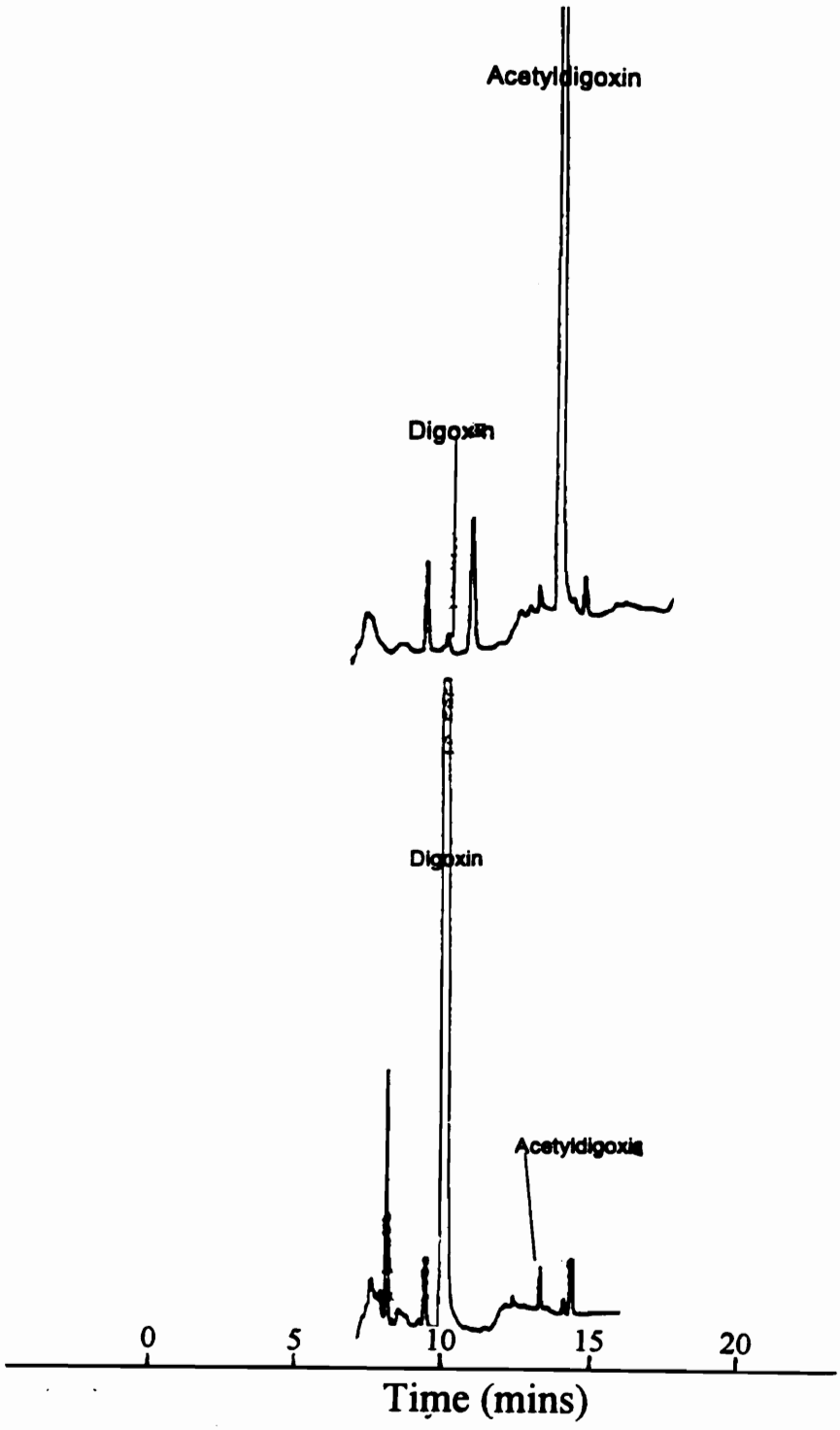


Figure 31. HPLC chromatogram of SF extract of the macerated, lyophilized leaf shown before (top) and after (bottom) base hydrolysis of the acetyldigoxin.

Several factors seemed to be important here. First, the initial stage of the extraction seems to be limited largely by solubility and to a smaller degree by analyte/matrix active site interaction. This is perhaps not as surprising as initially believed since the soak step prior to SFE probably allowed for the analyte/matrix bonds to be broken consequently allowing the analytes to be released to the leaf surface making them available for removal via the supercritical fluid. The fact that approximately 25% more analyte was recovered after allowing the previously extracted, wetted leaf to sit for an extended period of time, however, seemed to indicate a fraction of the digoxin is diffusion-limited. Therefore, it seemed reasonable that the two areas of the extraction which needed to be investigated were modifier amount, for optimized solubility and suppression of matrix effects, and extraction temperature, for optimized diffusion.

Modifier Amount and Method of Introduction

In-Line Addition and Amount

The effect of modifier on solvating power is well known; however, modifiers also play other very important roles especially for matrices where the analyte is bound through chemisorption and/or physisorption. Modifier, while increasing the solvating power of the fluid, also helps cover any active sites on the matrix surface, thereby displacing the analyte to be extracted.

Another advantage realized by employing a polar liquid modifier such as water or methanol is the swelling of the matrix which occurs, thereby increasing the amount of surface area accessible to the SF mixture. It has been shown that solid matrix materials exhibited a higher degree of swelling when a liquid was used as the swelling agent as opposed to when a gas or supercritical fluid was used (43). As a general rule, it was observed that the swelling power of a liquid decreased as the dielectric constant of the liquid decreased. This correlation held true in a pea leaf extraction as water proved to be the most effective swelling agent which was employed. However, for our work, water has been shown to have a detrimental effect on SFE efficiencies.

Modifier can be introduced either into the CO_2 (in-line) or the matrix (off-line). To study the effect of in-line addition, methanol amounts of 10, 15, 20, and 25 percent by volume were tested with all other conditions remaining the same as in the previous experiments. The leaf was singly extracted for 15 minutes in the static mode, followed by 45 minutes in the dynamic mode. A plot of percent recovery versus in-line modifier amount can be seen in **Figure 32**. As can be observed, the percent recovery increases dramatically from 10 to 20%, but begins to decrease after 20%. This trend can be explained by the fact that 20% methanol modified-carbon dioxide is sufficient to displace and solubilize the amount of free digoxin and acetyldigoxin. Amounts less than 20% modifier lack sufficient solvating power to remove these components. While recoveries for this technique at the given conditions are not as high as the conventional liquid extraction method, the precision of the technique is much improved as relative

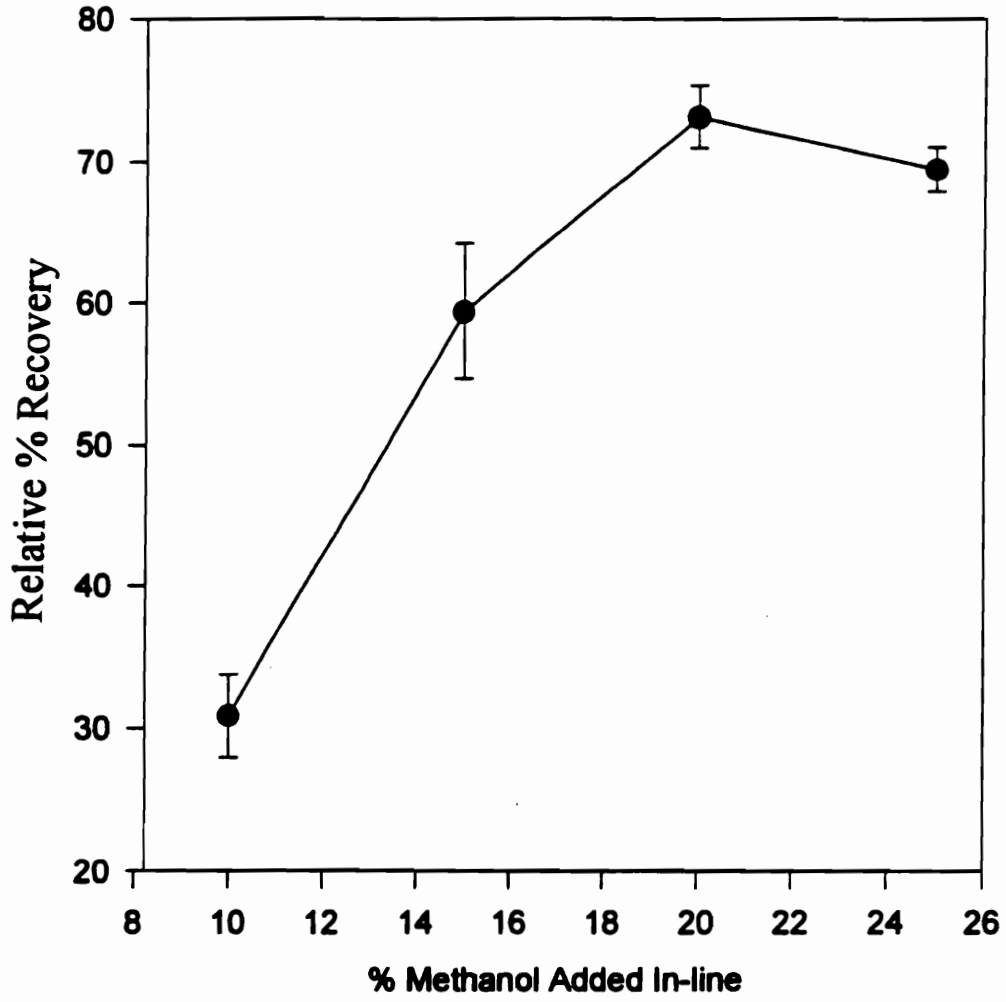


Figure 32. Plot of percent recovery of digoxin versus the amount of methanol modifier added in-line. Pressure: 383 bar; temperature: 40°C; liquid flow: 1 mL/min; time: 15 minute static, 45 minute dynamic; trap: stainless steel beads at 80°C; restrictor: 60°C. Extractions were performed in triplicate.

standard deviations for the SFE technique are less than 5%, while relative standard deviations for the conventional liquid extraction technique are greater than 10%.

Spike Addition and Amount

Since the majority of the initial digoxin recovered was believed to be solubility limited, it was theorized that perhaps by spiking a specific quantity of liquid methanol into the vessel prior to extraction, digoxin could be extracted much quicker than was realized with the 0.2 mL of methanol/minute used in the in-line extraction. The effect of four different spike volumes on percent recovery of digoxin from the soaked, dried leaf was investigated. Methanol modifier (0, 0.5, 1.0, and 1.5 mL) was spiked onto the leaf just prior to extraction with the same conditions as in the initial experiment. As can be seen in **Figure 33**, increased amounts of spiked methanol in the extraction vessel prior to SFE resulted in a decreased recovery of digoxin. This trend is not surprising as it further supports the conclusions of the trapping study (Chapter 3). At high spike modifier volumes, the trapping efficiency is decreased due to aerosol formation and modifier trap elution. This trend could also be theoretically explained by the fact that at higher ratios of liquid modifier a loss in diffusion could occur as the overall fluid becomes more liquid-like

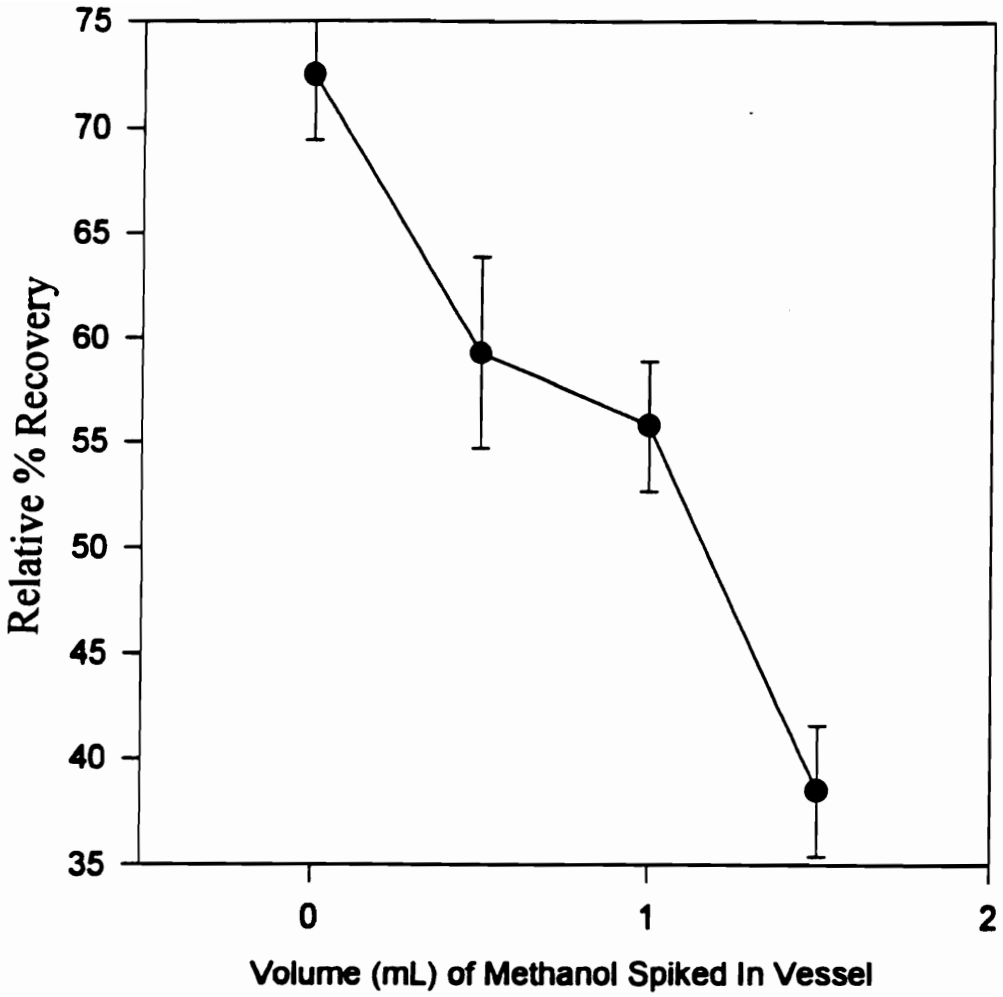


Figure 33. Plot of percent recovery of digoxin as a function of the amount of methanol modifier added via spiking directly into the vessel. Pressure: 383 bar; temperature: 40°C; liquid flow: 1 mL/min; time: 15 minute static, 45 minute dynamic; trap: stainless steel beads at 80°C; restrictor: 60°C. Extractions were performed in triplicate.

and less SF-like, but as a result of our previous trapping study it was believed that the majority of the problem was stemming from trapping inefficiencies.

It should be noted here that there appears to be a strong correlation between extraction reproducibility and trapping inefficiency. At high modifier levels and conditions where trapping problems are known to be prevalent, the precision of the SFE technique suffers greatly yielding high relative standard deviations. However, as trapping conditions are optimized so as to maximize the effectiveness of the trapping setup, the precision of the technique improves dramatically. These high relative standard deviations appear to be indicative of trapping problems, and therefore should be used to predict whether analytes are being trapped effectively.

At this point, density, flow rate, trapping parameters, modifier amount and method of modifier introduction, and trap rinse parameters had been optimized. The only parameter yet to be optimized was the temperature of the extraction.

Temperature Effects: Diffusion

It is known that while a temperature increase at fixed pressure results in a decreased density in the SF, the diffusivity increases and the viscosity decreases. In many cases, especially in leaf and soil matrices where a portion of the analytes must first undergo a diffusion-limited process, temperature increases may have a positive effect on extraction efficiencies. While a loss in solvating power is realized as the temperature is

raised, the presence of the large amount of polar modifier (20% volume) should act to keep the solvating power at an acceptable level.

Three different temperatures were investigated and their effect on percent recovery noted. As is seen in **Figure 34**, even though the CO_2 density decreases as the temperature increases, an increase in the amount of digoxin recovered increases from 77% to approximately 100% recovery (relative to the 0.25% recovered with the current liquid extraction method) on going from 40°C to 100°C. Even though the extraction fluid at these conditions is slightly subcritical (below the critical temperature) and our ability to vary solvating power of the fluid has diminished, the advantages of high diffusion and low viscosity remain. In addition, the technique under these conditions yields good precision with relative standard deviations less than 5%. Again, the extraction conditions remained the same as previously outlined with the exception of oven temperature and elimination of the static step. Therefore, 100% recovery of digoxin relative to the liquid extraction method was achieved in 45 minutes with 20% methanol-modified carbon dioxide at an extraction temperature of 100°C and a pressure of 383 bar. This extraction was exhaustive as the method was repeated once more on the raffeinate and no additional digoxin was detected in the assay.

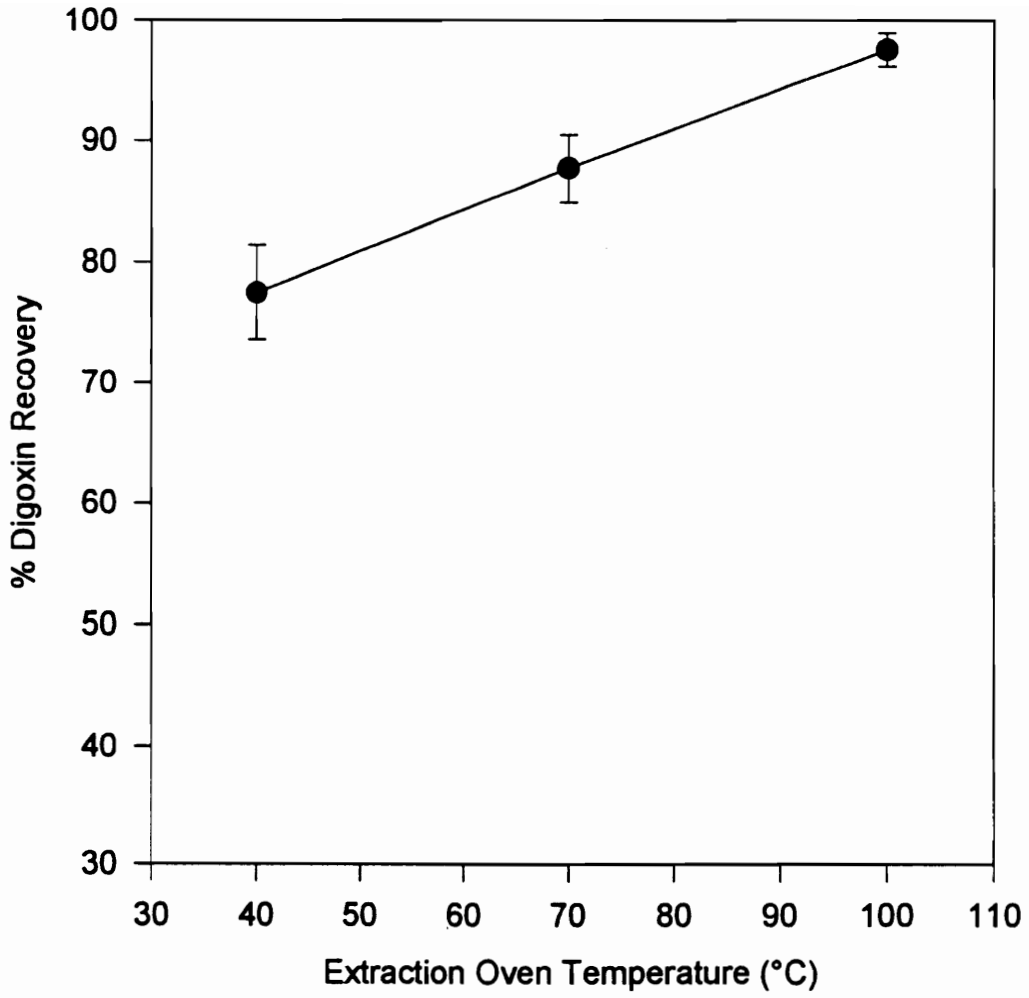


Figure 34. Plot of the percent recovery of digoxin as a function of the extraction temperature. Pressure: 383 bar; liquid flow: 1 mL/min; fluid: 20% methanol-modified CO_2; time: 15 minute static, 45 minute dynamic; trap: stainless steel beads at 80°C; restrictor: 60°C. Extractions were performed in triplicate.

Extraction Reproducibility

With all parameters optimized, five replicate extractions were performed with two different type traps, stainless steel beads, an inert solid, and octadecylsilica, a solid sorbent chromatographic stationary phase which is believed to have better trapping power than stainless steel.

Extraction conditions employed in this reproducibility study were: sample size: 200 mgs; pressure: 383 bar; temperature: 100°C; liquid flow: 1.0 mL/min; fluid: 20% methanol-modified carbon dioxide; trap temperature: 80°C; extraction time: 45 minutes; thimble volumes: 8.3, trap: C-18 or stainless steel beads at 80°C; rinse solvent: 4.5 mLs of methanol; trap temperature during rinse: 25°C.

The stainless steel trap resulted in recoveries of 105% with a 9% RSD relative to the liquid extraction method, the C-18 trap resulted in 95% recoveries with a 3% RSD. While the C-18 trap method exhibits a tighter recovery range and a cleaner extract than the stainless steel beads, the stainless steel trap tends to resist plugging to a better extent. Either of the traps were sufficient, and the one employed depends on whether one wants to sacrifice minimized trap plugging over extended periods of time with the stainless steel or lower RSD's with the C-18. For clarity, the entire extraction procedure for the direct SFE of the leaf has been outlined below.

An HPLC trace of the extract achieved with direct SFE of the soaked, lyophilized leaf is shown in **Figure 35** and compared with that of a chromatogram from the conventional liquid extraction of the supernate method. As might have been expected, the liquid extract of the supernatant is much cleaner than the SFE trace. This difference can be attributed to the matrix which is being extracted in the two cases. For the liquid extraction, the supernatant liquid is being extracted, however in the SFE case, the soaked leaf plus dried supernatant is being extracted. The SFE of the leaf was attempted first in our work as this presented the easiest and least complex of the possible pathways for isolation of the digoxin via SFE. However, since the SFE extract is considerably more complex than that of the liquid extract, another SFE method was developed.

Procedure for the SFE of the Soaked, Lyophilized Leaf

A. Weigh 10.0 grams of milled *Digitalis* leaf into a 500 mL round bottom flask.

B. Prepare 500 mL of 20% aqueous solution of ethyl alcohol by adding 100 mL of ethanol to 400 mLs of warm tap water.

C. Pour enough of the aqueous solution of alcohol into the 500 mL round bottom to saturate the 10 grams of Digitalis leaf. (Approximately 200 mL)

D. Cap the flask and allow to sit at room temperature for 24 hours while stirring once every 5-6 hours with a glass stirring rod to ensure that all of the leaf has sufficient contact time with the aqueous alcoholic solution.

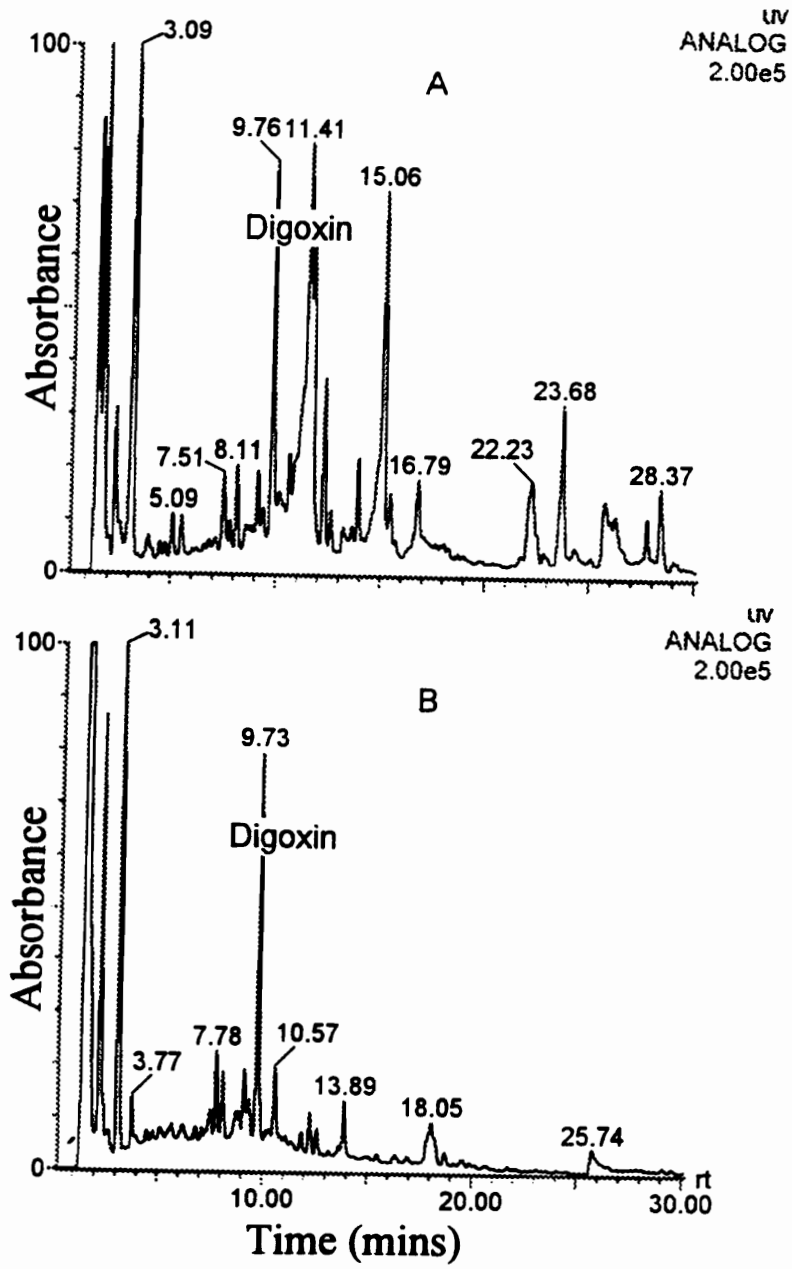


Figure 35. HPLC chromatogram of the extract achieved via SFE of the macerated, lyophilized leaf (A) compared with that of one achieved via the conventional liquid extraction of the supernate from the soaked leaf method (B).

.E. After 24 hours, place the 500 mL flask on a Lyophilizer for 10 hours to remove both the water and alcohol from the leaf.

F. Weigh 200 mg of the soaked/dried leaf into a 7 mL SFE extraction vessel which has been filled approximately half full with Ottawa Sand standard.

G. After adding 200.0 mg of the leaf to the extraction vessel, fill the remaining vessel volume with the Ottawa Sand standard with leaving approximately 5-10% of the vessel volume empty for swelling and expansion of the leaf.

H. Extract the leaf for 45 minutes with 20% methanol modified-CO_2 at a pressure of 383 bar and an oven temperature of 100°C at a liquid flow rate of 1 mL/min. The trap consists of either stainless steel beads or octadecylsilica stationary phase held at 80°C and the restrictor temperature is set at 60°C during the extraction. Following the extraction the restrictor and trap are cooled to 25°C and rinsed with 1.5 mLs of methanol into 3 separate vials, resulting in a total rinse volume of 4.5 mLs.

I. The extract solution is then transferred to a large mouth 20 mL vial for pH adjustment.

J. The pH of the extract is raised to 12.0 with dilute NaOH and held for 15 minutes. The pH is then lowered to 6.0 with dilute HCl. The resulting solution is then diluted to a total volume of 10 mLs with methanol and assayed with HPLC-UV.

SFE of the Liquid Supernatant from the Soak Step

While SFE of the leaf yielded comparable recoveries with that of the conventional liquid extraction method, reduced the time required, and eliminated the chlorinated solvents, the extracts achieved are more complex when compared with the conventional extraction method as seen in the HPLC-UV chromatogram in **Figure 35.**

Therefore, another SFE method was investigated which would allow the same advantages as the prior SFE procedure, while hopefully allowing a comparably clean extract relative to the liquid extraction method. The optimum extraction parameters employed in the direct SFE of the leaf were again used for this procedure as well. However, rather than freeze-drying the *D. lanata* leaf and extracting with SFE, the liquid supernatant was filtered from the leaf following the soak step, rotary evaporated down to a volume of 3 mLs, and spiked onto Celite® within the extraction vessel which filled approximately 90% its volume. An HPLC chromatographic trace compared with the conventional liquid extraction trace can be seen in **Figure 36.** As can be seen, the second SFE method which involved the extraction of only the supernatant from the solid-liquid extraction (soak) step was much cleaner than the first SFE method and was comparable to the chromatographic trace achieved with the liquid extraction technique. **Figure 37** is a flow diagram which shows the steps involved in the three different extraction methods. **Table 1** shows a comparison of the two SFE methods

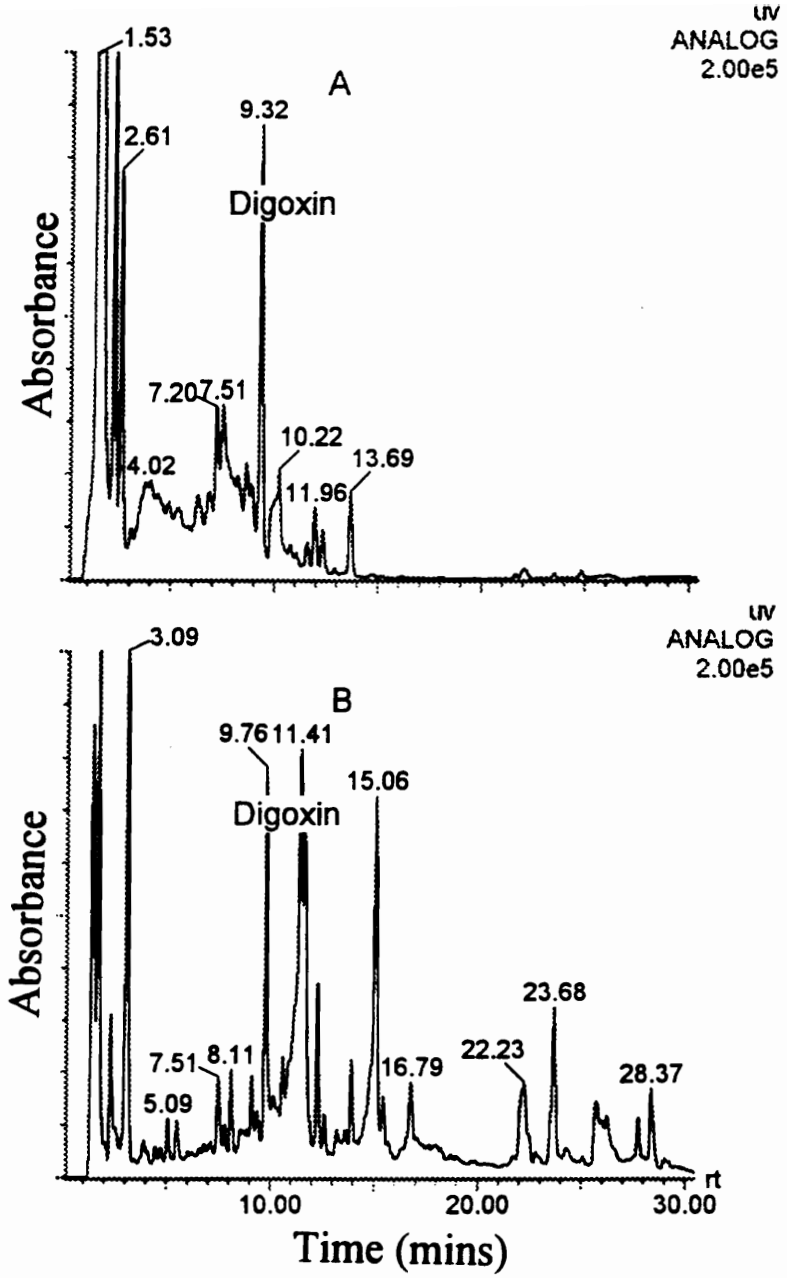


Figure 36. HPLC chromatogram obtained via the second SFE method (A) which involved extracting the supernatant from the soak step from a Celite bed. The chromatogram (B) from the initial SFE method #1 is also shown for comparison purposes.

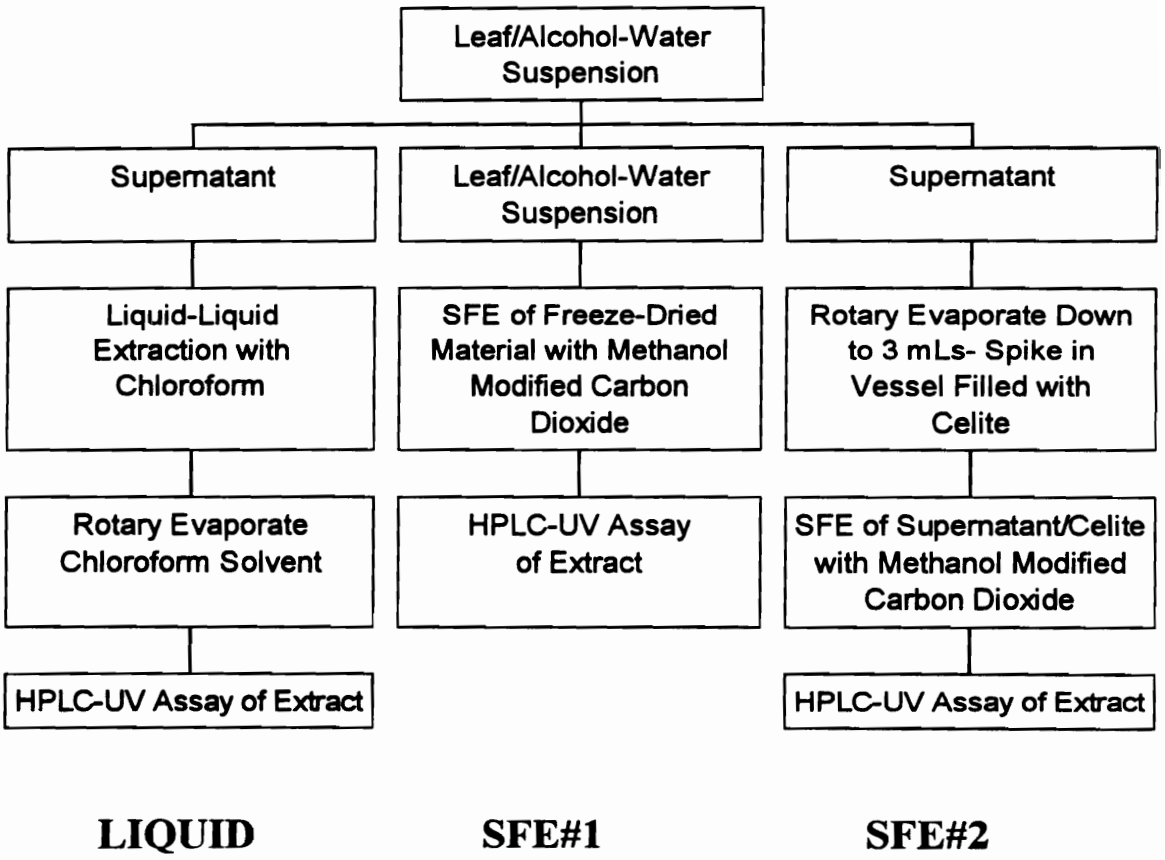


Figure 37. Flow diagram of the steps involved for the three different extraction methods investigated.

Table 1. Comparison of the three extraction methods investigated.

	LIQUID (10g leaf)	SFE#1 (200mg leaf)	SFE#2 (200mg leaf)
Time	**20 hrs**	**7 hrs**	**6 hrs**
Total Liquid Solvent Volume	**315 mL**	**75 mL**	**150 mL**
Chlorinated Solvent Volume	**270 mL**	**0 mL**	**0 mL**
Automation	**No**	**Partial**	**Partial**
Sample Handling	**3 hrs**	**0.25 hr**	**1.5 hrs**
Weight % Recovered	**0.25% (14% RSD) n=3**	**0.24% (3.8% RSD) n=3**	**0.27% (5.2% RSD) n=3**

and the currently employed liquid extraction method. As can be seen, the SFE methods far exceed the conventional method in many areas.

SUMMARY

The objective of this project was to replace the current liquid-liquid extraction method with an SFE technique in order to eliminate large amounts of hazardous liquids and associated waste disposal costs, and decrease the extraction time involved for the isolation of digoxin. This goal was achieved with the first SFE method which involved the extraction of the freeze-dried leaf and resulted in equivalent recoveries of digoxin from the leaf relative to the current extraction method. The technique of lyophylization worked well at setting up a favorable partition of digoxin into the SF. Temperature was shown to have a dramatic effect on extraction efficiency as the extraction of a portion of the leaf was proven to be diffusion limited. Two traps were employed and both performed well under the optimized trapping conditions. SFE of the freeze-dried leaf resulted in an HPLC trace which was much more complex when compared with that of the liquid extraction method. Therefore, the SFE of the supernatant was carried out. This method retained the same advantages of the first SFE method, while resulting in a cleaner extract. SFE of the digitalis lanata leaf as a way of isolating the polar digoxin and acetyldigoxin has proven to

be a reproducible sample preparation technique which yields comparable recoveries relative to the conventional extraction method.

Chapter V

An Investigation of Three Strategies To Improve Selectivity in Supercritical Fluid Extraction

INTRODUCTION

It is widely known that the solvating strength of a nonpolar fluid such as carbon dioxide can be increased through the addition of a polar liquid thereby increasing the extraction recoveries of polar analytes. However, as the solvating strength of a medium increases, there is a corresponding decrease in the extraction selectivity. As shown in Chapter IV (**Figure 35**), while increasing the recovery of digoxin and acetyldigoxin from the *D. lanata* leaf, methanol modifier acted to solubilize and remove unwanted coextractable materials which contributed to the complexity of the assay.

The focus of this project was to explore several techniques which are inclusive to the SFE process (i.e. do not require additional techniques) that may lead to a reduction in the amount of unwanted coextractable materials present in an extract solution. Coextractives can sometimes coelute with peaks of interest thereby causing an inability to perform accurate quantitative measurements. In addition, "dirty" extracts often translate into additional sample cleanup steps such as solid phase

extraction and increased costs due to more frequent replacement of the chromatography columns which are employed.

This chapter investigates and compares three alternatives available to the SFE user which may possibly improve selectivity. The success of each strategy; 1) alternative fluids, 2) pre-extraction fractionation, and 3) selective trap rinsing, is demonstrated for the extraction of digoxin and acetyldigoxin from the soaked, freeze-dried leaf matrix, as this system is well understood. Each technique will be presented separately and conclusions drawn concerning each ones ability to reduce the amount of unwanted materials present in the extract solution. The success of each technique was measured using reversed-phase liquid chromatography with ultraviolet detection at 218 nm.

INTRODUCTION-*ALTERNATIVE FLUIDS*

The tremendous popularity of carbon dioxide as a medium for supercritical fluid extraction can be attributed to its low toxicity, chemical inertness, easily accessible critical parameters, lack of flammability, and low cost. Numerous applications in the literature exist, however, regarding the use of nonideal solvents such as supercritical water, ammonia, nitrous oxide, pentane, and sulfur hexafluoride to name a few (44-49).

Recently, there have been a number of applications involving the use of the fluorinated hydrocarbon trifluoromethane (CHF_3) as an extraction medium in SFE, and an interest also has developed for the possibility of using 1,1,1,2-tetrafluoroethane (CH_2FCF_3) as well. Not only do these solvents possess relatively low critical parameters and lack flammability, but they also possess significant dipole moments as opposed to CO_2 which has no dipole moment. Various fundamental studies and applications in the literature deal with these fluorinated fluids. In 1980, Stahl et al. found that for a group of nitrogen-containing alkaloids, CHF_3 was a better solvent than either CO_2 or N_2O (50). King and coworkers reported that CHF_3 was able to extract comparable amounts of pesticides from chicken tissue relative to CO_2, while exhibiting a 100 fold decrease in the amount of unwanted coextractable fat removed when compared with CO_2 (51). Ashraf-Khorassani, Combs, and Taylor have used pure and methanol-modified CHF_3 for the extraction of polar sulfonamides from a variety of food-based matrices. CHF_3 extracted comparable or greater amounts of polar analytes of interest while extracting less of the nonpolar fatty material, relative to CO_2 (52). Also, Howard and coworkers reported higher recoveries with CHF_3 compared to CO_2 for the extraction of polar sulfonyl ureas, yet the trend was reversed for the extraction of the less polar PAHs with CO_2 performing as the better solvent (53). Blackwell and Cantrell have reported on the characterization of 1,1,1,2-tetrafluoroethane as an eluent in packed column SF chromatography, and their results show a dramatic decrease in the capacity factor (k') of a series of polar naphthalene

derivatives, when compared to both pure and 5% methanol-modified carbon dioxide. Consequently, interest in this fluid as an extraction medium has been sparked as well (54).

The aforementioned results all support the idea that fluoroform is a more selective solvent than CO_2 for the extraction of polar compounds. Moreover, this fluorinated fluid shows an inability to extract the lower polarity compounds. It is therefore conjectured that perhaps CHF_3 and CH_2FCF_3 will act as a more selective SFE solvent by extracting less low polarity materials from the leaf such as terpenes and oils thereby yielding a cleaner extract. In addition to the selectivity issue, the use of the polar fluid may help achieve an indirect benefit by reducing the amount of polar methanol modifier needed to achieve 100% recovery as the result of the greater degree of polar character both CHF_3 and CH_2FCF_3 possess. This reduction in the amount of liquid modifier employed would not only decrease the risk of trapping problems and reduce the costs of the extraction, but should also aid in chemical selectivity as well.

EXPERIMENTAL-*ALTERNATIVE FLUIDS*

Supercritical Fluid Extraction and Quantitation

All extractions for this chapter were performed on the AutoPrep 44 (Suprex, Pittsburgh, PA) which allows 44 extractions to be performed in sequence. This extractor pumps the fluid via a dual head reciprocating pump which requires that the fluid be in the liquid state prior to arrival at the pump heads. This is ensured as the fluid within the cylinder is compressed with helium headspace. The AP44 is capable of achieving an extraction temperature and pressure of 150°C and 500 atm, respectively. It employs an automated variable restrictor which allows decompression of the SF and regulates the back pressure to keep the fluid supercritical. In addition, this automated feature allows one to decouple the extraction pressure and flow rate. Extraction vessels (Keystone Scientific Inc., Bellefonte, PA) with a 5 mL volume were used for each extraction. SFE/SFC grade fluoroform and 1,1,1,2-tetrafluoroethane both with helium headspace (Air Products and Chemicals Inc., Allentown, PA) were used for all extractions. All modifier was HPLC grade and was obtained from EM Science (Gibbstown, NJ). All extractions used the same optimum conditions found within the *SFE of the Soaked, Lyophilized Leaf Extraction* portion of Chapter IV. The extraction conditions were: pressure: 400 atm, temperature: 100°C, liquid flow rate: 1 mL/min, time: 45 minutes, trap material: ODS, extraction trap temperature:

80°C, rinse trap temperature: 25°C, extraction restrictor temperature: 60°C, rinse restrictor temperature: 25°C, rinse solvent: 5 mLs methanol. The only variables for this experiment were fluid type and modifier percentage.

All SFE extracts in this chapter were assayed by HPLC with UV detection at 218nm. The same quantitative analysis method reported in Chapter IV was employed for each of these studies.

RESULTS AND DISCUSSION-*ALTERNATIVE FLUIDS*

Fluoroform

CHF_3, which has a high dipole moment of 1.65 debye, possesses a critical temperature and pressure of 26.3°C and 46.9 atmospheres, respectively. Its critical density is 0.52 g/mL compared with the critical density of CO_2, 0.47 g/mL. Since the extraction temperature and pressure are 100°C and 400 atmospheres, we know that the CHF_3 is supercritical with a calculated density of 0.917 g/mL. (55)

The macerated, freeze-dried leaf was extracted at the above conditions with fluoroform and 1,1,1,2- tetrafluoroethane and a graph of percent recovery of digoxin versus methanol-modifier percentage (volume %) for the fluids, CO_2,CHF_3, and CH_2FCF_3 was plotted (**Figure 38**). With 0% modifier, we see that

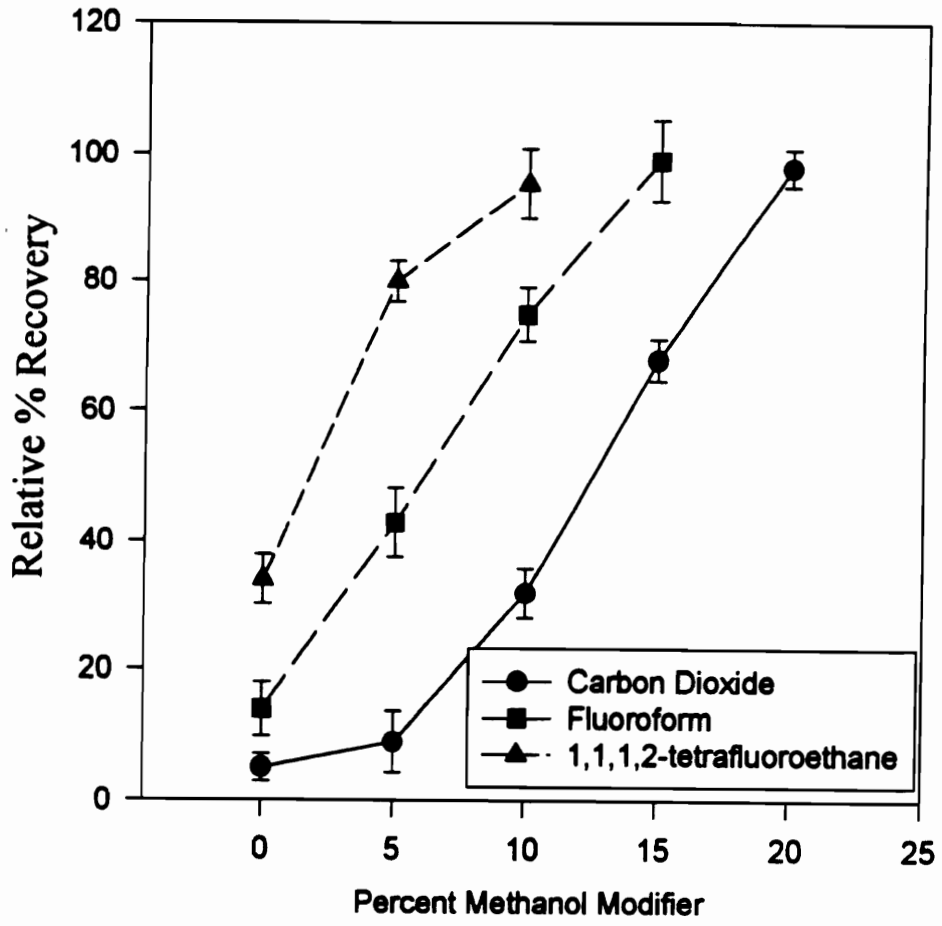


Figure 38. A comparison of the three fluids studied for the extraction of digoxin from the soaked, freeze-dried leaf. Extraction pressure: 400 atm, temperature: 100°C, flow rate: 1 mL/min, time: 45 minutes, extraction trap temperature: 80°C, rinse trap temperature: 25°C, trap material: octadecylsilica, rinse solvent: methanol, rinse temperature: 25°C.

CHF_3 is able to extract approximately 10% more digoxin. While it is easy to state that CHF_3 possesses a greater solvent strength than CO_2, this term is rather ambiguous and does not give a true representation of what is occurring at the molecular level. Solvating strength is related to a number of properties, but essentially arises from the ability of a solvent molecule or cluster of solvent molecules to interact, electrostatically, with the solute of interest. Many factors can contribute to this interaction, including, London-dispersion forces, dipole-induced dipole, ion-induced dipole, dipole-dipole, hydrogen bonding, and ion-dipole, in the order of increasing bond strength. A hydrogen bond is an electrostatic bond between a partially positive hydrogen atom and an electronegative atom, such as N or O, having lone pairs. CHF_3 contains a partially positive hydrogen atom, while the glycosides contain electronegative oxygen atoms therefore having the potential to form a hydrogen bond (C-H--O). Proof exists for the formation of the C-H--O hydrogen bond in solution as Jeng and Ault found that the strength of the bond was dependent on the basicity of the analyte interacting with the fluoroform (56).

Conversely, CHF_3 contains three electronegative fluorine atoms which have lone electron pairs, and the glycoside hydroxyl groups contain acidic hydrogens. Therefore, with this type of solvent-solute interaction, the formation of an O-H---F hydrogen bond seems feasible. Work by Johnston et al. has proven however that the CHF_3 molecule acts best as an electron acceptor, rather than an electron donator (57). We feel that in our case as well, fluoroform is able to interact with the glycoside

hydroxyl groups yielding the desired result: the dissolution of the glycoside compounds.

Additional evidence of hydrogen bonding has been reported by Blackwell and Schallinger as naphthalene with a hydroxyl group substituted in the 1- position exhibited a 50% decrease in the capacity factor (k') with CHF_3 compared to pure CO_2 (58). They attributed this observation to fluoroform's ability to interact via hydrogen bonding interaction. They concluded that other types of dipole interactions were less important, and that functional groups capable of interacting with CHF_3 through hydrogen bonding were affected to a greater extent than those which were polarizable, but unable to hydrogen bond. Work by Brennecke and coworkers further supports this claim as they observed through fluorescence spectroscopy that CHF_3 exhibited strong hydrogen bonding interaction with carbazole (59).

As can be seen, at equal levels of modifier, methanol-modified fluoroform is able to extract approximately 25% more digoxin than methanol-modified carbon dioxide at the same modifier percentage. CHF_3 is able to achieve 100% recovery of the digoxin compound relative to the conventional liquid extraction, yet requires only 15% methanol (vol %), 5% less than that of the nonpolar carbon dioxide. This improvement in recovery with methanol-modified CHF_3 relative to methanol-modified CO_2 has also been observed for the extraction of sulfonamides from chicken liver (52).

The use of CHF_3 not only results in the direct benefit of requiring less liquid modifier for quantitative recoveries of digoxin, but also results in a decreased amount

of liquid modifier cascading onto the trap per unit time, thereby decreasing the chance for inefficient trapping.

Figure 39 shows the HPLC-UV traces of the SF extract for pure CO_2 and CHF_3. As can be seen, the pure CHF_3 fluid results in a cleaner chromatogram relative to the pure CO_2 fluid in the later portion of the trace where the nonpolar compounds are known to elute. This further supports the theory that CO_2 seems to be more efficient at removing nonpolar analytes, such as the terpenes and oils found within the leaf matrix. It is believed that CHF_3, which is polar, is able to form hydrogen bonds with the polar compounds of the leaf, while its ability to extract lower polarity analytes is decreased due to the decreased solvent-solute interaction.

Figure 40 shows the HPLC-UV traces of the SF extract for the optimally modified fluids, 20% methanol-modified CO_2 and 15% methanol-modified CHF_3. As can be seen in the chromatograms for the modified fluids, the selectivity advantage realized for the pure fluid is lost upon the addition of large amounts of methanol modifier, as both fluids exhibit comparable chromatograms. It appears that the 5% decrease from 20% to 15% methanol is not sufficient to increase the selectivity enough

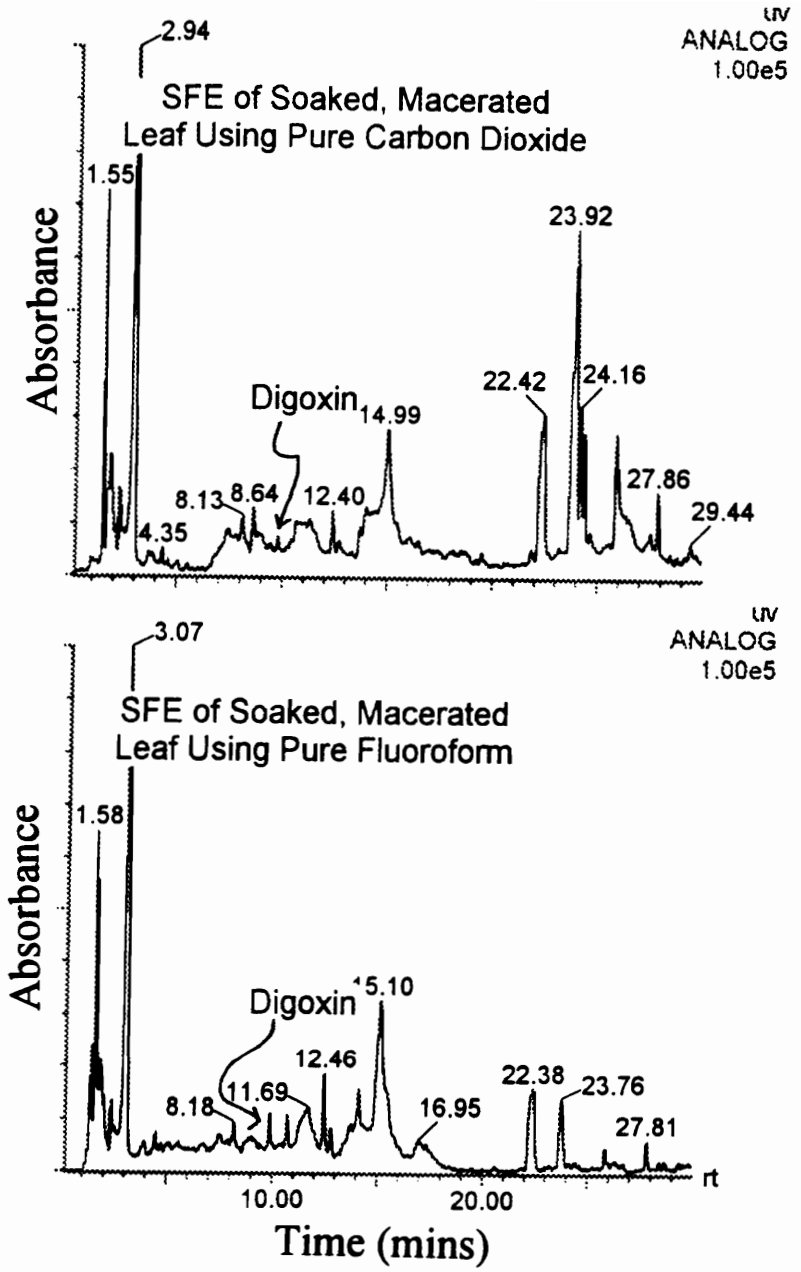


Figure 39. HPLC-UV traces at 218nm of the SF extract obtained with pure CO_2 and pure CHF_3 following base hydrolysis. Extraction pressure: 400 atm, temperature: 100°C, flow rate: 1 mL/min, time: 45 minutes, extraction trap temperature: 80°C, rinse trap temperature: 25°C, trap material: octadecylsilica, rinse solvent: methanol, rinse temperature: 25°C.

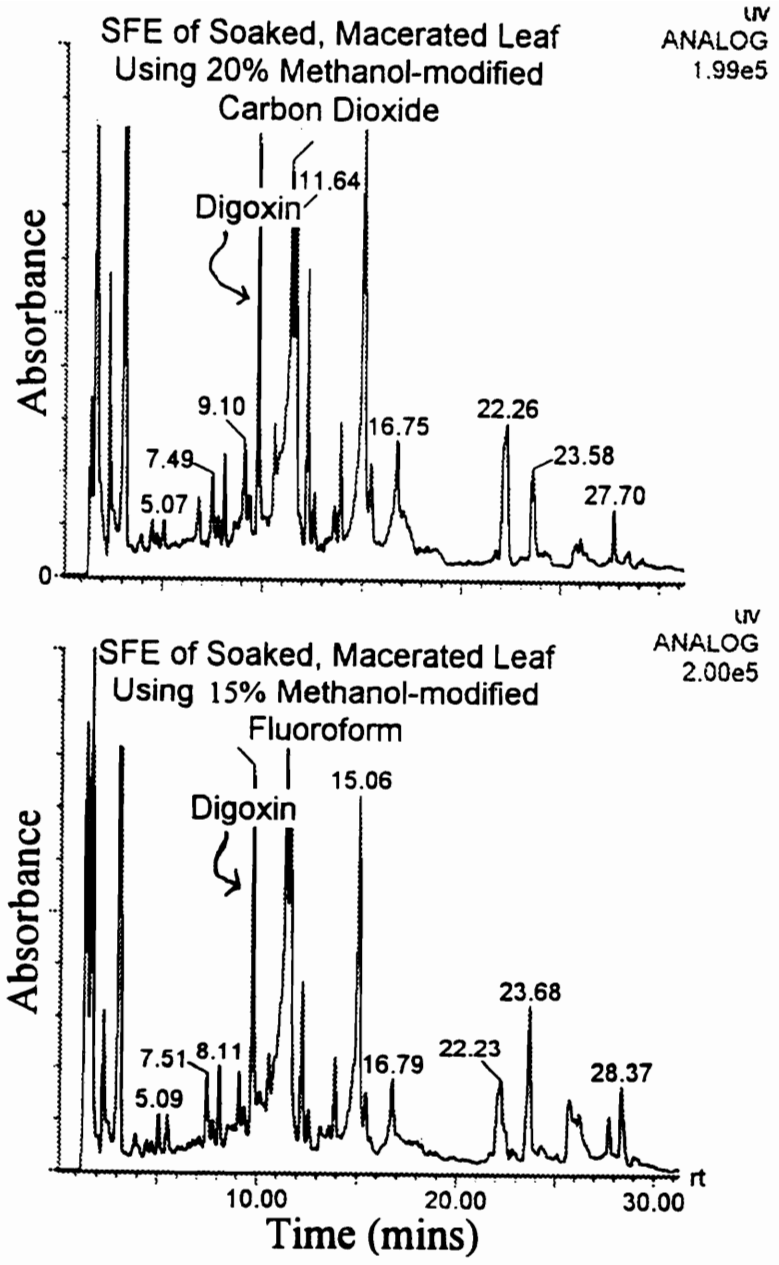


Figure 40. HPLC-UV traces at 218 nm of the SF extract obtained with 20% methanol-modified CO_2 and 15% methanol-modified CHF_3 following base hydrolysis. Extraction pressure: 400 atm, temperature: 100°C, flow rate: 1 mL/min, time: 45 minutes, extraction trap temperature: 80°C, rinse trap temperature: 25°C, trap material: octadecylsilica, rinse solvent: methanol, rinse temperature: 25°C.

to yield a cleaner extract. Therefore, at high levels of modifier, the only advantage of CHF_3 is the utilization of 5% less modifier with little or no differences in selectivity.

1,1,1,2-tetrafluoroethane

CH_2FCF_3 possesses a larger dipole (2.1 debye) than CHF_3 and is more polarizable. It has a low critical pressure of 40.07 atmospheres and a critical temperature of 101.1°C; its corresponding critical density is 0.46g/mL. Therefore, with extraction conditions of 400 atmospheres and 100°C, we are borderline sub/supercritical with a density of 0.94g/mL.

Again, the macerated, freeze-dried leaf was extracted at the same conditions with 1,1,1,2-tetrafluoroethane (**Figure 38**). The same trend for the higher molecular weight CH_2FCF_3 is observed as for the CHF_3 solvent. With no modifier present CH_2FCF_3 is able to extract an even greater percentage of the digoxin and acetyldigoxin compounds, than either CO_2 or CHF_3. This observation can be explained in much the same way as the increase in recovery on going from CO_2 to CHF_3. While CH_2FCF_3 is more polarizable and possesses a higher dipole moment than CHF_3, it also possesses a greater number of sites through which it can form hydrogen bonds (4 fluorines, 2 hydrogens). These physical properties effectively increase the amount, and therefore rate, at which the analytes of interest can be extracted.

For the modified fluids, CH_2FCF_3 is able to achieve 100% recovery of the digoxin compound at 10% methanol, half the volume of the liquid modifier required for the CO_2 extraction and 5% less modifier than is required for the extraction of digoxin with fluoroform. Again, this improvement in recovery for the CH_2FCF_3 can be explained through the increased solvent-solute interactions allowing CH_2FCF_3 to more effectively solubilize the digoxin compound, thereby resulting in less modifier required.

Figure 41 shows the HPLC-UV traces for the extract obtained with pure CH_2FCF_3 versus CO_2. Again the two different fluids exhibit different selectivities as the compounds extracted from the leaf differ in amount. The fluorinated fluid is cleaner than that of the pure CO_2 extract in the nonpolar region (later portion of the trace). However, the early eluting peaks (<15 minutes) which are believed to be the polar compounds of the leaf, exhibit higher recoveries than for the corresponding CO_2 trace. The compounds which elute later in the chromatography method from the reversed-phase column are believed to be from the class of terpene compounds which exhibit a lack of polar character and are extracted to a less degree with the fluorinated solvent.

Figure 42 shows the HPLC-UV traces for optimally modified CH_2FCF_3 and CO_2. A different trend is noted here with respect to the trend observed previously with the modified CHF_3/CO_2 comparison in **Figure 40**. The 10% methanol-modified CH_2FCF_3 not only results in comparable recoveries of digoxin with respect to the 20%

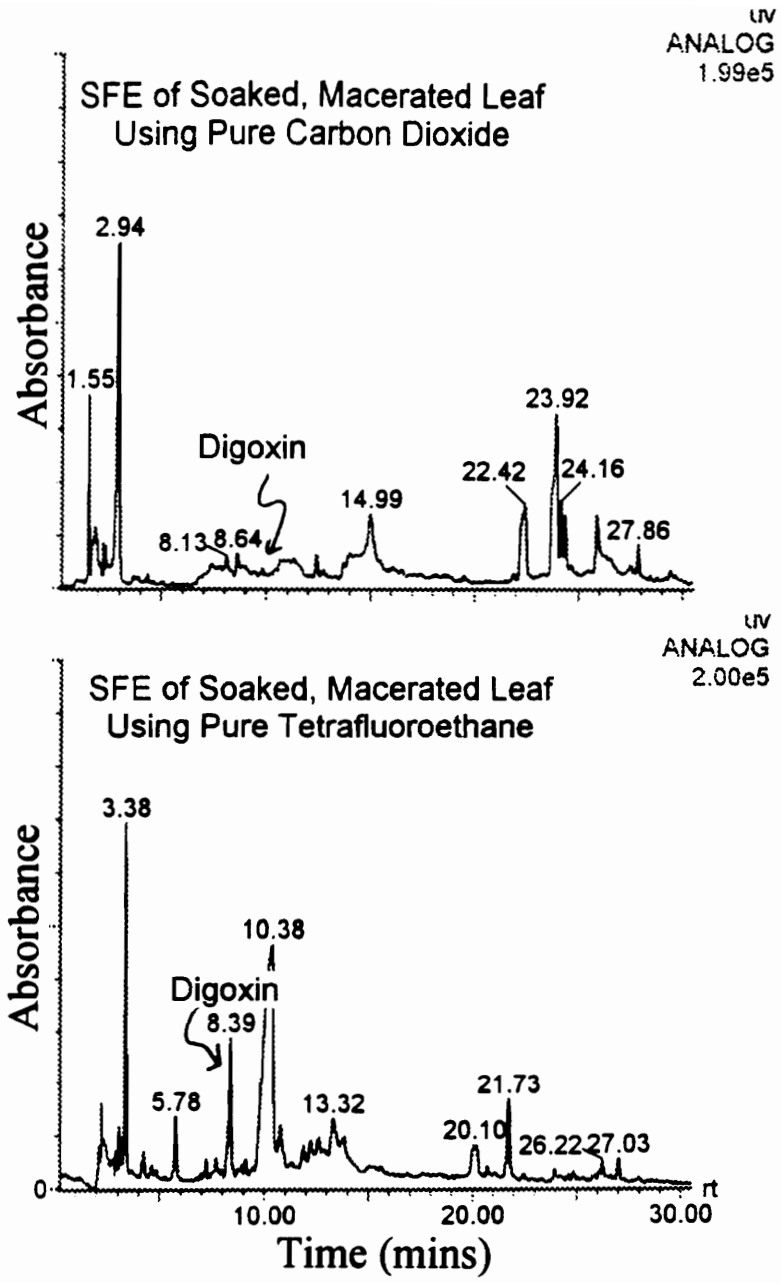


Figure 41. HPLC-UV traces at 218 nm of the extracts obtained using pure CO_2 and CH_2FCF_3 for the extraction of the soaked, freeze-dried leaf. Extraction pressure: 400 atm, temperature: 100°C, flow rate: 1 mL/min, time: 45 minutes, extraction trap temperature: 80°C, rinse trap temperature: 25°C, trap material: octadecylsilica, rinse solvent: methanol, rinse temperature: 25°C.

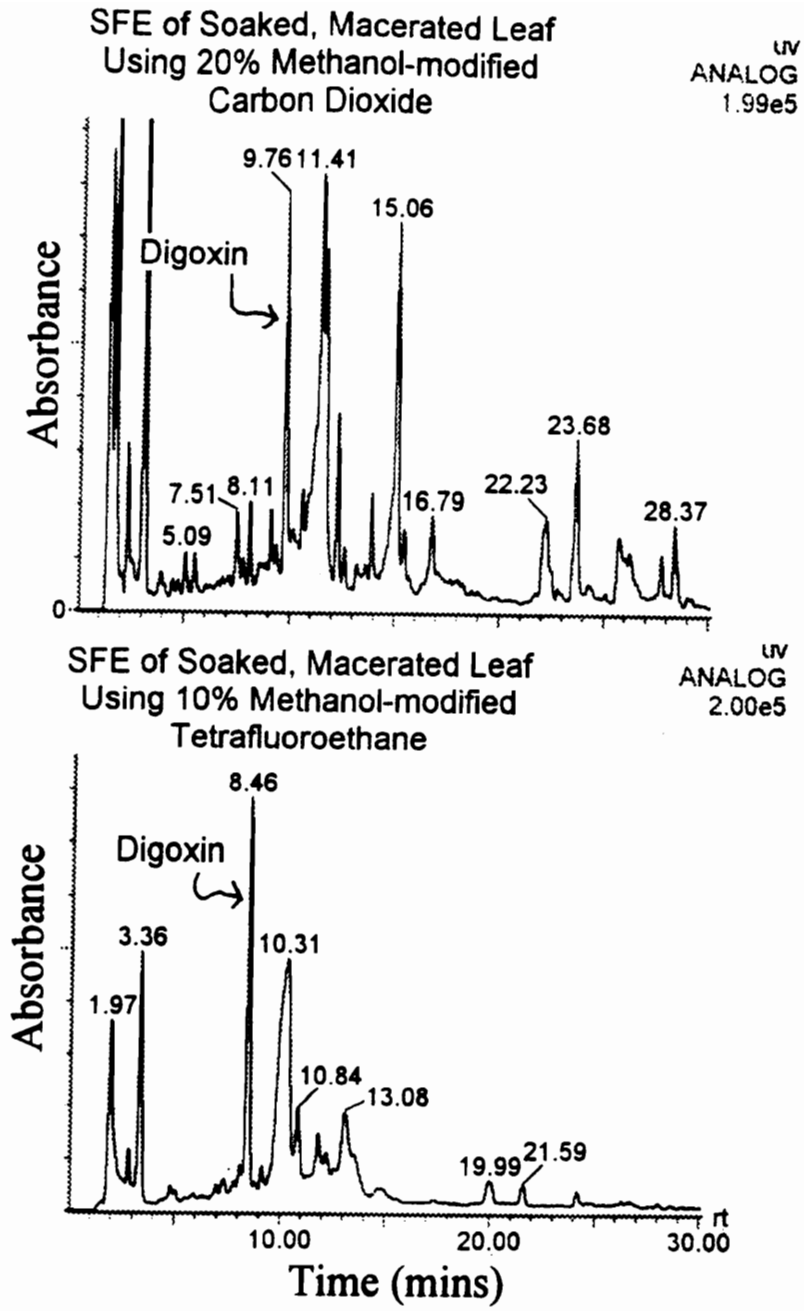


Figure 42. HPLC traces at 218 nm of the extracts obtained using 10% methanol-modified tetrafluoroethane and 20% methanol-modified CO_2 for the extraction of the soaked, freeze-dried leaf. Extraction pressure: 400 atm, temperature: 100°C, flow rate: 1 mL/min, time: 45 minutes, extraction trap temperature: 80°C, rinse trap temperature: 25°C, trap material: octadecylsilica, rinse solvent: methanol, rinse temperature: 25°C.

methanol-modified CO_2, but also yields an extract which is much cleaner. This increase in selectivity can be explained by the idea that the 10% decrease in the amount of methanol employed results in a more selective fluid as the lower percentage of methanol used relative to either the CHF_3 or CO_2 extraction is not enough to overcome the innate selectivity characteristic which these fluorinated fluids seem to exhibit.

SUMMARY-*ALTERNATIVE FLUIDS*

The objective of this section was to investigate fluoroform and 1,1,1,2-tetrafluoroethane as alternative fluids for the SFE of polar glycosides from a leaf matrix as a means of improving selectivity. It has been shown that by employing either of these fluorinated solvents, the amount of methanol required to recover equivalent amounts of digoxin with respect to carbon dioxide can be substantially reduced. Both pure fluorinated fluids exhibited different selectivities when compared to pure CO_2, due to the different mechanisms through which the solvent interacts with the solute. In addition, while 15% methanol-modified CHF_3 fluid exhibited a comparable selectivity when compared with the optimally methanol-modified CO_2 fluid, the 10% methanol-modified CH_2FCF_3 did exhibit greater selectivity and helped reduce the amount of unwanted coextracted materials present in the leaf extract.

INTRODUCTION- *PRE-EXTRACTION FRACTIONATION*

Since the fluid used for extraction is compressible, the solvating strength can be altered to achieve a medium (e.g. threshold density) which is selective for a particular class of compounds. For example, at the critical temperature of a fluid, small increases in pressure above the critical pressure result in significant changes in solvent density (solvating power). This phenomenon is very useful for producing successive extractions which can fractionate (selectively isolate) classes of compounds. Furthermore, the utilization of modifiers, which can have a profound effect on extraction efficiencies, helps to increase the possibilities for achieving better selectivity, as the solvating strength can be altered even further. Selective extractions can be obtained in which low polarity and high polarity materials can be fractionated.

In the following section, this property which is so unique to SFE, will be exploited in an attempt to regain some of the selectivity which can be lost as a result of employing the high levels of liquid methanol modifier. While it is known that the leaf contains a large number of nonpolar compounds which should have significant solubility in nonpolar CO_2, it has been proven (Chapter IV) that digoxin and acetyldigoxin have limited extractability in pure CO_2 (<3% relative to the liquid extraction method) (60). Consequently, it was proposed that the leaf be extracted in

an attempt to remove some of the interfering coextractable material prior to extracting the digoxin and acetyldigoxin with the 20% methanol-modified fluid.

RESULTS AND DISCUSSION-*PRE-EXTRACTION FRACTIONATION*

To ensure that all pre-extracted materials were exhaustively removed from the leaf, and since we were not interested in trapping the interfering components, the extraction flow rate was set at 5 mL/minute, the upper limit of the extraction system. The leaf (200mg) was pre-extracted with SF CO_2 at 400 atmospheres and 40°C. The extraction time was set at 30 minutes. Following the pre-extraction step, the leaf was extracted with the optimum conditions established in Chapter IV. **Figure 43** reveals the chromatographic trace achieved employing the pre-extraction experiment, and the complexity of the chromatogram is greatly reduced compared with the extraction in which the leaf was not pre-extracted, while still achieving similar amounts of the digoxin. Many of the coextracted compounds are greatly reduced in the later region of the chromatographic trace. In addition, the extracts appeared much cleaner visually than without the pre-extraction step.

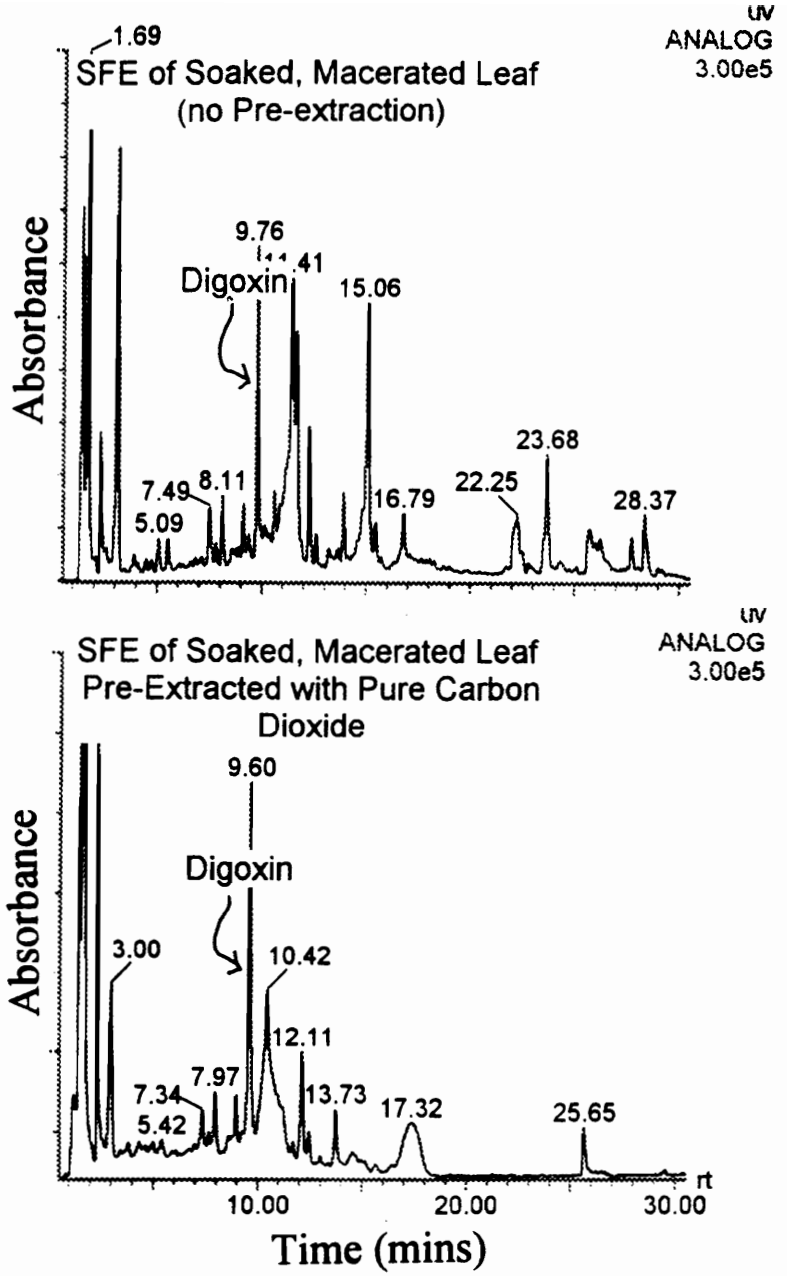


Figure 43. Chromatographic trace (bottom) at 218nm of the soaked, freeze-dried leaf which has been pre-extracted with pure CO_2 compared with trace (top) which has not been pre-extracted. Extraction pressure: 400 atm, temperature: 100°C, flow rate: 1 mL/min, fluid: 20% methanol/CO_2, time: 45 minutes, extraction trap temperature: 80°C, rinse trap temperature: 25°C, trap material: octadecylsilica, rinse solvent: methanol, rinse temperature: 25°C.

SUMMARY-*PRE-EXTRACTION FRACTIONATION*

Fractionation is a characteristic which is inherent to the SFE process and as seen in **Figure 43** can greatly improve the quality of the extract and help reduce the level of background with the chromatographic trace. While simple to perform, the ability to easily vary the extraction parameters and fluid characteristics without additional and time consuming manual steps can greatly help to improve the selectivity or cleanliness of an extraction.

INTRODUCTION-*SELECTIVE RINSING*

SFE typically employs one of two general types of trapping schemes, solid phase trapping or liquid trapping. While liquid trapping may be mechanically more simple to perform, solid phase trapping possesses some unique and beneficial characteristics. If a solid phase trap is employed, SFE possesses the ability to achieve greater selectivity by selectively rinsing the trap by varying the strength of the rinse solvent employed. In addition, one also has the ability to vary the solid phase used. Many conventional liquid extraction techniques such as liquid and Soxhlet, typically require additional cleanup steps such as solid phase extraction, as a result of the inability to vary the solvating strength of the fluid. However, due to extraction setup

in SFE, it is mechanically simple to perform a selective rinse of the solid phase trap. Therefore, this section was designed to test the successfulness and ease of application of selective rinsing (solid phase extraction) of the solid phase trap following SFE of the soaked, lyophilized leaf, by varying the strength of the rinse solvent, in order to improve the selectivity of the SFE process.

RESULTS AND DISCUSSION-*SELECTIVE RINSING*

Several rinse solvents ranging in strength were made to mimic the HPLC mobile phase in order to selectively remove the polar glycosides from the chromatographic stationary phase, while leaving behind the more nonpolar species. These rinse solvents consisted of varying ratios of acetonitrile to water (3:7, 2:8, and 1:9). **Figure 44** shows the results of the selective rinse experiment. Each of the chromatographic traces are shown with respect to the methanol rinse employed earlier (chapter IV). As can be seen, the strength of the rinse solvent can greatly affect the cleanliness of the chromatogram. Each of the rinses yielded 100% recovery of digoxin relative to the liquid extraction method. While the early portion of the chromatogram is relatively unchanged, the later portion (after digoxin) is changed greatly, as much of the coextracted unwanted material is left behind on the octadecylsilica stationary phase.

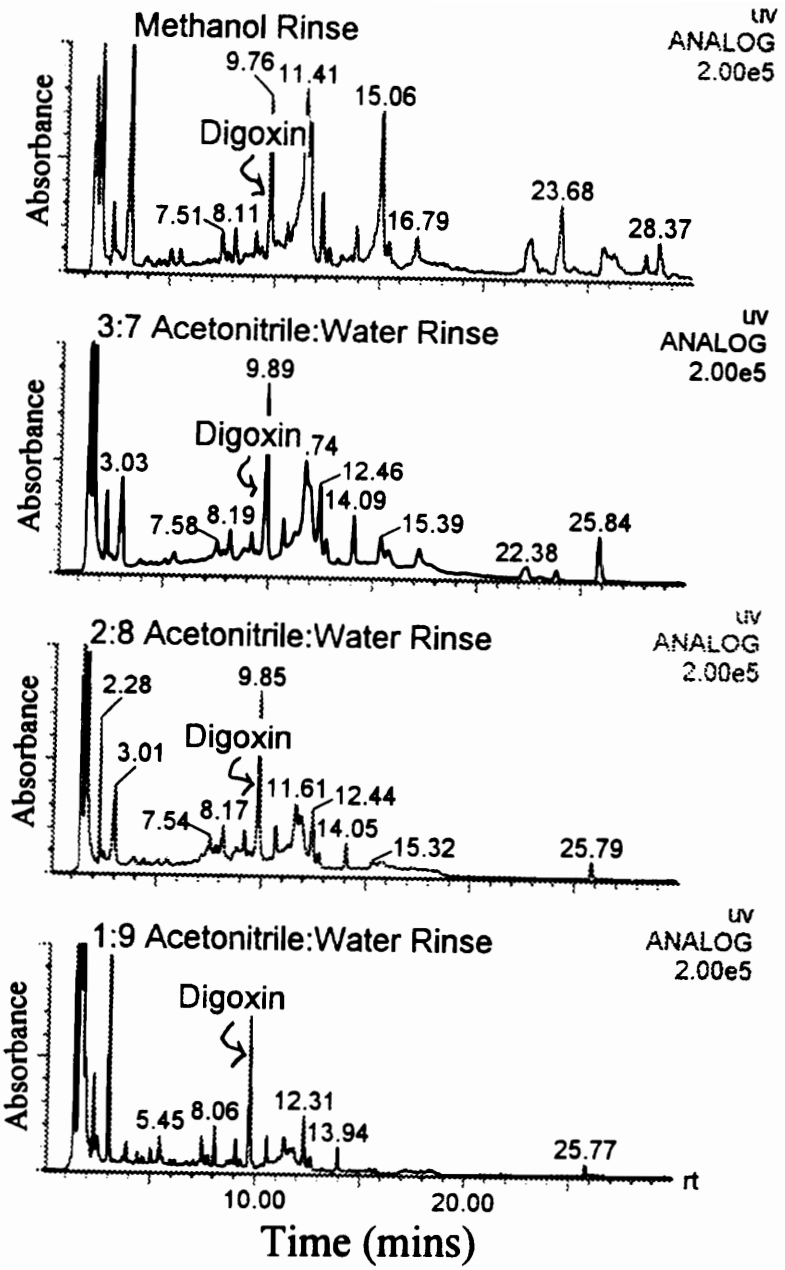


Figure 44. Chromatographic traces at 218nm of the extracts obtained following selective rinsing of the solid phase trap with various solvents. Extraction pressure: 400 atm, temperature: 100°C, flow rate: 1 mL/min, fluid: 20% methanol/CO_2, time: 45 minutes, extraction trap temperature: 80°C, rinse trap temperature: 25°C, trap material: octadecylsilica, rinse solvent: varied, rinse temperature: 25°C.

SUMMARY-*SELECTIVE RINSING*

Selective rinsing is a simple technique which can be optimized quickly and can considerably improve the quality of the final extract. It is a step which must be performed in the SFE process, and it is proven here that it is worth taking the time to develop the correct elution solvent so as to maximize selectivity.

PROJECT SUMMARY

The focus of this chapter was to examine several alternatives which were inclusive to the SFE process and may lead to a reduction in the amount of unwanted coextractives present in the extract solution, therefore resulting in an improvement in the selectivity which had been lost as the result of employing high levels of liquid modifier. While the alternative fluids helped regain some of this loss selectivity, perhaps its most important advantage is in reducing the amount of modifier which is needed for the extraction process. For selectivity improvement however, it seems clear that the other two techniques would prove much more reasonable for cost effectiveness as the fluorinated fluids are costly. Each of these techniques have their own advantages such as reduction in modifier amount needed, ease of use, etceteras, they should be used in conjunction with one another to achieve the cleanest possible extract.

Chapter VI

CONCLUSION

The focus of this research was to expand the applicability of supercritical fluid extraction (SFE) to include analytes which possess considerable polar character by attempting to overcome several of the recognized limitations of the technique.

Consequently, in the first phase of the work, an inverse SFE method was developed for the highly polar amino acid complex, polymyxin B sulfate, and was proven to be successful even at the low concentration of 0.08% present in the solid-like ointment formulation. Recoveries were found to be similar to solid phase extraction with low RSD's. In addition, a method was developed for the liquid-like cream matrix in which the concentration of polymyxin B sulfate was 0.16%.

The second portion of the work was undertaken in an attempt to better understand the role which modifier plays in the trapping step of SFE. Three issues were of interest: 1) to try and understand the effect of the mode of modifier introduction on solid phase trapping, 2) to try and identify the various types of mechanisms which could lead to analyte loss at the solid phase trap when high levels of modifier are employed, and 3) investigate whether a tandem trapping system employing a liquid trap would help to trap the extracted analytes as the pass through the solid phase portion of the tandem trapping system. The mode of modifier introduction in solid phase trapping was found to greatly

affect ones ability to trap the extracted materials. It was learned that spiking high levels of modifier into the vessel, while possibly resulting in a quicker extraction of the analytes of interest, caused severe trapping problems which resulted in low recoveries. In addition, several mechanisms were found which were responsible for sample loss at the solid phase trap. These mechanisms included: 1) aerosol formation, 2) modifier elution, and 3) blow-by. Each of these mechanisms were believed to dominated at certain flow rate and trap temperature conditions. Moreover, it was discovered that for highly modified SF extractions, the optimum trapping conditions were at high trap temperatures (above the boiling point of the modifier) and lower flow rates. It was also discovered that a liquid trap placed in tandem with the solid phase trap did trap some of the materials which were not retained on the solid portion, however, the effectiveness of this liquid trap was determined to be negligible, except for low flow, low trap temperature extractions in which modifier elution was believed to be occurring. At these conditions, the tandem liquid trap was found to be a must.

The third goal in this work, was to apply what was learned in the second portion by developing the quickest and easiest SFE method possible for the extraction and isolation of digoxin and acetyldigoxin from their leaf matrix. This goal was achieved with two separate SFE methods, the first of which involved the SFE of the soaked, freeze-dried leaf, the second of which involved the SFE of the supernatant from the soak step. Many factors were found to be important in the course of this project. Extraction temperature was shown to have a dramatic effect on extraction efficiency as a portion of the glycosides

were determined to be diffusion-limited. In addition, two different solid phase traps were tested and both performed well under the optimized conditions. The two methods helped reduce the time, sample handling, and costs associated with the conventional liquid extraction method, while eliminating the chlorinated solvents totally.

The final portion of this study investigated three different ways in which selectivity could be improved in SFE, should it be lost as the result of employing the high levels of modifier. Previous work on the extraction of the glycosides compounds from the *Digitalis lanata* leaf proved that at high levels of methanol modifier, many unwanted materials were extracted from the leaf due to the high solvent strength. This observation was taken advantage of in an attempt to demonstrate the relative successfulness of the three cleanup strategies employed: 1) alternative fluids, 2) pre-extraction, and 3) selective rinsing of the solid phase trap. While the alternative fluids helped regain some of the selectivity which had been lost, their real advantage was in the reduction of the liquid polar modifier which was required. This reduction in methanol not only reduced the costs of the extraction, but also reduced the risk for trapping problems as discussed in Chapter 3. For selectivity improvement, it seemed clear that the other two techniques are much more useful. While each of the techniques resulted in an improvement in the quality of the chromatogram, it was determined that they should be used in conjunction with one another in order to achieve the greatest improvement in selectivity.

The work presented here proved the feasibility of using SFE as a way of isolating polar pharmaceutical compounds, even at very high levels of modifier. While the

mechanisms proven to cause problems for trapping on solid phases when high levels of modifier are employed were identified and suggestions made for ways to overcome them, further work needs to be done on the trap design utilized in SFE. Also, while the SFE method for extracting digoxin and acetyldigoxin has been proven to be successful on a small scale, further work should be pursued in order to expand its usefulness to include larger preparative scale processes for isolating large quantities of drugs.

Chapter VII

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Chapter VIII

Future Work

The work presented here proved the feasibility of using SFE as a way of isolating polar pharmaceutical compounds, even at very high levels of modifier. While the mechanisms proven to cause problems for trapping on solid phases when high levels of modifier are employed were identified and suggestions made for ways to overcome them in the thesis, further work needs to be done on the trap design utilized in SFE. Perhaps better engineered trapping setups would allow for less problems when high levels of liquid modifier are used. Also, while the SFE method for extracting digoxin and acetyldigoxin has been proven to be successful on a small scale, further work should be pursued in order to expand its usefulness to include larger preparative scale processes for isolating large quantities of the drugs.

The initial results achieved with the fluorinated fluids have stimulated significant interest. Tetrafluoroethane and fluoroform both exhibit unique selectivity characteristics relative to carbon dioxide and appear to solubilize polar compounds to a greater extent. More work should be done with these two fluids in an effort to better pin down the hydrogen bonding theory both with pure and modified fluids. A study should be performed which will test the hydrogen bonding idea with other models containing different functional groups. Also, different matrices should be tested in order to better

understand the selectivity characteristics of the two fluids. Furthermore, the quest for other feasible fluids should continue.

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