THE INVESTIGATION OF PRIMARY AND SECONDARY MODIFIERS IN THE EXTRACTION AND SEPARATION OF NEUTRAL AND IONIC PHARMACEUTICAL COMPOUNDS WITH PURE AND MODIFIED CARBON DIOXIDE

Phyllis R. Eckard

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Larry T. Taylor, Chairman
Neal Castagnoli, Jr.
James O. Glanville
Gary L. Long
Harold M. McNair
Gregory C. Slack

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Blacksburg, Virginia

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THE INVESTIGATION OF PRIMARY AND SECONDARY MODIFIERS IN THE EXTRACTION AND SEPARATION OF NEUTRAL AND IONIC PHARMACEUTICAL COMPOUNDS WITH PURE AND MODIFIED CARBON DIOXIDE

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(ABSTRACT)

A successful supercritical fluid extraction method includes removal of the analyte from the matrix into the bulk fluid as well as trapping or concentration of the analyte prior to analysis. In the first phase of this research, the trapping capacities of three solid-phase traps (glass beads, 50/50 (w/w) glass beads/octadecylsilica), 50/50 (w/w) Porapak Q®/glass beads) were determined as a function of trap composition for a mixture of components varying in polarity and volatility. The Porapak Q®/glass beads mixture was found to be the most successful solid-phase investigated exhibiting the highest trapping capacity. The use of the Porapak Q®/glass beads as a solid-phase trap was investigated in later extraction studies in this dissertation.

The extraction of highly polar, multifunctional analytes may not be completely successful with modified carbon dioxide, therefore, a secondary modifier (i.e. additive) may be added directly to the extraction fluid in hopes of improving the recoveries. In the second phase of this research, the effect of secondary modifiers in the subcritical fluid extraction of lovastatin from in-house prepared tablet powder mixtures and MEVACOR® tablets was investigated. The effect of in-line methanol-modifier percentage, additive type (acidic, basic, neutral) to the in-line methanol, and additive concentration on the extraction efficiency were examined. The extraction recoveries of lovastatin from MEVACOR® tablets were shown to be highly dependent on methanol concentration and additive type. Isopropylamine was shown to be the most successful additive investigated. An optimized and reproducible extraction method was developed.

The extraction of ionic compounds with carbon dioxide may be difficult due to the high polarity of the compounds. In the third phase of this research, the addition of ion-pairing additives to the matrix in hopes of forming an ion-pair complex of reduced analyte polarity was investigated. Therefore, a screening study consisting of a fractional-factorial design was performed in order to identify the factors which contribute most to the recovery of an anionic species, triphenylphosphinetrisulfonate (TPPTS), from a spiked-sand surface employing supercritical fluid extraction with carbon dioxide. The experimental parameters investigated were: type of ion-pairing additive (i.e. tetralkylammonium hydrogen sulfates) and its concentration, carbon dioxide density, extraction temperature, static extraction time, CO_2 mass used, liquid CO_2 flow rate, and the volume of methanol spiked into the matrix prior to extraction. Of the eight factors investigated, four factors were identified as significantly affecting the recovery of the anionic species. They were: 1) ion-pairing reagent added to the spiked sand surface and its concentration; 2) static extraction time; and 3) volume of methanol present in the extraction vessel. The experimental parameters and settings identified as influential by the statistical approach were later shown in concert to yield 100% recovery of TPPTS from the spiked-sand.

In the fourth phase, the extraction of a cationic species, pseudoephedrine hydrochloride, from spiked-sand and Suphedrine tablets, with pure and methanol-modified CO_2 was examined. Once the extraction was shown to feasible, several strategies were compared in terms of their effectiveness in enhancing the analyte's extractability. The first strategy involved the addition of ion-pairing additives. Several sodium salts of alkylsulfonic acids varying in lipophilicity and concentration were investigated. The addition of 1-heptanesulfonic acid, sodium salt, in methanol, in a 5:1 mole ratio of reagent to analyte was shown to be the most useful in recovering the drug from the spiked-sand. The second strategy considered the influence of acids and bases and other modifier compositions such as a methanol/water mixture with or without 1-heptanesulfonic acid, sodium salt, on the pseudoephedrine recovery. The recoveries obtained from the drug

spiked-sand were shown to comparable in the presence of a methanol/water solution, a tetrabutylammonium hydroxide in methanol solution, and a methanol solution with 1-heptanesulfonic acid, sodium salt. Next the extraction of pseudoephedrine hydrochloride from Suphedrine tablets was performed with pure and modified CO_2. Similar to the sand-spike studies, the effect of the addition of the ion-pairing reagent and other in-cell modifiers were examined. Once again, the recoveries obtained when the matrix was in the presence of a methanol/water mixture and a methanol solution containing 1-heptanesulfonic acid, sodium salt were similar. Finally, the identity of the extracted analyte was determined via IR analyses, and it was shown that pseudoephedrine hydrochloride was indeed extractable from the tablets with in-line modified CO_2 in the absence of any in-cell modifier.

In the last phase of this research, a supercritical fluid chromatographic separation with evaporative light scattering detection was developed for the separation of five phospholipids varying in polarity and ionic characteristics. Several parameters were investigated and shown to be influential in the separation. They were: 1) stationary phase composition, 2) addition of an acidic additive and its concentration, 2) mobile phase ramp rate, and 4) column outlet pressure.

This dissertation is dedicated to the memory of my mother and grandfather,

Ruth Ann Williams Eckard and William L.L. Williams, Jr.

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CHAPTER I

INTRODUCTION

In 1822, Baron Cagniard de la Tour first observed the appearance of a supercritical phase by observing the disappearance of two distinct gas and liquid phases into one visual phase by increasing the temperature of a material in a closed glass container. [1] This early discovery marks the first notation of a supercritical fluid.

A supercritical fluid (SF) is defined as any substance that is above its critical pressure and temperature. The critical pressure is the highest pressure at which a liquid can be converted into a gas by an increase in temperature, while the critical temperature is the highest temperature at which a gas can be converted into a liquid by an increase in pressure. Therefore in the critical region there is only one phase, and this phase possesses both gas and liquid-like properties. The solvating power of the supercritical fluid can be influenced by a change in pressure or temperature. While in the supercritical state, at constant pressure, the solvating power of the fluid decreases with increasing temperature. Likewise, at constant temperature, the solvating power of the fluid increases with increasing pressure. This solvating power can be maximized by changing the density of the SF by manipulating both temperature and pressure. This property allows one to adjust the density so as to solubilize certain types of compounds in a selective fashion. For example, low polarity analytes may be solubilized at low densities and more polar analytes at higher densities.

Supercritical fluids make ideal extraction solvents and chromatographic mobile phases because of their high mass transport properties. As compared to normal liquids, SFs exhibit higher diffusivities, lower viscosities, and near zero surface tension. These gas-like properties allow fast mass transfer into and out of complex matrices. Also when

[1] *C. Cagniard de la Tour*, Ann. Chim. Phys., **21** (1822) 127, 178.

used as mobile phases, the lowered viscosity, as compared to high performance liquid chromatographic (HPLC) mobile phases, allows the use of higher flow rates, due to a decreased pressure drop along the length of the column. This may allow separations to be achieved in significantly less time than with normal liquids without a large loss in efficiency.

The most commonly used supercritical fluid as an extraction solvent and mobile phase is carbon dioxide (CO_2). It is relatively inert, non-flammable, non-toxic, is readily attainable in high purity, has easily accessible critical parameters (P_c = 72.9 atm, T_c = 31 °C), and is considered environmentally friendly. Also CO_2 is a gas under ambient conditions. Its use as an extraction solvent and mobile phase may be advantageous. For extraction purposes, it is possible that no solvent concentration following the extraction and prior to analysis will be needed, in direct contrast to most liquid-liquid and liquid-solid extractions. During chromatography, little waste disposal is needed since the gas can be vented directly into the atmosphere.

Carbon dioxide is a non-polar fluid, and its solvating power is comparable to liquid hexane. [2] For these reasons, CO_2 has been used primarily in the analysis of nonpolar to relatively polar compounds. In order to overcome this limitation, a modifier, consisting of small volumes of organic solvents such as methanol, may be added directly to the fluid or matrix, or a more polar fluid such as ammonia may be used. Ammonia is rarely used due to its toxicity, reactivity, and extreme critical parameters (P_c = 111 atm, T_c = 132 °C). For this reason, many have used modified CO_2.

Modifiers generally serve two functions: a) increase the solvating power of the SF and b) facilitate the disruption of analyte-matrix interactions. The addition of a modifier to either the SF or to the matrix prior to SFE may not be sufficient for the extraction and separation of multifunctional, highly polar, and ionic/ionizable mixtures such as many pharmaceuticals. A secondary modifier (i.e. additive) may be added to the primary

[2] *C.R. Yonker, R.D. Smith*, Supercritical Fluid Extraction and Chromatography, *B.A. Charpentier, M.R. Sevenants, Eds.*, ACS Symposium Series, Volume 366, American Chemical Society, Washington, DC (1989) 52.

modifier to achieve successful analyte extraction or separation. The additives typically consist of organic acids, bases, and ion-pairing reagents, and may be added directly to the primary modifier or to the matrix.

Since it is expected that the extraction or the separation of pharmaceutical compounds may be difficult because of low analyte solubility in the supercritical fluid or severe matrix interaction, the extraction or chromatographic conditions may be improved by several means. Several strategies are reported in this dissertation including: ion-exchange, ion-suppression, and ion-pairing. Ion-exchange refers to the displacement of an acidic, a basic, or an ionic species from highly active matrix sites or stationary phase with a "stronger" modifier. Ion-suppression involves charge neutralization by the addition of an appropriate acid or base, while ion-pairing represents the electrostatic interaction of two species of opposite charge with one another to form an ion-pair-analyte complex.

For an extraction method to be deemed successful, both the removal of the analyte from the matrix and the trapping or concentration of the analyte prior to analysis must be optimized. There are generally three-types of off-line trapping systems used today. They include: 1) analyte precipitation onto a cryogenically cooled inert material such as glass beads, 2) analyte precipitation onto a solid-phase sorbent such as octadecyl silica, and 3) analyte collection into a liquid solvent. The main drawback of using liquid collection is that relatively low flow rates of approximately 1 mL/min. (liquid CO_2) or 500 mL/min. (decompressed gas) must be used because of possible solvent evaporation and violent bubbling which may lead to analyte loss. On the other hand with solid-phase trapping, higher flow rates may be used, and the need for preconcentration of the collected extract prior to analysis may be reduced. One of the major drawbacks of solid-phase trapping is the capacity of the solid-phase or the amount of analyte that the solid-phase can retain before analyte breakthrough occurs.

In the following chapters, both the role of trapping and strategies for improving the extraction of neutral and ionic pharmaceutical compounds will be explored. In

Chapter II, the trapping capacities of three solid-phases as a function of analyte polarity and volatility with CO_2 as an extraction fluid will be determined. In Chapter III, the effect of acidic, basic, and neutral secondary modifiers (i.e. additives) in the extraction of lovastatin from MEVACOR® tablets will be investigated. In Chapter IV, the extraction of an anionic compound, triphenylphosphinetrisulfonate, from spiked-sand with CO_2 aided by various ion-pairing additives will be investigated. In Chapter V, the effect of both primary modifiers and additives including ion-pairing reagents, acids, and bases on the extraction of a cationic species, pseudoephedrine hydrochloride, from spiked-sand and Suphedrine tablets will be investigated. In Chapter VI, the separation of neutral and ionic phospholipids via supercritical fluid chromatography as a function of stationary phase, addition of an acidic additive and its concentration, modifier ramp rate, and column outlet pressure will be presented.

CHAPTER II

TRAPPING CAPACITIES OF THREE SOLID-PHASES FOR SUPERCRITICAL FLUID EXTRACTION WITH PURE CARBON DIOXIDE

2.1 INTRODUCTION

A successful off-line supercritical fluid extraction experiment may be divided into two main processes. First, if we assume that the analyte is soluble in the supercritical fluid and the interaction between the analyte and matrix can be overcome, the analyte is removed from its matrix. Second, upon removal of the analyte from its matrix, the analyte is trapped or concentrated prior to analysis. Currently there are three main trapping systems commonly used today. The first and second involve the concentration of the analyte onto a solid surface. This solid surface is either a solid sorbent such as the chromatographic bonded phase octadecyl silica or an inert solid substrate such as stainless steel balls. The third trapping system commonly involves the collection of the analyte directly into a liquid solvent such as methanol. There have been numerous reports illustrating the importance of the solid-phase or liquid-phase composition, trapping temperature, flow rate, and the supercritical fluid composition (unmodified versus modified) on trapping. [1-15]

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[1] *L.J. Mulcahey, J.L. Hedrick, L.T. Taylor,* Anal. Chem., **63** (1993) 2225.
[2] *L.J. Mulcahey, L.T. Taylor,* Anal. Chem., **64** (1992) 2352.
[3] *B.W. Wright, C.W. Wright, R.W. Gale, R.D. Smith,* Anal. Chem., **59** (1987) 38.
[4] *P.G. Thompson, L.T. Taylor,* J. High Resolut. Chromatogr., **17** (1994) 759.
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[10] *N.L. Porter, J.A. Swanson, R.B. Nielson, A.R. Rynaski, E.R. Campbell, M. Saunders, B.J. Murphy, B.E. Richter,* J. Chromatogr. Sci., **30** (1992) 367.
[11] *M. Ashraf-Khorassani, R.K. Houck, J.M. Levy,* J. Chromatogr. Sci., **30** (1992) 361.
Footnotes continued on next page

As SFE gains popularity and becomes a common industrial sample preparation technique, the optimization of the extraction and trapping parameters will continue to play an important role. If solid-phase trapping is to be used successfully on the preparative scale, it is important to determine the amount of solid-phase that is needed to retain all extracted analyte. This Chapter outlines the experimental determination of the trapping capacity of three solid-phases. A test mixture consisting of five different chemical classes which varied in polarity and volatility was spiked onto sand and extracted under identical conditions at three different spike masses. **Table 2.1** lists the analytes investigated as well as their corresponding molecular weights, boiling points, and melting points. [16] A tandem liquid trap was used to retain any analyte exiting from the solid-phase trap. Percent recoveries for the solid-phase rinses and tandem liquid traps at all spike masses are reported. Analyte breakthrough as a function of trap composition will be compared and discussed.

2.2 EXPERIMENTAL

Extraction

Extractions were performed on the Isco/Suprex Prepmaster (Lincoln, NE). Carbon dioxide (SFE/SFC grade) with helium headspace was donated by Air Products and Chemicals Co. (Allentown, PA).

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[12] *J.M. Levy, R.M. Ravey, R.K. Houck, M. Ashraf-Khorassani*, Fresenius J. Anal. Chem., **344** (1992) 577.
[13] *J.M. Levy, R.K. Houck*, Amer. Lab., **25** (1993) #4, 36L.
[14] *D.J. Miller, S.B. Hawthorne, M.E. McNally*, Anal. Chem., **65** (1993) 1038.
[15] *P.G. Thompson, L.T. Taylor, B.E. Richter, N.L. Porter, J.L. Ezzel*, J. High Resolut. Chromatogr., **16** (1993) 713.
[16] *R.C. Weast, M.J. Astle, W.H. Beyer, Eds.*, Handbook of Chemistry and Physics, 65th edition, CRC Press, Inc., Boca Raton (1984).

Table 2.1. Molecular weights, boiling points, and melting points of compounds investigated*

Compound	Molecular Weight, amu	Boiling Point, °C	Melting Point, °C
Acetophenone	120.16	202.6	20.5
N,*N*-Dimethylaniline	121.18	194	2.45
Naphthalene	128.19	218	80.5
2-Naphthol	144.19	288	96.0
n-Tetracosane	338.67	391.3	54

*Information taken from reference 16

A polarity test mixture consisting of acetophenone, *N*,*N*-dimethylaniline, naphthalene, 2-naphthol, and *n*-tetracosane (C_{24}) was prepared in methylene chloride. Approximately 5.4 grams of Ottawa Cement Testing sand (Fisher Scientific, Raleigh, NC) was placed in a 3.5 mL Keystone Scientific (Bellefonte, PA) extraction vessel. The test mixture was spiked onto the sand and extracted at three different total spike masses: 2.5 mg, 10.0 mg, and 20.0 mg or 0.5 mg, 2.0 mg, and 4.0 mg per component.

The three solid-phases investigated in this study were 80-100 mesh glass beads (Applied Science Laboratories, Inc., State College, PA), a 50/50 (w/w) mixture of 30 μm octadecyl silica (Keystone, Bellafonte, PA) and glass beads, and a 50/50 (w/w) mixture of 100-120 mesh Porapak Q® (Alltech, Deerfield, IL) and glass beads. Each solid-phase trap was prepared by filling the empty container provided by the vendor to approximately 90% with the appropriate phase(s). The glass beads trap contained approximately 1.5 g of glass beads, the 50/50 (w/w) octadecyl silica/glass beads trap contained approximately 0.5 g of octadecyl silica and 0.5 g of glass beads (total solid-phase mass of 1.0 g), and the 50/50 (w/w) Porapak Q®/glass beads trap contained approximately 0.4 g of Porapak Q® and 0.4 g of glass beads (total solid-phase mass of 0.8 g). A tandem liquid trap, **Figure 2.1,** consisting of 12 mL methylene chloride was employed to trap any unretained analytes exiting from the solid-phase trap. Upon completion of the extraction, approximately 4 mL of methylene chloride remained in the tandem liquid trap collection vial. All extractions were performed in two steps, at an oven temperature of 35 °C and at a liquid flow rate of 1.0 mL/min. The total mass of CO_2 delivered was 96.8 g (i.e. 39.8 g at a CO_2 pressure of 220 atm (0.88 g/mL CO_2 density) and 57.0 g at 340 atm (0.95 g/mL CO_2 density)). The solid-phase trap was cooled to +5 °C while the restrictor temperature was maintained at 100 °C. The tandem liquid trap was maintained at room temperature. Upon completion of the extraction, the trap temperature was raised to 25 °C while the restrictor temperature was lowered from 100 °C to 30 °C. The solid-phase was then rinsed twice with 5 mL aliquots of methylene chloride.

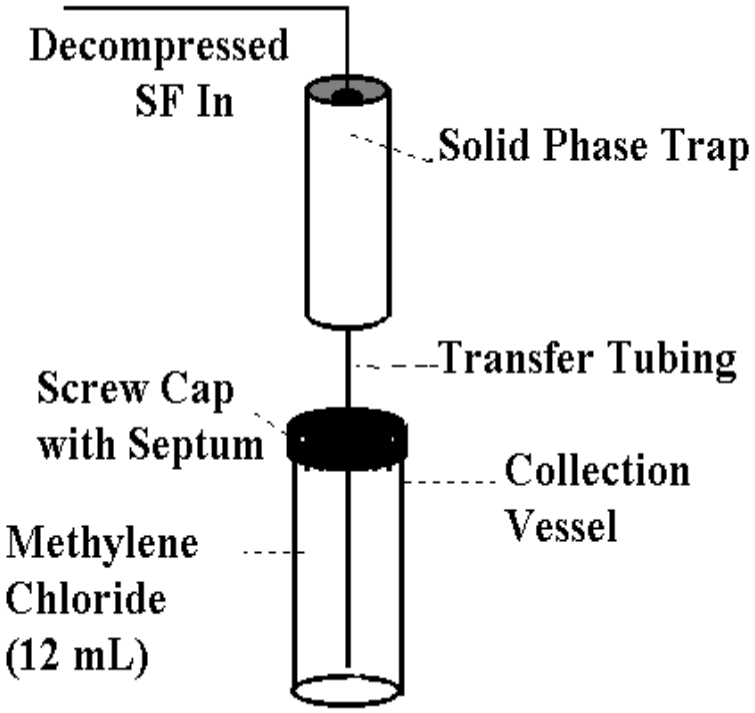

Figure 2.1. Tandem solid-phase/liquid trap

Extract Analysis

After completion of the extraction the solid-phase rinses and the tandem liquid trap were sonicated for 5 minutes to aid in the removal of any dissolved carbon dioxide. A 20,000 ppm pyrene internal standard (100 μL) was then added to the solid-phase rinses and the tandem liquid trap, and a portion of each solution was transferred to GC vials for analysis. The purpose of adding internal standard to the trap rinse and tandem liquid trap was to ensure good quantitation in the case of variation in solid-phase trap rinse volumes or evaporation loss in the tandem liquid trap during the extraction. Values corresponding to 100% recovery were obtained by adding 100 μL of the appropriate test mixture solution and 100 μL of internal standard to an empty collection vial and diluting to 5 mL with methylene chloride. Each solution corresponding to 100% recovery was run three times and the peak area ratios (peak area of analyte/peak area of internal standard) were averaged. Experimental percent recoveries were then calculated and reported.

A Hewlett Packard (Wilmington, DE) 5890 Series II GC equipped with a 7673 autosampler, a flame ionization detector, and a Vectra QS/20 Chemstation were used for all extract analyses. A DB-5 MS (5 % phenylmethylsiloxane) column (30 m long X 0.25 mm i.d.) with a film thickness of 0.25 μm was used. A 1 μL splittless injection was performed. The initial oven temperature was held at 50 °C for one minute and ramped to 140 °C at 15.0 °C/min., then ramped to 300 °C at 30.0 °C/min. and held for 3.0 min. The injector was maintained at 275 °C, and the flame ionization detector was maintained at 325 °C.

2.3 RESULTS AND DISCUSSION

There was several experimental difficulties encountered while performing this study. A one step extraction at high density was initially attempted; however, variable restrictor (100 °C) plugging resulted due to the high amounts of initially extracted analyte,

therefore, a two step extraction was warranted which resulted in a long extraction time. The extraction was first performed at low densities to remove an initial amount of analyte followed by the second step at higher densities to remove the remaining analytes. The time needed to completely remove the analytes at the higher spike masses was 1.75 hours. Initially the trapping capacity at 1.0 mL/min and 4.0 mL/min. were to be determined; however, at a liquid flow rate of 4.0 mL/min (measured at the pump) complete loss of the tandem liquid trap solvent as well as restrictor plugging were observed. The flow rate was thus limited to 1.0 mL/min., resulting in an additional experimental difficulty.

With these restrictions, the trapping capacity or the total amount of analyte that is able to be retained on a solid-phase before significant breakthrough occurs has been examined for three different solid-phases. **Table 2.2** lists the recoveries obtained for the solid-phase rinses and tandem liquid traps for the 50/50 (w/w) octadecyl silica/glass beads trap. Each entry represents the average of at least two replicates. The solid-phase trapping capacity was shown to be a function of the chosen analyte. All test analytes showed quantitative recoveries of greater than 90% at the 2.5 mg total spike mass. At a total spike mass of 10.0 mg significant breakthrough from the solid-phase trap into the tandem liquid trap of a more volatile analyte, naphthalene, occurred; however, significant breakthrough of all other test analytes was not seen. Breakthrough or the appearance of analyte in the tandem liquid trap was seen for all analytes at the 20.0 mg total spike mass. Assuming that the trapping capacity of the glass beads is minimal at +5 °C, it would appear that the trapping capacity of octadecyl silica for all analytes except naphthalene was less than 4 mg/g of octadecylsilica. Since these five analytes were co-extracted, the trapping capacity might be higher if each analyte had been individually extracted. A lower solid-phase trap temperature may also lead to a greater net trapping capacity.

A glass beads trap was then investigated to better determine the role of the octadecyl silica phase on the trapping of the analytes on the octadecylsilica/glass beads

Table 2.2. Analyte percent recovery for the 50/50 (w/w) octadecylsilica/glass beads trap (1.0 g)

Solid-Phase Rinse**

Total Spike Mass, mg	Acetophenone	Dimethylaniline	Naphthalene	2-Naphthol	*n*-Tetracosane
2.5	96.2	90.0	96.1	100.4	94.7
10.0	87.8	86.2	34.0	82.1	88.7
20.0	75.8	47.9	17.0	89.0	86.6

Tandem Liquid Trap***

Total Spike Mass, mg	Acetophenone	Dimethylaniline	Naphthalene	2-Naphthol	*n*-Tetracosane
2.5	ND*	ND	ND	ND	ND
10.0	ND	ND	56.3	ND	ND
20.0	22.0	40.1	75.6	0.3	9.3

Mass Balance****

Total Spike Mass, mg	Acetophenone	Dimethylaniline	Naphthalene	2-Naphthol	*n*-Tetracosane
2.5	96.2	90.0	96.1	100.4	94.7
10.0	87.8	86.2	90.3	82.1	88.7
20.0	97.8	88.0	92.6	89.3	95.9

* ND indicates none detected
** Percent of extracted analyte retained on the solid-phase material
*** Percent of extracted analyte collected in tandem liquid trap
**** Percent retained on solid-phase + tandem liquid trap

SFE Conditions: CO_2 Pressure: 220 atm (step 1), 340 atm (step 2); Mass of CO_2 Delivered: 39.8 g (step 1), 57.0 g (step 2); Oven Temperature: 35 °C; CO_2 Liquid Flow Rate: 1.0 mL/min.; Restrictor Temperature: 100 °C; Solid-phase Collection Temperature: 5 °C; Tandem Liquid Trap: 12 mL Methylene Chloride (room temperature); Solid-Phase Desorption Temperature: 30 °C; Solid-Phase Rinse: 5 mL Methylene Chloride (2 times)

Assay - see Experimental

glass beads trap. **Table 2.3** lists the recoveries for the solid-phase rinses and tandem liquid traps for the glass beads trap. Acetophenone, *N*,*N*-dimethylaniline, and naphthalene could not be successfully retained on the glass beads trap at any spike mass, indicating their retention on the octadecylsilica/glass beads phase was aided by their interaction with the octadecylsilica material. The less volatile analytes 2-naphthol and *n*-tetracosane were retained successfully on the glass beads trap showing quantitative recoveries of greater than 92% at all spike masses. The total trapping capacity of the glass beads trap for the three test analytes was therefore less than 0.5 mg/1.5 g under the extraction and trapping conditions chosen. For 2-naphthol and *n*-tetracosane, the glass beads function as well or better than the octadecylsilica/glass beads trap (e.g. > 2 mg/1.5 g of glass beads).

The Porapak Q®/glass beads trap was shown to be in general the most successful solid-phase exhibiting the best trapping capacity of all three solid sorbents investigated. **Table 2.4** lists the recoveries for the solid-phase rinse and tandem liquid trap at all spike masses. Recoveries of greater than 88% on the solid-phase alone were seen for all test analytes at 0.5, 2.0, and 4.0 mg per analyte. Considering that the mass of Porapak Q® was 0.4 g in the trap, the trapping capacity for each analyte was at least 10 mg/g of Porapak Q®. Identical extraction and trapping conditions were used for all three solid-phases investigated. The appearance of test analytes in the tandem liquid trap after the Porapak Q®/glass beads at all spike masses was believed to be due to non-optimized trapping conditions rather than exceeding the solid-phase's trapping capacity. Higher spike masses could not be performed due to extraction difficulties such as restrictor plugging.

The trapping of the five test analytes when employing 100% CO_2 was shown to be easily achieved at the lower spike masses; however, the addition of modifier may be required for many real world analytes/matrices. It is believed that modified fluids will have an effect on the trapping capacity of both solid-phase and liquid phase traps. Numerous studies have shown that high trap temperatures and low flow rates favor successful trapping when employing modified fluids. On the other hand, liquid phase trapping must

Table 2.3. Analyte percent recovery for the 100% glass beads trap (1.5 g)

Solid-Phase Rinse**

Total Spike Mass, mg	Acetophenone	Dimethylaniline	Naphthalene	2-Naphthol	*n*-Tetracosane
2.5	2.5	1.4	3.1	92.1	93.5
10.0	1.7	1.0	4.6	94.2	98.6
20.0	4.3	3.1	19.9	92.5	93.7

Tandem Liquid Trap***

Total Spike Mass, mg	Acetophenone	Dimethylaniline	Naphthalene	2-Naphthol	*n*-Tetracosane
2.5	106.7	74.1	106.8	1.6	1.4
10.0	101.1	96.6	97.8	1.7	0.7
20.0	99.7	98.2	83.3	5.9	7.2

Mass Balance****

Total Spike Mass, mg	Acetophenone	Dimethylaniline	Naphthalene	2-Naphthol	*n*-Tetracosane
2.5	109.2	75.5	109.9	93.7	94.9
10.0	102.8	97.6	102.4	95.9	99.3
20.0	104.0	101.3	103.2	98.4	100.9

** Percent of extracted analyte retained on the solid-phase material
*** Percent of extracted analyte collected in tandem liquid trap
**** Percent retained on solid-phase + tandem liquid trap

SFE Conditions: CO_2 Pressure: 220 atm (step 1), 340 atm (step 2); Mass of CO_2 Delivered: 39.8 g (step 1), 57.0 g (step 2); Oven Temperature: 35 °C; CO_2 Liquid Flow Rate: 1.0 mL/min.; Restrictor Temperature: 100 °C; Solid-phase Collection Temperature: 5 °C; Tandem Liquid Trap: 12 mL Methylene Chloride (room temperature); Solid-Phase Desorption Temperature: 30 °C; Solid-Phase Rinse: 5 mL Methylene Chloride (2 times)

Assay - see Experimental

Table 2.4. Analyte percent recovery for the 50/50 (w/w) Porapak Q®/glass beads trap (0.8 g)

Solid Sorbent Rinse**

Total Spike Mass, mg	Acetophenone	Dimethylaniline	Naphthalene	2-Naphthol	*n*-Tetracosane
2.5	96.5	95.8	97.8	102.3	88.6
10.0	93.8	93.7	97.2	89.3	90.7
20.0	89.8	90.2	93.7	95.4	90.5

Tandem Liquid Trap***

Total Spike Mass, mg	Acetophenone	Dimethylaniline	Naphthalene	2-Naphthol	*n*-Tetracosane
2.5	6.5	4.9	3.4	2.4	1.7
10.0	11.1	7.8	4.3	6.4	7.1
20.0	7.3	6.9	3.5	1.9	6.9

Mass Balance****

Total Spike Mass, mg	Acetophenone	Dimethylaniline	Naphthalene	2-Naphthol	*n*-Tetracosane
2.5	103.0	100.7	101.2	104.7	90.3
10.0	104.9	101.5	101.5	95.7	97.8
20.0	97.1	97.1	97.2	97.3	97.4

** Percent of extracted analyte retained on the solid-phase material
*** Percent of extracted analyte unretained on the solid-phase and collected into tandem liquid trap
**** Percent retained on solid-phase + tandem liquid trap

SFE Conditions: CO_2 Pressure: 220 atm (step 1), 340 atm (step 2); Mass of CO_2 Delivered: 39.8 g (step 1), 57.0 g (step 2); Oven Temperature: 35 °C; CO_2 Liquid Flow Rate: 1.0 mL/min.; Restrictor Temperature: 100 °C; Solid-phase Collection Temperature: 5 °C; Tandem Liquid Trap: 12 mL Methylene Chloride (room temperature); Solid-Phase Desorption Temperature: 30 °C; Solid-Phase Rinse: 5 mL Methylene Chloride (2 times)

Assay - see Experimental

also be reoptimized with modified fluids although the protocols have not been set forth. Trapping capacity was determined to be a function of trap composition and is analyte dependent. Although high trap temperatures favor successful trapping when employing modified fluids, it is possible that the more volatile analytes will be lost from the solid-phase at elevated trapping temperatures, therefore decreasing the trapping capacity. If one lowers the trapping temperature to favor trapping of the more volatile analytes, loss of analyte may still result due to the mechanical rinsing of analyte off the solid-phase with the condensed modifier. It may be possible to increase the trapping capacity of the solid-phases by altering several parameters such as the trapping temperature and flow rate.

2.4 SUMMARY

The trapping capacities of three solid-phase traps consisting of either a 50/50 (w/w) mixture of octadecyl silica and glass beads, glass beads, or a 50/50 (w/w) mixture of Porapak Q® and glass beads for a mixture of components representing five different chemical classes were determined. It was seen that analyte breakthrough varied with trap and analyte composition. The trapping capacity for the octadecylsilica/glass beads trap was determined to be less than 8 mg/g of octadecylsilica for four analytes and less than 4 mg/g of octadecylsilica for naphthalene. The glass beads trap was less successful for the trapping of the three more volatile analytes resulting in a capacity of less than 0.5 mg/1.5 g. Consequently, the retention of acetophenone, dimethylaniline, and naphthalene on the octadecylsilica trap was due to adsorption on the octadecylsilica material. However, the glass beads are an effective trapping material for the two less volatile, higher molecular weight components, 2-naphthol and *n*-tetracosane. A trapping capacity for these two analytes was comparable to the octadecylsilica/glass beads trap of > 2 mg/1.5 g of glass beads. In this case, their retention was attributed to cryogenic freezing on the glass beads surface. Finally, the most successful trapping material was shown to be

the mixture of Porapak Q® and glass beads resulting in a trapping capacity of at least 10 mg/g of Porapak Q® per analyte.

CHAPTER III

INVESTIGATION OF PRIMARY AND SECONDARY MODIFIERS FOR THE SUBCRITICAL EXTRACTION OF LOVASTATIN FROM MEVACOR® TABLETS WITH CARBON DIOXIDE

3.1 INTRODUCTION

Past studies in both super/subcritical fluid extraction (SFE) and chromatography (SFC) have primarily utilized carbon dioxide as the supercritical fluid (SF) because it is relatively inert, highly pure, nontoxic, exhibits readily attainable critical parameters (T_c=31 °C, P_c=71 atm), and has solvent power equivalent to common organic solvents such as hexane. Super/subcritical carbon dioxide has been proven to be an efficient medium in the extraction of non-polar and moderately polar compounds; however, its solvating power may be insufficient for the extraction of highly polar compounds such as most pharmaceuticals. As a result, the pharmaceutical industry has been reluctant to accept CO_2 as an extraction fluid. This limitation may be overcome by either using a more polar SF like ammonia, or by adding small amounts of polar organic solvents (i.e. modifiers) to carbon dioxide. A polar SF such as ammonia is rarely used due to its toxicity, reactivity, and extreme critical parameters (T_c=132 °C, P_c=111 atm). As a result, most pharmaceutical applications have utilized modified carbon dioxide. [1-4]

Modifiers generally serve two functions: a) increase the solvating power of the SF and b) facilitate the disruption of analyte-matrix interactions. [5] For instance, felodipine, an antihypertensive basic drug, was found to be soluble in 100% CO_2; however, when

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[1] *W.N. Moore, L.T. Taylor*, Anal. Chem., **67** (1995) 2030.
[2] *N.N. Dulta, A.P. Baruah, P. Phukan*, CEW, **26** (1991) 25.
[3] *A.L. Howard, M. Shah, P.I. Dominic, M.A. Brooks, J.T.B Strode, L.T. Taylor*, J. Pharm. Sci., **83** (1994) 1537.
[4] *K.A. Larson, M.L. King*, Biotech. Prog., **2** (1986) 73.
[5] *J.M. Levy, L. Dolata, R.M. Ravery, E. Storozynsky, K.A. Holowczak*, J. High Resolut. Chromatogr., **16** (1993) 368.

extracting a sustained-release tablet containing felodipine, only 60% was recovered with pure carbon dioxide under similar conditions. [3] To achieve quantitative extractions (97-103%), 8% (v/v) methanol-modified CO_2 was needed apparently to disrupt analyte/matrix interactions. Alternatively, the modifier may be more effectively used by introducing it directly to the matrix prior to extraction with CO_2.

The addition of a modifier to either the SF or to the matrix prior to SFE may not be sufficient for the extraction of multifunctional, highly polar, and ionic compounds. A secondary modifier (i.e. additive) may be added to the primary modifier to achieve successful analyte extraction or separation. The additives typically consist of relatively strong organic acids or bases and are usually added directly to the primary modifier (0.1%-5% (v/v)) rather than to the fluid or matrix. [6] The general guideline for the use of additives in SFC and SFE is that acidic analytes require acidic additives and basic analytes require basic additives. Additives have been used recently to improve peak shape and enhance separation in supercritical fluid chromatography of polar compounds. [6-8] Berger and Deye have demonstrated that compounds containing more than two carboxylic acid groups on a benzene ring could not be eluted from a sulphonic acid column with less than 20% methanol-modified CO_2; however when an additive such as citric acid was added to methanol-modified CO_2, benzene mono-, di-, and tricarboxylic acids could be separated and eluted. [9] In this case, the citric acid was said to cover active sites (exposed silanols) on the stationary phase surface, thus retention of the highly polar carboxylic acids was greatly reduced. Additives have also found some use in SFE, specifically in the extraction of basic polar compounds. A mebeverine alcohol metabolite was successfully isolated from dog plasma onto a SPE cartridge and extracted from the cartridge with SFE using methanol-modified CO_2 and isopropylamine. [10] Also, cocaine from human hair was

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[6] *T.A. Berger, J.F. Deye*, J. Chromatogr. Sci., **29** (1991) 26.
[7] *T.A. Berger, J.F. Deye*, ACS Symp. Ser., **488** (1992) 132.
[8] *T.A. Berger, W.H. Wilson*, J. Chromatogr. Sci., **31** (1993) 127.
[9] *T.A. Berger, J.F. Deye*, J. Chromatogr. Sci., **29** (1991) 141.
[10] *H. Liu, L.M. Cooper, D.E. Raynie, J.D. Pinkston, K.R. Wehmeyer*, Anal. Chem., **64** (1992) 802.

extracted using CO_2 modified with water and triethylamine. [11] In both cases, the basic additive served as an ion-suppresser thus favoring the extraction of the free bases. Therefore it was concluded that additives serve two main purposes including acting as ion-suppressers as well as reducing the activity of the stationary phase or matrix by covering active sites.

Although the solubility of an analyte may be high in the fluid, successful extraction of the analyte from a complicated matrix such as a tablet may be problematic due to large analyte/matrix interactions. It was, therefore, the objective of this study was to investigate the role of secondary modifiers (i.e. additives) for the extraction of a commonly used pharmaceutical compound from a complicated matrix such as a tablet. In this study, the analyte being extracted was neutral and contained no ionizable functionalities. Although it was expected that ion-suppression would not play a role in this study, it was of interest to examine the effect of additive type (acidic, basic, neutral) on its ability to cover active matrix sites and thus displace the polar analyte from the complicated tablet matrix.

The study was divided into four phases utilizing lovastatin, an antihypercholesterolemic drug, (active ingredient in MEVACOR® tablets) as the prototype drug. Phase A determined the effect of methanol on the extractability of lovastatin from an in-house prepared tablet mixture. The role of additive type (acidic, basic, and neutral) was then investigated in Phase B. The effect of additive and modifier concentration was investigated in Phase C. Once an optimum additive concentration was chosen (Phase D), the usefulness of the additive with methanol versus methanol-modified CO_2 alone in terms of overall extraction recovery and time needed to extract lovastatin directly from commercially available MEVACOR® tablets was examined. Finally, the reproducibility of the optimum SFE method was demonstrated.

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[11] *J.F. Morrison, W.A. MacCrehan,* Presented at the 5th International Symposium on SFE/SFC, January, 1994.

3.2 EXPERIMENTAL

All extractions were performed on the Isco Suprex Prepmaster (Lincoln, NE). Carbon dioxide (SFE/SFC grade) with helium headspace was donated by Air Products and Chemicals, Inc. (Allentown, PA). For all tablet extractions, either approximately 100 mg of in-house prepared tablet powder (Phases A-C) containing 10 mg of lovastatin (Merck Research Laboratories, West Point, PA) or a crushed MEVACOR® tablet (Phase D) containing 10 mg lovastatin was placed into an extraction vessel (5 mL volume (Phase A-B) or 3.5 mL volume (Phase C-D), Keystone Scientific, Bellefonte, PA) containing cotton balls. Cotton balls were used to reduce the dead volume of the vessel. The extraction and trapping conditions for this study are found in **Table 3.1.**

Extract Analysis

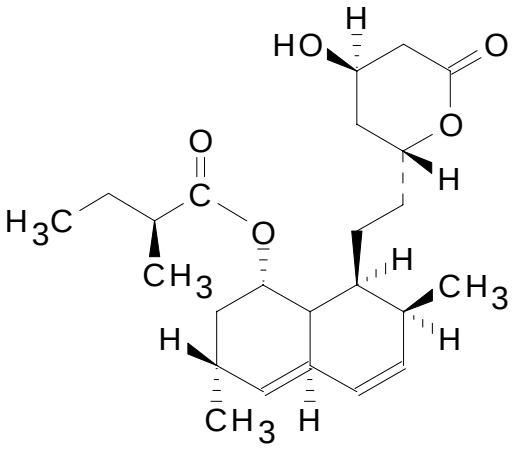
After the extraction, trapping, and recovery steps, approximately 0.4 mg of 17-α-hydroxyprogesterone (**Figure 3.1**) was added to the combined liquid trap/solid-phase trap rinses as an internal standard. The purpose of adding internal standard to the trap rinse and tandem liquid trap was to ensure good quantitation in case there were variations in solid-phase trap rinse volumes as well as evaporation losses in the tandem liquid trap during the extraction. A portion of each solution was then transferred to SFC vials for analysis.

A prototype of the Hewlett Packard Model G1205 SFC system (Little Falls, DE) was used for Phase A, B, and D (15% methanol-modified CO_2 mixtures and reproducibility) analyses. All other analyses were performed on the Gilson SF3™ SFC system (Middleton, WI). All separations were performed isocratically with a Hypersil®

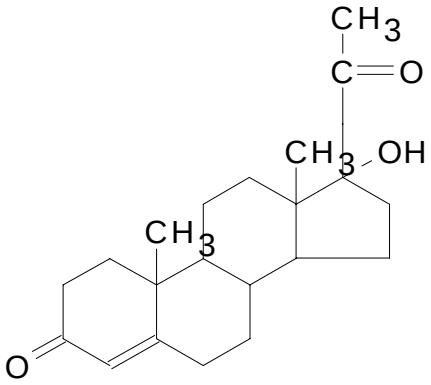
Table 3.1. Extraction and Trapping Conditions Used for Phases A-D

CO_2 Pressure	400 atm
Oven Temperature	40 °C
Liquid Flow Rate	2.0 mL/min.
Restrictor Temperature	50 °C
Solid Phase Trap	50/50 (w/w) Porapak Q/Glass Beads
Liquid Tandem Trap	Methanol
Liquid Tandem Trap Volume	5 mL, room temperature, 7 mL, room temperature (Phase D, reproducibility)
Collection Temperature (solid-phase)	40 °C
Desorption Temperature (solid-phase)	40 °C
Solid-Phase Trap Rinse Solvent/Volume	Methanol, 2.0 mL* (Phases A-D) Methanol, 5.0 mL (Phase D, reproducibility)
Rinsing Flow Rate	1.0 - 2.0 mL/min. (Phase D (reproducibility)

*Solid-phase trap was rinsed directly into liquid tandem trap following each dynamic extraction step when constructing extraction profiles.



(a)



(b)

Figure 3.1. Chemical Structures for Lovastatin (a) and 17α-Hydroxyprogesterone (b)

silica (4.6 mm i.d. X 25 cm, 3 μm particle diameter) column (Keystone Scientific, Bellefonte, PA) and a mobile phase consisting of 6% (v/v) (0.5% (v/v) trifluoroacetic acid) methanol-modified CO_2 at a pressure of 230 bar and a liquid flow rate of 2.0 mL/min. The purpose of the trifluoroacetic acid was to eliminate peak tailing of a possible degradation product, hydroxy acid lovastatin. The peak shape of lovastatin was not affected by the addition of the acidic additive to the mobile phase. [12] The column was maintained at 45 °C. The injection solvent consisted of methanol at a volume of 5 μL. Detection was by UV at 230 nm. A UV flow cell maintained at room temperature with 10 μL volume was used.

Traditional Liquid Extraction

Approximately 100 mg of the in-house tablet powder mixture or a MEVACOR® tablet was placed into a 50 mL volumetric flask. Then, 10 mL of an acetic acid- sodium acetate buffer (pH=4.0) was added to the flask, and the solution was sonicated until the tablet powder/tablet was fully disintegrated (15 minutes). Next, 35 mL of acetonitrile was added to the flask, and the solution was sonicated for 20 minutes. After cooling to room temperature (30 minutes), the solution was diluted to 50 mL with acetonitrile. Analyses were performed by SFC on the resulting solutions.

In-House Tablet Powder Mixture

The in-house tablet powder mixture was prepared by mixing all the ingredients except for lovastatin in a round bottom flask. Lovastatin was then dissolved in methanol (50 mL) and added to the flask with stirring. The tablet mixture was refrigerated overnight. The methanol was then removed by rotary evaporation. The concentration of

[12] *J.T.B. Strode, L.T. Taylor, A.L. Howard, D. Ip, M.A. Brooks*, "Analysis of Lovastatin by Packed Column Supercritical Fluid Chromatography", submitted for publication.

lovastatin in the in-house tablet powder mixture was 10 mg lovastatin/100 mg tablet powder. Four samples were taken to test uniformity of the tablet mixture (**Table 3.2**) using the traditional liquid extraction method, followed by SFC analysis.

MEVACOR® Tablet Crushing Method

Each commercially prepared MEVACOR® tablet (Merck Research Laboratories, West Point, PA) was placed on top of a piece of weighing paper which was loose in a mortar cup. A pestle was placed on top of the tablet, and pressure was applied until the tablet particles appeared evenly dispersed as a powder. The weighing paper was carefully removed and the complete crushed tablet was poured into the extraction vessel filled approximately 3/4 with a cotton ball. The weighing paper, mortar, and pestle were wiped clean with an additional small piece of cotton. This particular piece of cotton was then placed on top of the other cotton ball inside the extraction vessel. More cotton was added to fill approximately 90% of the vessel volume. The extraction vessel was then sealed.

3.3 RESULTS AND DISCUSSION

Phase A - Effect of Methanol-Modifier Concentration

Lovastatin, an antihypercholesterolemic drug (**Figure 3.1**), was chosen as the test analyte because it is relatively polar and exhibits marginal solubility (0.04 % (w/w) at 5000 psi, 40 °C) in 100% CO_2. [4] Larson and King found that the solubility was dramatically increased to 0.4% (w/w) with the incorporation of 5% (w/w) methanol-modified CO_2 from a pre-mixed tank. The increased solubility of lovastatin in the methanol-modified CO_2 versus pure CO_2 was attributed to the ability of the dilute methanol to hydrogen bond with the polar lovastatin. Consequently, a series of extractions (**Table 3.1**) were performed to determine the extractability of lovastatin from the in-house prepared tablet

Table 3.2. In-house Prepared Tablet Powder Mixture Uniformity With Liquid Extraction Method

Sample #	mg Lovastatin/100 mg tablet powder
1	9.78
2	9.33
3	9.50
4	9.60
Average	9.55
% RSD	2.0

SFC Conditions Used for Tablet Powder Uniformity. Column: Hypersil® silica (4.6 mm i.d. X 25 cm, 3 μm particle diameter), Mobile Phase: 6% (v/v) (0.5% (v/v) trifluoroacetic acid) methanol-modified CO_2; Pressure: 230 bar; Column Temperature: 45 °C; Liquid Flow Rate: 2.0 mL/min; Injection solvent: methanol; Injection Volume: 5 μL; Detection: UV at 230 nm. UV Flow Cell Volume, Temperature: 10 μL, room temperature.

Liquid-solid extractions - see Experimental for procedure

powder mixture with methanol-modified CO_2. Experiments were designed in such a way that an extraction profile could be constructed from the data (**Figure 3.2**) in order to examine the effect of methanol concentration and to investigate the extraction kinetics of lovastatin. A series of dynamic mini-extraction steps followed by trap rinsing and assay was employed. The tablet powder mixture was first allowed to equilibrate with the fluid for a certain period of time (static time) followed by continuous CO_2 flow for an additional period of time (dynamic time). The constructed extraction profiles consisted of several dynamic ministeps whereby the extraction was placed in static mode after a certain dynamic period, and the solid-phase trap was rinsed and assayed for percent recovery for that particular dynamic period. A tandem solid-phase/liquid trap was employed to ensure quantitative trapping recovery. Lovastatin recoveries were found to be low over the first forty minutes where only 58% was extractable with 1% (v/v) methanol-modified CO_2, and 77% was extractable with 5% (v/v) methanol-modified CO_2. When utilizing 1% and 5% (v/v) methanol-modified CO_2, the extraction profile suggested that most of the extractable lovastatin was removed during the first 20 minutes of dynamic extraction. During this period, the extraction appeared to be dependent upon the solubility of the analyte in the methanol-modified CO_2. After 20 minutes, the extraction process appeared to be limited by the diffusion of the analyte from the matrix into the SF. [13] Quantitative recoveries from the in-house prepared tablet powder mixture of greater than 97% were, however, achieved with 10% (v/v) methanol-modified CO_2 employing dynamic extraction mini-steps (**Figure 3.2).** Since trapping becomes more difficult with modifier concentrations greater than 2%, it was of interest to determine if the addition of a secondary modifier could reduce the primary modifier concentration.

[13] *K.D. Bartle, A.A. Clifford, S.B. Hawthorne, J.J. Langenfield, D.J. Miller, R. Robinson*, J. Supercrit. Fluids, **3** (1990) 143.

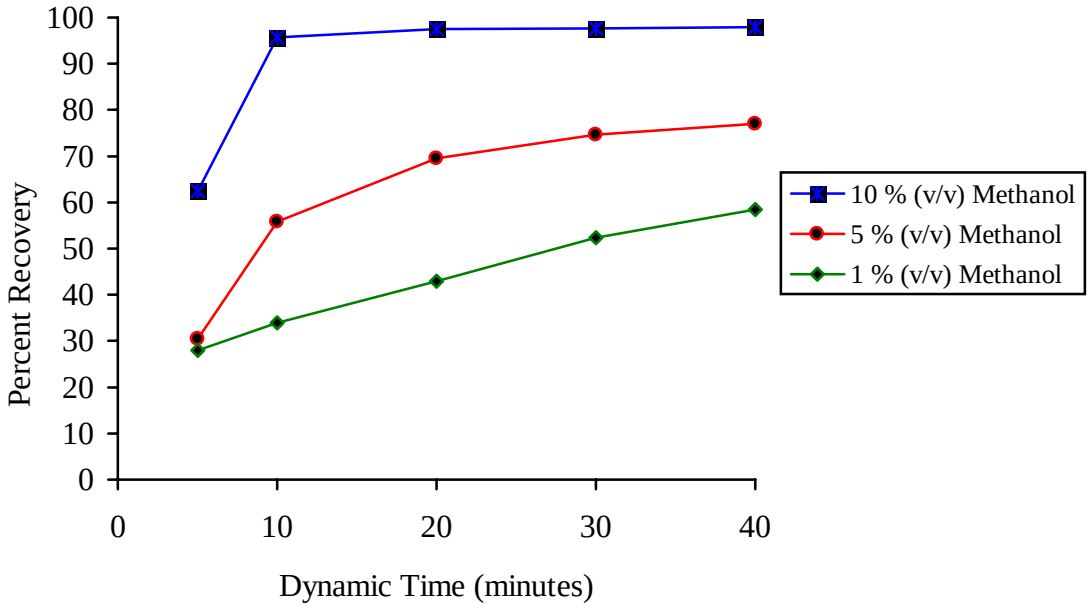


Figure 3.2. Effect of Methanol-Modifier Concentration on Lovastatin Recoveries (n=3) From In-House Prepared Tablet Powder Mixture

SFE Conditions. CO_2 Pressure: 400 atm; Oven Temperature: 40 °C; Liquid Flow Rate: 2.0 mL/min.; Restrictor Temperature: 50 °C; Solid-Phase Trap: 50/50 (w/w) Porapak Q/Glass Beads; Liquid Tandem Trap: Methanol; Liquid Tandem Trap Volume: 5 mL; Collection Temperature (Solid-Phase): 40 °C, Desorption Temperature (Solid-Phase); 40 °C; Solid-Phase Rinse Solvent/Volume: Methanol, 2.0 mL, Solid-Phase Rinsing Flow Rate: 1.0 mL/min.; Initial Static Time: 3.0 min.; Dynamic Time: 40.0 min. (total of 5 dynamic mini-steps); Static Time During Trap Rinsing: 2.0 min. *Solid-phase trap rinsed directly into tandem liquid trap following each dynamic step.

Sample - 100 mg tablet powder mixture containing 10 mg lovastatin

Phase B - Effect of Additive Composition

After determining the effect of methanol on extraction efficiency, the role of the secondary modifier (i.e. additive) type was investigated. Methanol-modified CO_2 (1% (v/v)) was chosen as the extraction fluid in Phase B due to the limited extractability of lovastatin under these conditions so that the apparent effect of each additive on the extractability could be ascertained. Each additive was introduced directly to the methanol as 1% (v/v). The total additive concentration introduced corresponds to 0.0001%. It can be seen in **Figure 3.3** that isopropylamine was the only additive that significantly improved the extractability of lovastatin over time from the prepared tablet powder mixture. In fact, similar extraction recoveries utilizing all three additives (i.e. acid, neutral, base) were observed during the first 20 minutes of the extraction. During this time, the extraction was apparently governed simply by the solubility of the lovastatin in the 1% (v/v) methanol-modified CO_2. The extractability of lovastatin, however, increased from 58% with 1% (v/v) methanol-modified CO_2 to 71% with 1% (v/v) (1% (v/v) isopropylamine) methanol-modified CO_2 after 40 minutes. T-tests were performed in order to statistically compare the average extraction recoveries after 40 minutes of all three additives with methanol vs methanol-modified CO_2 alone. With a 95% confidence interval, it was shown that the extraction recoveries of lovastatin (e.g. after 40 minutes)
were statistically greater with the use of isopropylamine rather than with trifluoroacetic acid and tributylphosphate or no additive at all.

The increased extractability of lovastatin with the secondary modifier, isopropylamine, after 40 minutes can not simply be explained by enhanced solubility, but by a combination of solubility and analyte displacement from the matrix. Excluding the active drug substance, common tablet ingredients include filling agents such as cellulose and starch as well as lubricants and coloring agents. Cellulose, for example, contains free methoxy and hydroxy acidic sites which contribute to the “activity” of the matrix. Lovastatin, containing a lactone ring (cyclic ester), may be considered basic due to

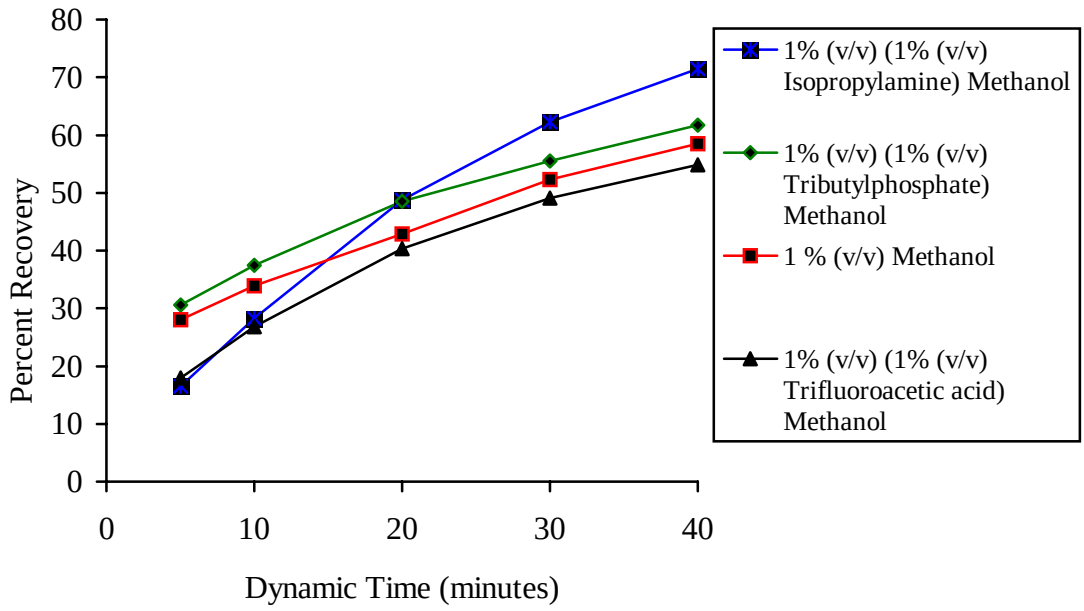
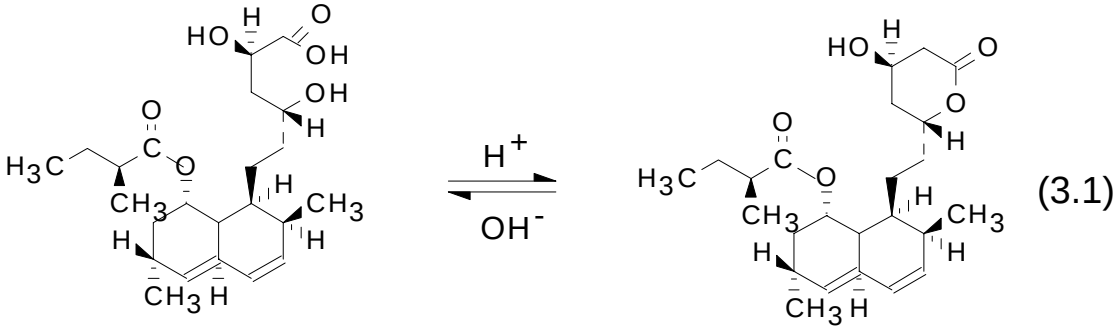


Figure 3.3. Effect of Additive Type on Lovastatin Recoveries (n=3) From In-House Prepared Tablet Powder Mixture

SFE Conditions. CO_2 Pressure: 400 atm; Oven Temperature: 40 °C; Liquid Flow Rate: 2.0 mL/min.; Restrictor Temperature: 50 °C; Solid-Phase Trap: 50/50 (w/w) Porapak Q/Glass Beads; Liquid Tandem Trap: Methanol; Liquid Tandem Trap Volume: 5 mL; Collection Temperature (Solid-Phase): 40 °C, Desorption Temperature (Solid-Phase); 40 °C; Solid-Phase Rinse Solvent/Volume: Methanol, 2.0 mL, Solid-Phase Rinsing Flow Rate: 1.0 mL/min.; Initial Static Time: 3.0 min.; Dynamic Time: 40.0 min. (total of 5 dynamic mini-steps); Static Time During Trap Rinsing: 2.0 min. *Solid-phase trap rinsed directly into tandem liquid trap following each dynamic step

Sample - 100 mg tablet powder mixture containing 10 mg lovastatin

unshared pairs of electrons on the oxygen in the lactone ring, as well as its ability to accept protons. When treated with base, lactone rings are known to open up due to hydrolysis of the cyclic ester. Specifically, Larson et al. report the conversion of lovastatin in fermentation broth to its hydroxyacid form when in the presence of 3% methanol-modified CO_2 and t-butylamine (**Equation 3.1**). [4]



(3.1)

Hydroxyacid lovastatin Lovastatin

Knowing that lovastatin is basic and that the tablet matrix contains many acidic sites, the enhanced extractability of lovastatin from the tablet powder mixture with the basic additive can be explained by displacement. In this case, when the basic additive was introduced, the stronger base, isopropylamine, preferentially adsorbed to the matrix thus displacing the basic analyte, lovastatin, from any acidic sites on the tablet powder matrix. The conversion of lovastatin to its hydroxyacid degradate during the extraction with isopropylamine was indeed a concern. However, when the SFC analysis which has the capability to separate lovastatin and its hydroxyacid degradate was performed, no additional chromatographic peaks were detected. Therefore, it was assured that the extracted lovastatin was present in the lactonized form. This was expected due to the low amounts of isopropylamine used (0.0001% (v/v)).

Phase C - Effect of Additive Concentration

Since the lovastatin extraction recoveries from the tablet powder mixture were shown to be statistically greater with isopropylamine than the extraction recoveries achieved with the other additives and methanol-modified CO_2 alone, the effect of isopropylamine concentration at various methanol-modified CO_2 concentrations was investigated further in Phase C. Surprisingly, increased additive concentrations (0.5%, 1.0%, 2.0% (v/v) in 1% (v/v) methanol-modified CO_2) at a constant modifier concentration did not affect the extraction recoveries or the extraction rate. It was believed that all matrix acidic sites were occupied by isopropylamine at a concentration of 0.5% (v/v) in methanol; therefore increased additive concentrations would not further increase lovastatin extractability.

The usefulness of the isopropylamine additive at various methanol-modified CO_2 concentrations can be also observed in **Figure 3.4**. T-tests were performed to compare the average extraction recoveries after 40 minutes with and without isopropylamine in 5% (v/v) methanol-modified CO_2. With a 95% confidence interval, it was shown that the extraction recoveries of lovastatin (e.g. after 40 minutes) were statistically greater when isopropylamine was used. Once again lovastatin extraction recoveries at various modifier concentrations were significantly enhanced with the presence of isopropylamine. Overall extraction recoveries over 40 minutes increased from 77% with 5% (v/v) methanol-modified to 86% with 5% (v/v) (1% (v/v) isopropylamine) methanol-modified CO_2.

Phase D - Extraction of MEVACOR® Tablets

A MEVACOR® tablet containing 10 mg of lovastatin was crushed, placed in an extraction vessel filled with cotton, and extracted under similar conditions as described in Phases A-C. The total extraction time was extended to 87 minutes (17 minutes total static time, 70 minutes total dynamic time) for 5% (v/v) methanol-modifier with and without

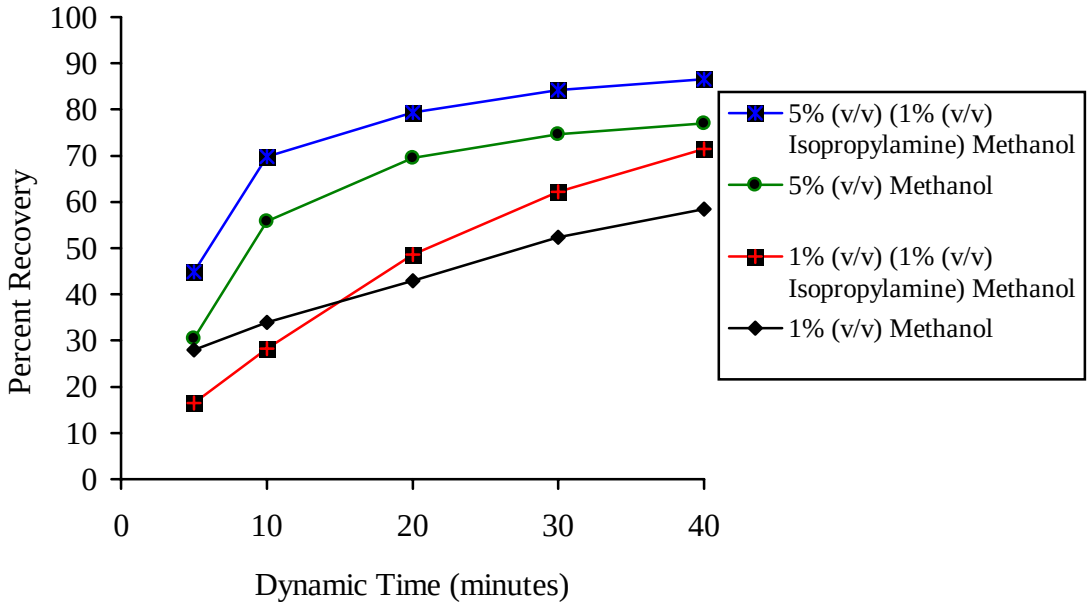


Figure 3.4. Effect of Methanol-Modifier Concentration on Lovastatin Recoveries (n=3) From In-House Prepared Tablet Powder Mixture

SFE Conditions. CO_2 Pressure: 400 atm; Oven Temperature: 40 °C; Liquid Flow Rate: 2.0 mL/min.; Restrictor Temperature: 50 °C; Solid-Phase Trap: 50/50 (w/w) Porapak Q/Glass Beads; Liquid Tandem Trap: Methanol; Liquid Tandem Trap Volume: 5 mL; Collection Temperature (Solid-Phase): 40 °C, Desorption Temperature (Solid-Phase); 40 °C; Solid-Phase Rinse Solvent/Volume: Methanol, 2.0 mL, Solid-Phase Rinsing Flow Rate: 1.0 mL/min.; Initial Static Time: 3.0 min.; Dynamic Time: 40.0 min. (total of 5 dynamic mini-steps); Static Time During Trap Rinsing: 2.0 min. *Solid-phase trap rinsed directly into tandem liquid trap following each dynamic step.

Sample - 100 mg tablet powder mixture containing 10 mg lovastatin

isopropylamine (**Figure 3.5**). Since the additive concentration in methanol had no statistical effect on recovery, 1% (v/v) isopropylamine was chosen. An overall recovery of only 84% was achieved with 5% (v/v) (1.0% (v/v) isopropylamine) methanol-modified CO_2 within an extraction time of 40 minutes (dynamic); however, 106% was recovered within 70 minutes (dynamic). A MEVACOR® tablet was also extracted with 5% methanol-modified CO_2 (e.g. no isopropylamine) where only 74% was recovered within 70 minutes (dynamic). The advantages of the addition of isopropylamine as an additive when extracting from the MEVACOR® tablet were clearly shown.

Although quantitative lovastatin recoveries from MEVACOR® were achieved with 5% (v/v) methanol (1 % (v/v) isopropylamine), the time required for the extraction was 87 minutes (17 min. total static time, 70 min. total dynamic time). A dynamic extraction without trap rinsing between dynamic mini-steps as well as an extraction time of approximately 30 minutes was desired for the final optimized SFE method. Similar to the previous studies, extraction profiles consisting of alternating static/dynamic steps with trap rinsing in between each dynamic step were performed in order to compare overall extraction recoveries achieved and time needed versus the various modifier and additive percentages. A modifier percentage of 10% (v/v) methanol with and without isopropylamine was then investigated (**Figure 3.6**). Overall extraction recoveries (n=1) of 95 and 88% were achieved with 10% methanol with and without isopropylamine respectively, but the time needed was 50 minutes (6 dynamic mini-steps). Further attempts were made to increase the extraction recovery to 100% and to reduce the time needed to approximately 30 minutes. Therefore, 15% (v/v) methanol with and without isopropylamine was investigated (**Figure 3.7**). Once again an enhancement was observed when isopropylamine was employed where 102% and 91% with and without isopropylamine respectively was recovered (n=1), and in this case, the extraction time needed with the isopropylamine was 35 minutes (static and dynamic). Percent lovastatin recovery comparisons as a function of methanol-modifier percentage, dynamic time needed, and addition of isopropylamine are found in **Table 3.3**.

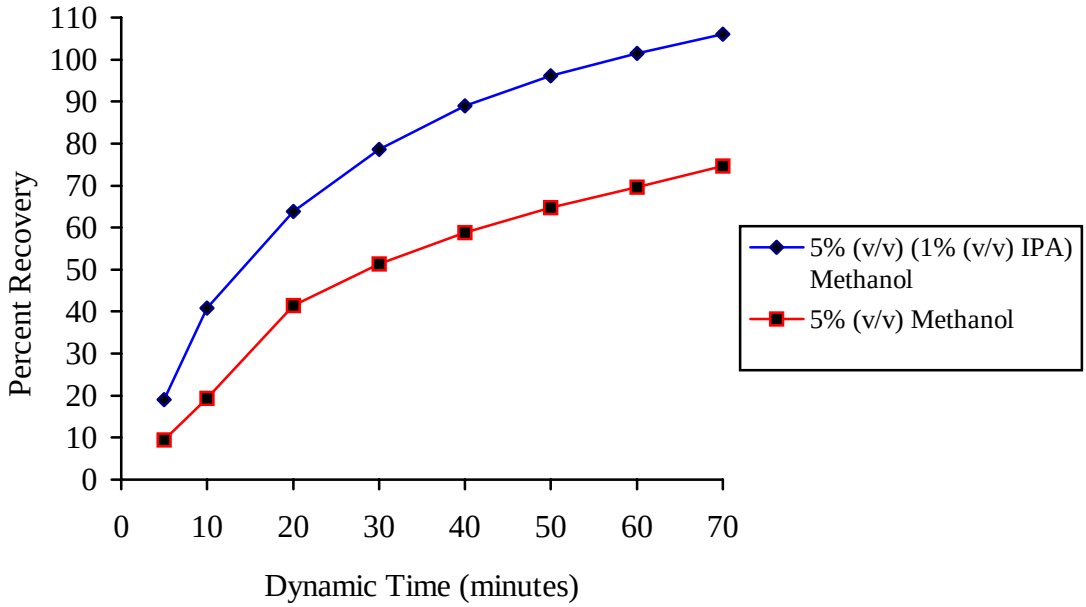


Figure 3.5. Subcritical Fluid Extraction (n=1) of Lovastatin From MEVACOR® Tablets at Various Additive/Modifier Concentrations

SFE Conditions. CO_2 Pressure: 400 atm; Oven Temperature: 40 °C; Liquid Flow Rate: 2.0 mL/min.; Restrictor Temperature: 50 °C; Solid-Phase Trap: 50/50 (w/w) Porapak Q/Glass Beads; Liquid Tandem Trap: Methanol; Liquid Tandem Trap Volume: 5 mL; Collection Temperature (Solid-Phase): 40 °C, Desorption Temperature (Solid-Phase); 40 °C; Solid-Phase Rinse Solvent/Volume: Methanol, 2.0 mL, Solid-Phase Rinsing Flow Rate: 1.0 mL/min.; Static Time: 3.0 min.; Dynamic Time: 70.0 min. (total of 8 dynamic mini-steps); Static Time During Trap Rinsing: 2.0 min. *Solid-phase trap rinsed directly into tandem liquid trap following each dynamic step.

Sample - 1 crushed MEVACOR® tablet containing 10 mg lovastatin

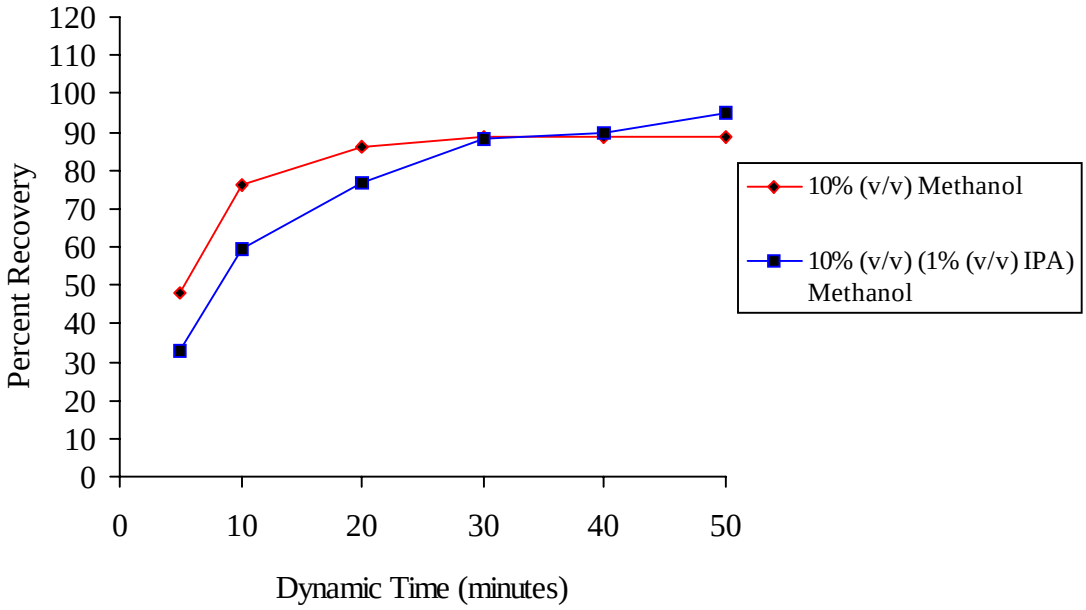


Figure 3.6. Subcritical Fluid Extraction (n=1) of Lovastatin From MEVACOR® Tablets at Various Modifier Concentrations With and Without isopropylamine

SFE Conditions. CO_2 Pressure: 400 atm; Oven Temperature: 40 °C; Liquid Flow Rate: 2.0 mL/min.; Restrictor Temperature: 50 °C; Solid-Phase Trap: 50/50 (w/w) Porapak Q/Glass Beads; Liquid Tandem Trap: Methanol; Liquid Tandem Trap Volume: 5 mL; Collection Temperature (Solid-Phase): 40 °C, Desorption Temperature (Solid-Phase); 40 °C; Solid-Phase Rinse Solvent/Volume: Methanol, 2.0 mL, Solid-Phase Rinsing Flow Rate: 1.0 mL/min.; Static Time: 3.0 min.; Dynamic Time: 50.0 min. (total of 6 dynamic mini-steps); Static Time During Trap Rinsing: 2.0 min. *Solid-phase trap rinsed directly into tandem liquid trap following each dynamic step.

Sample - 1 crushed MEVACOR® tablet containing 10 mg lovastatin

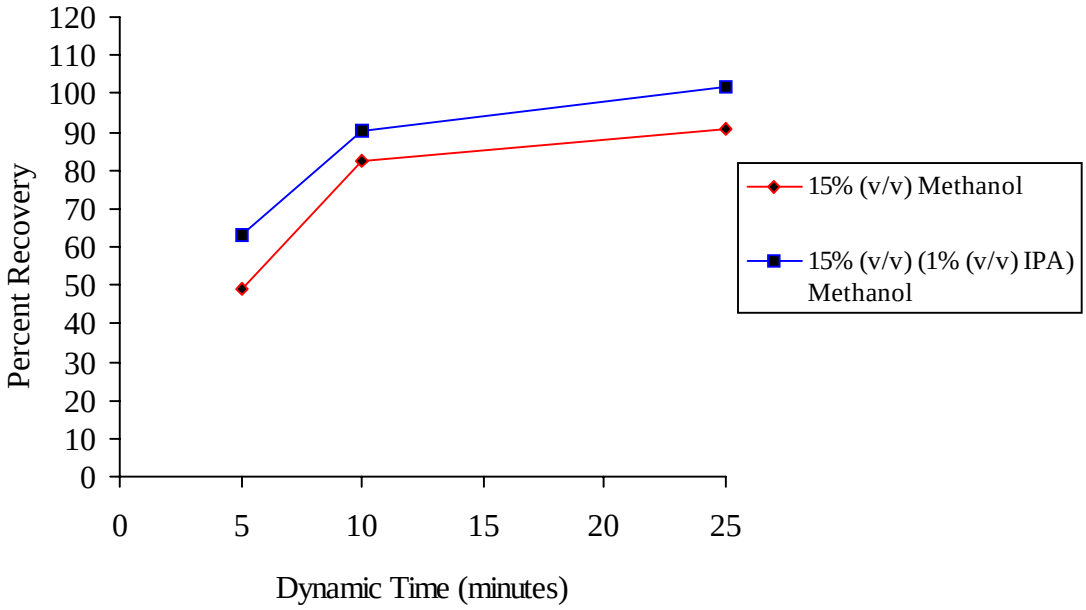


Figure 3.7. Subcritical Fluid Extraction (n=1) of Lovastatin From MEVACOR® Tablets at Various Modifier Concentrations With and Without isopropylamine

SFE Conditions. CO_2 Pressure: 400 atm; Oven Temperature: 40 °C; Liquid Flow Rate: 2.0 mL/min.; Restrictor Temperature: 50 °C; Solid-Phase Trap: 50/50 (w/w) Porapak Q/Glass Beads; Liquid Tandem Trap: Methanol; Liquid Tandem Trap Volume: 5 mL; Collection Temperature (Solid-Phase): 40 °C, Desorption Temperature (Solid-Phase); 40 °C; Solid-Phase Rinse Solvent/Volume: Methanol, 2.0 mL, Solid-Phase Rinsing Flow Rate: 1.0 mL/min.; Static Time: 3.0 min.; Dynamic Time: 25.0 min. (total of 3 dynamic mini-steps); Static Time During Trap Rinsing: 2.0 min. *Solid-phase trap rinsed directly into tandem liquid trap following each dynamic step.

Sample - 1 crushed MEVACOR® tablet containing 10 mg lovastatin

Table 3.3. SFE of MEVACOR® Tablets as a Function of Methanol-Modifier Percentage and Addition of Isopropylamine

% Methanol-Modified CO_2	Dynamic Time (min.)	% Recovery no Isopropylamine (n=1)	% Recovery (1% (v/v) Isopropylamine in Methanol) (n=1)
5%	70	84	106
10%	50	88	95
15%	25	91	102

SFE Conditions. CO_2 Pressure: 400 atm; Oven Temperature: 40 °C; Liquid Flow Rate: 2.0 mL/min.; Restrictor Temperature: 50 °C; Solid-Phase Trap: 50/50 (w/w) Porapak Q/Glass Beads; Liquid Tandem Trap: Methanol; Liquid Tandem Trap Volume: 5 mL; Collection Temperature (Solid-Phase): 40 °C, Desorption Temperature (Solid-Phase); 40 °C; Solid-Phase Rinse Solvent/Volume: Methanol, 2.0 mL, Solid-Phase Rinsing Flow Rate: 1.0 mL/min.; Static Time: 3.0 min.; Dynamic Time: see table; Static Time During Trap Rinsing: 2.0 min. *Solid-phase trap rinsed directly into tandem liquid trap following each dynamic step.

In the belief that an optimized method had been developed, 5 MEVACOR® tablets were then extracted with 15% (v/v) methanol with 1% (v/v) isopropylamine. The extraction method consisted of 3 dynamic mini-steps with a 2 minute static time added between each dynamic step to mimic trap rinsing as was used when constructing the previous extraction profiles. In this case, the solid-phase trap was not rinsed until the 35 min. extraction was completed. Average percent recoveries (n=5), standard deviations, and % RSDs are found in **Table 3.4**. It can be seen that 10 mg of lovastatin per tablet was fully recovered (99.5%) from the MEVACOR® tablets with a % RSD of 1.2% with 15 % (v/v) (1% (v/v) isopropylamine) methanol-modified CO_2 within 35 minutes. As compared to the traditional liquid extraction procedure (**Table 3.2**), the SFE method has been shown to be very advantageous (**Table 3.5**). The use of acetonitrile and buffer has been eliminated, and solvent consumption has been reduced from 95 mL to 17.5 mL of methanol consisting of: modifier (7.5 mL), tandem liquid trap (5 mL), and solid-phase rinsing (5 mL). Also many laborious and time consuming steps performed in the liquid extraction such as the addition of buffer and acetonitrile, mixing, sonicating, and cooling steps have been eliminated. As compared to the traditional liquid extraction procedure, the extraction time was reduced from over an hour to merely 35 minutes by using SFE. All that is required for the SFE method is crushing the tablet, placing it in the extraction vessel, and performing the one-step extraction.

Table 3.4. SFE Reproducibility for Lovastatin (10 mg) from MEVACOR® Tablets with 15 % (1.0% (v/v) isopropylamine) Methanol-Modified CO_2

Tablet #	Percent Recovery
1	98.4
2	101.0
3	98.7
4	98.7
5	100.5
Avg. Percent Recovery	99.5
RSD	1.2

SFE Conditions. CO_2 Pressure: 400 atm; Oven Temperature: 40 °C; Liquid Flow Rate: 2.0 mL/min.; Restrictor Temperature: 50 °C; Solid-Phase Trap: 50/50 (w/w) Porapak Q/Glass Beads; Liquid Tandem Trap: Methanol; Liquid Tandem Trap Volume: 7 mL; Collection Temperature (Solid-Phase): 40 °C, Desorption Temperature (Solid-Phase); 40 °C; Solid-Phase Rinse Solvent/Volume: Methanol, 5.0 mL, Solid-Phase Rinsing Flow Rate: 2.0 mL/min.; Static Time: 3.0 min.; Dynamic Time: 25.0 min. (total of 3 dynamic mini-steps); Static Time Between Dynamic Steps: 2.0 min.

Table 3.5. Traditional Liquid Extraction and SFE Solvent Use, Steps, and Time Comparisons

	Traditional liquid extraction	**SFE**
Volume of Solvent Used	95 mL acetonitrile/buffer	17.5 mL methanol total • 7.5 mL modifier • 5 mL tandem-liquid trap • 5 mL solid-phase rinse
Mixing	XXX	none
Sonication	XXX	none
Cooling	XXX	none
Tablet Crushing	none	XXX
Extraction Time Including Sample Preparation	>60 min.	35 min.

3.4 SUMMARY

The effect of primary and secondary modifiers (i.e. additives) on the subcritical fluid extraction of lovastatin from in-house prepared tablet powder mixtures and MEVACOR® tablets was investigated. Methanol-modifier percentage, additive type (acidic, basic, neutral) in methanol, and the effect of additive concentration on the extraction efficiency were examined. Extractability was shown to depend on modifier concentration and additive type. Isopropylamine was believed to be the most successful additive because of its ability to displace adsorbed lovastatin from the acidic tablet matrix sites, an effect not possible with methanol-modified CO_2 alone. An optimized extraction method was developed, and lovastatin recoveries of 99.5% with a RSD of 1.2% from MEVACOR® tablets with 15% (v/v) (1.0% (v/v) isopropylamine) methanol-modified CO_2 was achieved.

CHAPTER IV

INVESTIGATION OF ION-PAIRING ADDITIVES FOR THE SUPERCRITICAL FLUID EXTRACTION OF TRIPHENYLPHOSPHINETRISULFONATE, SODIUM SALT

4.1 INTRODUCTION

It was demonstrated in Chapter III that the extraction of a highly polar, nonionizable pharmaceutical, lovastatin, from a complicated matrix (i.e. MEVACOR® tablets) was indeed feasible using subcritical fluid extraction. The effect of the addition of methanol to the carbon dioxide was examined, and it was shown that the recoveries could be enhanced due to a subsequent increase in the solvating power of the fluid. Although an enhancement was observed, a secondary modifier (i.e. additive), specifically isopropylamine, was needed to possibly overcome significant analyte-matrix interactions. Due to the mildly basic characteristics of the drug, a stronger base was added to the fluid to facilitate displacement of the drug from any acidic sites on the tablet matrix. In this Chapter, the usefulness of other types of additives will be investigated for the extraction of an ionic compound.

As described previously, the use of SFE in the pharmaceutical field has been limited. Many pharmaceuticals are ionic/ionizable, water soluble, and highly polar. Due to the low dielectric constant (1.0 - 1.6) of supercritical CO_2, ionic compounds are considered insoluble in pure CO_2 alone, and are therefore unextractable.

A technique used in liquid-liquid extractions for the extraction of ionic compounds is ion-pair extraction. [1-2] The ionic species of interest, through electrostatic interactions, is essentially neutralized by combination with a counter-ion of opposite charge. The resulting ion-pair is then extracted.

Numerous reports have examined the role of ion-pairing reagents in the supercritical fluid extraction of anionic species. [3-9] Most commonly an ion-pairing reagent such as a tetralkylammonium salt [NR_4^+] is added in a known volume of solvent to the solid matrix to form an ion-pair [NR_4A] with the analyte [A^-]. Common extracted analytes include phenoxyacid herbicides, alkylsulfonates, and sulphonamides. Several papers have illustrated the combination of ion-pairing and alkylation. [3-7] Hawthorne et al. extracted a) spiked and native phenoxyacid herbicides from soils and sediments, b) microbial phospholipids, and c) phenols from solid sorbents as methylester derivatives using trimethylphenylammonium hydroxide utilizing this technique. [3]

In 1992, Field et al. reported on the extraction of secondary alkylsulfonate and linear alkylbenzensulfonate surfactants from sewage sludge as their tetrabutylammonium ion-pairs, followed by subsequent quantification by GC-MS. [4] The ion-pairs were said to form their corresponding butyl esters in the injection port of the gas chromatograph. The recoveries were shown to be enhanced by 2.5 fold with the presence of the ion-pairing reagent. Further studies describing the supercritical fluid extraction of a phenoxyacid herbicide (2,4-dichlorophenoxyacetic acid) from soil were described by Rochette et al. [5]

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[1] *R.W. Frei, J.F. Lawrence, Eds.*, "Chemical Derivatization in Analytical Chemistry, Volume 2: Separation and Continuous Flow Techniques", Plenum Press, New York (1982).
[2] *K. Blau, G.S. King, Eds*., "Handbook of Derivatives for Chromatography", Heyden & Son, Ltd., Philadelphia (1978).
[3] *S.B. Hawthorne, D.J. Miller, D.E Nivens, D.C. White,* Anal. Chem., **64** (1992) 405.
[4] *J.A. Field, D.J. Miller, T.M. Field, S.B. Hawthorne, W. Giger,* Anal. Chem., **64** (1992) 3161.
[5] *E.A. Rochette, J.B. Harsh,* Talanta, **40** (1993) 147.
[6] *V. Lopez-Avila, N.S. Dodhiwala,* J. Agric. Food Chem., **41** (1993) 2038.
[7] *T.S. Reighard, S.V. Olesik,* Anal. Chem., **69** (1997) 566.
[8] *M.T. Tena, M.D. Luque de Castro, M. Valcárcel,* Chromatographia, **40** (1995) 197.
[9] *N. Din, K.D. Bartle, A.A. Clifford,* J. Chromatogr. Sci., **35** (1997) 31.

Several strategies were studied with the herbicide including silylation, methyl esterification, ion-pairing, and ionic displacement. A solution of [*m*-(trifluoromethyl)phenyl]trimethylammonium hydroxide in methanol was added as the ion-pairing modifier; however, the recoveries were only seen to increase from 6% to 16% in the presence of the ion-pairing reagent versus methanol alone. Of the 4 strategies investigated, ionic displacement with a strong cation displacing agent, $CaCl_2 \cdot 2H_2O$, in methanol yielded the most quantitative recoveries (approximately 87%) from the very active soil matrix.

In 1993, Lopez-Avila et al. investigated SFE as a means of extracting seven chlorophenoxyacid herbicides from soil samples. [6] Several ion-pairing/derivatization agents were investigated including: trimethylphenylammonium hydroxide (TMPA), benzyltrimethylammonium chloride (BTMAC), benzyltriethylammonium chloride (BTEAC), and tetrabutylammonium hydroxide/methyl iodide (TBA/MI). The main purpose for adding these reagents was to form the methylester derivative during the extraction so that they subsequently could be analyzed by gas chromatography. When TMPA, BTEAC, and TMPA were compared in a series of spike studies from sand, it was shown that TMPA resulted in the most favorable recoveries (63 - 96%). Additional experiments were then performed with TMPA which indicated that the conversion from the chlorophenoxyacid herbicides to their corresponding methylesters was indeed occurring in the injection port of the GC rather than during the static SFE step.

The use of trimethylphenylammonium hydroxide (TMPA) in methanol added as a static modifier (ion-pairing/derivatizing agent) versus methanol-modified CO_2 was compared for the extraction of phenoxyacid herbicides from house dust. [7] First, the extraction recoveries were compared with 200 µL of methanol vs 200 µL of TMPA. Similar recoveries were achieved in both cases, therefore the addition of TMPA as an ion-pairing reagent was shown not to enhance the analyte extraction. Similar to the work of Lopez-Avila, the use of methanol-modified CO_2 (20 mol%) was shown to be a better

alternative than the use of the static modifiers, resulting in recoveries ranging from 83% to 95%.

The combination of ion-pairing followed by SFE was also investigated as a means of extracting sulphonamides from solid-supports and meat tissues. [8-9] Tena et al. compared the extraction efficiencies of 5 sulphonamides from diatomaceous earth within and in the absence of 1 mL of trimethylphenylammonium hydroxide in methanol. The use of TMPA was shown to be quite advantageous wherein the recoveries were enhanced from an average of 30% to 96%. Comparisons between pure and methanol-modified CO_2 were also made. Surprisingly, less than 20% of each sulphonamide was extractable in the absence of TMPA and less than 5% in the presence of TMPA with methanol-modified CO_2.

The extraction of sulphamethazine and its metabolites from parts-per-million spiked "wet" and freeze-dried swine liver and kidney was carried out with both methanol-modified CO_2 and in situ ion-pairing with tetramethylammonium hydroxide (TMA) in 1997 by Din et al. [9] Recoveries were very matrix dependent, with the lower polarity sulphonamides found to be easily extractable from wet kidney with 10% methanol-modified CO_2. Extraction of the freeze-dried tissues was problematic, this was postulated to be due to large sulphonamide-matrix binding. The recoveries of only 3 of the 5 more polar and ionic sulphonamides from ground freeze-dried kidney were enhanced in the presence of TMA. Enhancements ranged from 23% to 72%. The ineffectiveness of TMA for the other matrices was attributed to poor reagent to analyte site accessibility.

Previous results have been mixed, some reporting that the addition of an ion-pairing reagent directly to the matrix significantly enhances the extractability of an ionic species from a matrix. Others have reported no real improvements postulating high analyte-matrix binding, poor reagent to analyte site accessibility, or have shown that the use of modifiers (methanol-modified CO_2 versus pure CO_2) are just as useful or even better than results obtained using ion-pairing reagents. [3-9] The objective of our study was to fundamentally examine the extraction of an anionic species,

triphenylphosphinetrisulfonate, sodium salt, from a relatively uncomplicated matrix, sand, with supercritical CO_2 aided by various ion-pairing additives. It was of interest to examine the ion-pair extraction process in more detail than has been previously studied by others. For example, if tetralkylammonium hydrogen sulfate reagents were used as the ion-pairing reagent, would a tetrahexyl versus a tetramethyl ion-pair complex be more extractable? Would the recoveries be improved if the reagent was added in excess? Would the recoveries be enhanced in the presence of a greater amount of modifier? Is the extraction dependent upon the fluid's density, extraction temperature, flow rate, equilibration time, or amount of CO_2 used? It is this line of questioning we wished to investigate.

4.2 EXPERIMENTAL

Instrumentation

All extractions were performed on the Isco/Suprex Prepmaster (Lincoln, NE) consisting of a dual reciprocating pump, temperature-controlled oven, Duraflow® restrictor, and an Accutrap® solid-phase trap collection and rinsing device. A Hewlett Packard 1090 Liquid Chromatographic System (Wilmington, DE) was used for all extract analyses.

Reagents

Carbon dioxide (SFE/SFC grade) with approximately 2000 psi helium headspace was obtained from Scott Specialty Gases (Plumsteadville, PA). Triphenylphosphinetrisulfonate, sodium salt, was supplied by the DuPont Merck Pharmaceutical Company, Radiopharmaceuticals Division (N. Billerica, MA) and used as received. Caffeine, tetramethylammonium hydrogen sulfate, and tetrahexylammonium

hydrogen sulfate were obtained from Sigma Chemical Company (St. Louis, MO). Ottawa Cement Testing sand was supplied by Fisher Scientific (Raleigh, NC).

Extraction Conditions and General Procedure

A screening study consisting of a fractional-factorial design was implemented to investigate the effect of several ion-pairing parameters including ion-pairing reagent composition and mole ratio of ion-pairing reagent to TPPTS upon the extraction efficiency of TPPTS from a spiked-sand surface. Several extraction parameters including CO_2 density, extraction temperature, static extraction time, CO_2 mass, liquid CO_2 flow rate, and methanol in-cell spike volume were also investigated. RS1 Discover software (BBN Software Products Corp., Cambridge, MA) was used for setting up the experimental design as well as for data analysis. To simply experimental design and data interpretation, two assumptions were made: 1) a linear response existed for each factor investigated and 2) no interactions existed among the various controlled factors which influenced the results. A high and a low value were chosen for each controlled factor except for ion-pairing reagent composition and concentration (**Table 4.1**). If a full-factorial design had been performed with 8 controlled factors, 384 experiments would have resulted. Due to time constraints, a fractional-factorial experimental design was chosen wherein 21 individual experiments (n=3) were randomly investigated. The specific extraction methods utilized and the order in which they were conducted are found in **Table 4.2**.

The following general procedure was used for all studies. A 5.0 mL extraction vessel (Keystone Scientific, Bellefonte, PA) was filled to approximately 90% with Ottawa Cement Testing Sand (Fisher Scientific, Raleigh, NC). Triphenylphosphinetrisulfonate, sodium salt, was dissolved in methanol to prepare a 6000-ppm solution which was used to spike (50 μL) the sand (300 μg spike mass) contained in the vessel. Either pure methanol, or a solution of the appropriate ion-pairing

Table 4.1. High/Low Extraction Parameters Investigated*

	Low	High
Mole Ratio (Ion-Pairing Reagent:TPPTS)	3:1	15:1
Carbon Dioxide Density (g/mL)	0.71	0.85
Extraction Temperature (°C)	35	80
Static Extraction Time (min.)	0	30
Dynamic Mass (g of CO_2)	15	40
Flow Rate (mL/min.)	1.0	2.0
Methanol in-cell Spike Volume (μL)	75	200

* Ion-Pair Reagents were either Tetramethylammonium hydrogen sulfate or Tetrahexylammonium hydrogen sulfate

Table 4.2. Parameters Used for Fractional-Factorial Experiments

Extraction Order	Ion-Pair Reagent	Mole Ratio [Ion-Pairing Reagent]: [TPPTS]	CO_2 Density (g/mL)	Temp (°C)	Static Time (min.)	MeOH in-cell Spike Volume (mL)	Dynamic Mass (g CO_2)	Liquid Flow Rate (mL/min.)
1	hexyl*	1:1	0.71	35	30	75	15	2.0
2	hexyl	1:1	0.71	80	0	75	40	1.0
3	none***	0:1	0.85	35	0	200	40	1.0
4	none	0:1	0.71	80	30	200	40	2.0
5	methyl**	5:1	0.71	35	30	200	15	1.0
6	hexyl	1:1	0.85	80	0	200	15	1.0
7	methyl	1:1	0.71	35	0	75	40	1.0
8	none	0:1	0.85	35	0	75	15	2.0
9	methyl	5:1	0.85	80	30	75	15	2.0
10	methyl	5:1	0.85	80	0	200	40	2.0
11	methyl	1:1	0.85	35	30	200	40	2.0
12	none	0:1	0.85	35	30	75	15	1.0
13	hexyl	5:1	0.71	35	0	200	15	2.0
14	none	0:1	0.71	80	30	75	40	1.0
15	methyl	5:1	0.71	80	30	200	15	1.0
16	none	0:1	0.85	80	0	200	15	1.0
17	methyl	1:1	0.71	35	0	75	40	1.0
18	hexyl	5:1	0.85	80	30	75	40	1.0
19	hexyl	5:1	0.85	35	0	200	40	2.0
20	methyl	1:1	0.85	80	0	75	15	2.0
21	none	0:1	0.71	80	0	75	15	2.0

Static time - time allotted for equilibration between analyte and fluid
Dynamic mass - mass of CO_2 flowed at a continuous rate through extraction vessel
* denotes ion-pairing reagent -tetrahexylammonium hydrogen sulfate
** denotes ion-pairing reagent - tetramethylammonium hydrogen sulfate
*** denotes - no ion-pairing reagent added

reagent in methanol was then spiked onto the previously trisulfonate spiked-sand surface. The total methanol-spike volume into the vessel including TPPTS with or without ion-pairing reagent was 75 or 200 μL, depending on the extraction method. The extraction vessel was then sealed, shaken by hand vigorously, and immediately extracted. The spiked-sand surface was not allowed to dry prior to extraction.

Extraction Trapping Conditions

The solid-phase trap used in this study consisted of glass beads (80/100 mesh). To ensure proper trapping of all extracted analyte, a tandem-liquid trap consisting of 2.0 mL methanol immediately followed the solid-phase trap. The solid-phase trap and tandem-liquid trap were maintained at (-) 5 °C and room temperature, respectively during the extraction. Upon completion of the extraction, the solid-phase trap temperature was raised to 30 °C, and the trap was rinsed with 2.0 mL of methanol into an empty collection vessel. Both the solid-phase trap rinse and the tandem liquid trap were diluted with 3.0 mL of HPLC grade water and analyzed by HPLC separately.

Extract Analysis

Upon completion of the extraction, 50 μL of 6000-ppm caffeine in methanol (300 μg) was added to both the solid-phase trap rinse and tandem liquid trap as an internal standard. The purpose of adding internal standard to the trap rinse was to ensure good quantitation in case there were variations in solid-phase trap rinse volumes and/or evaporation losses in the tandem-liquid trap during the extraction. Analysis of the extract solutions was performed by HPLC. Values corresponding to 100% recovery were obtained by adding 50 μL of the TPPTS spiking-solution (6000-ppm) and 50 μL of the caffeine internal standard solution (6000-ppm) to an empty collection vial which was

diluted with 2.0 mL methanol and 3.0 mL of water. Percent recovery values for extracted TPPTS were calculated by direct comparison to the 100% standard.

Chromatographic Conditions

All separations were performed on a Cosmosil 5C18-MS (25 cm x 4.6 mm i.d., 10 μm) column (Waters, Milford, MA) with mobile phase A consisting of 95% 20mM phosphate buffer, 5% methanol, 0.05% triethylphosphite (v/v), and mobile phase B consisting of 95% methanol, 5% water, 0.05% triethylphosphite (v/v). The mobile phase gradient program was: 100% A for 2 min., ramped down to 66% A in 13 min., ramped down to 33% A in 17 min., ramped down to 5% A in 17.5 min. and held for 4.5 min. The system was then allowed to equilibrate for 7 min. with 100% A prior to the next injection. The column was maintained at 40 °C. A flow rate of 1.0 mL/min and injection volume of 10 μL was used. Detection was by UV at 260 nm.

4.3 RESULTS AND DISCUSSION

The goal of this research was to identify the main factors including both ion-pairing additive and SFE parameters that influenced the extraction of triphenylphosphinetrisulfonate (TPPTS) (**Figure 4.1**) from a relatively uncomplicated matrix such as sand. This was accomplished by performing a screening study that investigated ion-pairing reagent composition, the mole ratio of ion-pairing reagent to TPPTS in the extraction vessel, carbon dioxide density, extraction temperature, static extraction time, CO_2 mass used, and liquid CO_2 flow rate.

It was believed that ion-pairing reagent composition and the relative amount of reagent added would play a significant role in the extraction process. Two ion-pairing reagents which varied in lipophilicity were investigated: tetramethyl- and tetrahexylammonium hydrogen sulfate. It was believed at the outset that due to their

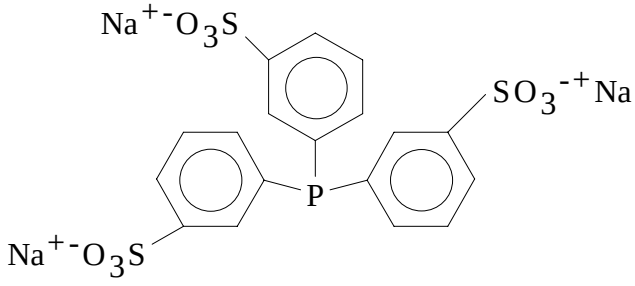


Figure 4.1. Chemical Structure of Triphenylphosphinetrisulfonate, Sodium Salt (TPPTS)

polarity, ionic compounds do not have sufficient solubility in the non-polar fluid carbon dioxide. In order to enhance the ionic compound's solubility and thus extractability, an ion-pairing reagent may be added to possibly neutralize the ionic compound's charge, and at the same time add sufficient lipophilicity to the compound. This would act to make it more non-polar, and thus more extractable with a nonpolar fluid such as CO_2. Our hypothesis was that the extraction efficiencies of TPPTS would be greatest with the more lipophilic ion-pairing reagent because of its reduced polarity . Secondly, the mole ratio of ion-pairing reagent to TPPTS was investigated. Complete ion-pair formation should be more favored when an excess of ion-pairing reagent was present.

$$R'SO_3^-Na^+ + R_4NHSO_4 \rightleftharpoons R'SO_3^-N^+R_4 + NaHSO_4 \quad (1)$$

Several SFE parameters were also investigated including carbon dioxide density, extraction temperature, static extraction time, mass of CO_2 used, and CO_2 liquid flow rate. It is well known that the solvating power of CO_2 is enhanced at a higher density. A higher extraction temperature may also increase the solubility of the analyte. The extraction efficiencies may be greater at a higher extraction temperature due to increased solubility as well as decreased analyte-matrix interactions. Static extraction time was also investigated. Higher extraction recoveries would be expected if more time was allowed for ion-pair formation to occur under supercritical conditions. The next parameter investigated was dynamic CO_2 mass. Larger amounts of CO_2 may be needed to extract all analyte. Lastly, flow rate was investigated. In the case of no static extraction time and at a higher flow rate, not enough time may be allowed for ion-pair formation to occur. Thus there would be less partitioning of the ion-pair into the fluid giving rise to lower recoveries.

Average percent recovery of TPPTS from a spiked-sand surface versus the various methods investigated is plotted in **Figure 4.2**. Error bars representing one standard

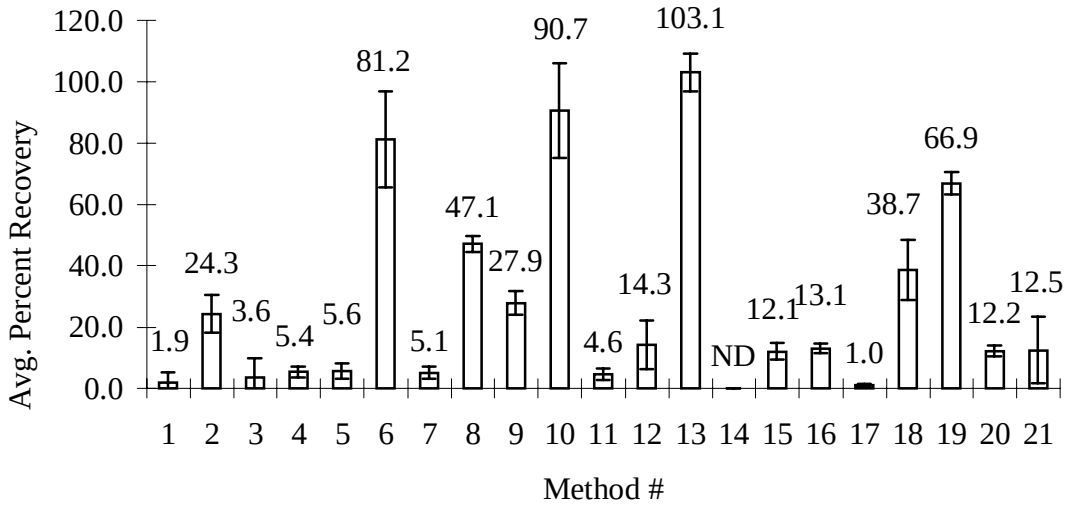


Figure 4.2. Average Percent Recovery (n=3) TPPTS vs Methods Investigated see Table 4.2 for Method # extraction conditions

deviation are also shown. No two methods were alike, therefore, the influence of each particular parameter could not be ascertained from this graph. It can be observed, however, that many methods resulted in very unfavorable recoveries, several methods resulted in adequate recoveries (> 60%), and one method resulted in a recovery of approximately 100%.

In order to evaluate if any of the ion-pairing and extraction parameters did indeed have a significant effect on the recovery of TPPTS from a spiked-sand surface, least square coefficients were determined for the screening study. The assumptions of this model were that a linear response existed among all the variables investigated and that no interactions existed among the factors investigated thus attributing to the response (recovery). Least squares coefficients are used to describe the relationship between the recovery and the settings of the factor so that a mathematical expression can be made. The sign and the magnitude of the least squares coefficients indicate what type of effect the variable has on the response (recovery). Furthermore, by examining the p-values or the significance values, one can determine if the factor investigated had a significant effect on the response (recovery). For this study, the null hypothesis was that each variable did not contribute to the overall recovery of the anionic species regardless of its setting. Specifically, the p-value, is the probability of making a Type I error, which is rejecting a true null hypothesis. For a p-value of 0.1 (90% confidence interval), there is a 10% probability that accepting the null hypothesis is incorrect. Therefore, if the controlled factor had a p-value of < 0.1, the null hypothesis that the variable did not significantly affect the recovery of the anionic species was rejected.

In this study, within a 90% confidence interval, the ion-pairing reagent composition, mole ratio of ion-pairing reagent to TPPTS, static time, and in-cell methanol spike volume were found to significantly play a role in the extraction process ($p < 0.1$). Therefore, CO_2 density, CO_2 mass used, and flow rate were deemed to be unimportant factors. These factors were removed from the screening study model, and the least squares coefficients, T-values, and p-values were fitted for the reduced model (**Table 4.3**).

Table 4.3. Least Square Coefficients, Reduced Fit, Response Percent Recovery

Term				
Constant	27.4 +/- 4.9			
	Slope	**Error of Slope**	**t-Value**	**p-Value**
IP Reagent Composition				0.0264
none	-14.1	6.7	2.11	0.0513
TetramethylAHS	-7.5	6.7	1.12	0.2783
TetrahexylAHS	21.6	7.3	2.98	0.0089
IP:TPPTS Mole Ratio				0.0571
3:1	-9.9	4.8	2.05	0.0571
15:1	9.9	4.8	2.05	0.0571
Static Time	-11.0	4.9	2.26	0.0384
In-Cell Methanol Spike Volume	8.7	4.8	1.81	0.0886

As previously stated, the sign and magnitude of the slope of the least squares coefficients can be used to visualize how the change in each parameter affects the overall response (recovery). For example, the term having the largest slope was the addition of tetrahexylammonium hydrogen sulfate (21.6 ± 7.3), thus indicating that the response was most affected by this addition. This must be confirmed by examination of the p-value. For this particular term, the p-value was 0.0089. As previously described, by having a p-value of less than 0.1, this effect on the recovery was shown to be statistical. The slope may also be negative. For example, the addition of tetramethylammonium hydrogen sulfate as well as static extraction time negatively impacted the response. However, by examining the p-values (<0.1), only static extraction time was shown to statistically decrease the response (recovery). The relative importance of each of these terms (parameters) will be discussed later.

The significant main effects on the recovery were next determined (**Figure 4.3**). The main effect for each influential parameter is the difference between the means of all the runs at the high setting and low setting for a particular parameter regardless of the other settings. The most influential parameter on the extraction recoveries was determined to be the ion-pairing reagent composition. First, by examining the p-values (**Table 4.3**), the recoveries were shown to be statistically affected by the addition of tetrahexylammonium hydrogen sulfate ($p < 0.1$). The addition of tetramethylammonium hydrogen sulfate apparently had no affect on the recovery of the anionic species, TPPTS, from the spiked sand surface. Once again, it was believed that two factors are operating: charge neutralization and the addition of lipophilic characteristics to the polar, ionic compound. Although the charge on TPPTS may be neutralized with the addition of any ion-pairing reagent, the compound may be still too polar to be extractable with methanol-modified CO_2. In the case of the addition of tetrahexylammonium hydrogen sulfate, the formed ion-pair complex would be more non-polar, and thus more soluble and extractable than when forming the tetramethylammonium ion-pair. On the average, it was seen that

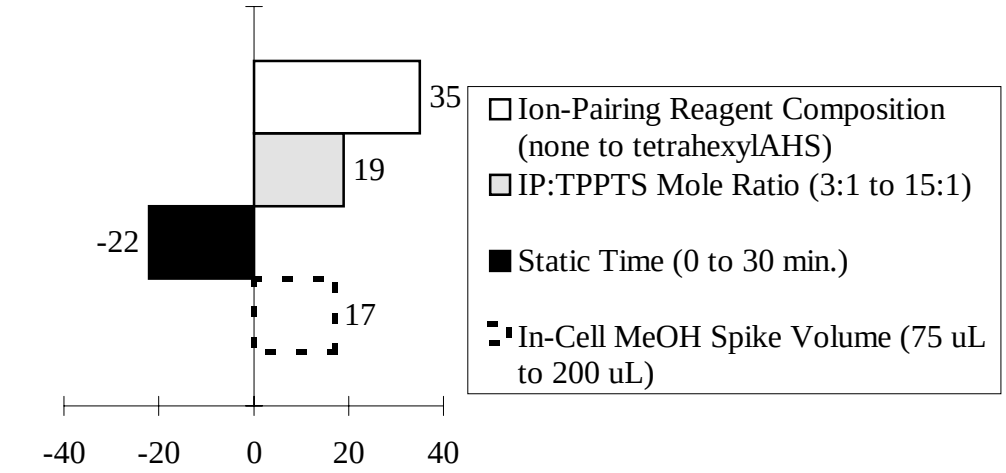


Figure 4.3. Significant Main Effects on Recovery

by simply adding tetrahexylammonium hydrogen sulfate to the sample matrix, the recoveries from the spiked-sand surface were increased by 35%.

In order to better visualize the effectiveness of the addition of tetrahexylammonium hydrogen sulfate, the recoveries of the 7 experimental methods that were performed without any ion-pairing reagent present were averaged. Once again, no 2 experimental methods were alike. On the average, under the various extraction methods, 14% of the trisulfonate was extracted from the spiked-sand surface. By simply adding the tetrahexyl ion-pairing reagent, the recoveries on the average were increased by 3.5 fold.

The second parameter found to be influential was the amount of ion-pairing reagent added or the mole ratio of ion-pairing reagent to analyte. For example, TPPTS contains three sulfonate groups, therefore, when placed in solution, the compound has a net charge of -3. Ion-pairing reagent was added in a mole ratio of 3:1 and 15:1 which would correspond to a 1:1 charge neutralization or in excess, a 5:1 charge neutralization. It was observed that by adding an excess of ion-pairing reagent (15:1), the recoveries increased by approximately 19% versus the 3:1 mole ratio. The increase in recovery can be simply explained by Le'Chatliers principle. When an excess of ion-pairing reagent was added, the equilibrium shifted towards the right favoring ion-pair formation (see Equation 1). In this case, it was assumed that at a 1:1 charge neutralization ion-pair formation was incomplete.

Static extraction time was the third parameter found to be influential. It was originally hypothesized that a static time would be beneficial for the formation of the ion-pair complex. However, this was not the case. The recoveries decreased by approximately 22% with a static time of 30 minutes versus no static time. Present in the extraction vessel was TPPTS, ion-pairing reagent, and a certain volume of methanol. The methanol was not predried and was assumed to contain about 0.1% (w/w) water. In the presence of carbon dioxide, water will react to form carbonic acid, thus the overall pH of the resulting solution may be decreased to as low as 3.0. [10] The pKa of

[10] *K.L. Toews, R.M. Shroll, C.M. Wai, N.G. Smart,* Anal. Chem., **67** (1995) 4040.

triphenylphosphinetrisulfonate is 2-3. If the pH of the resulting solution is decreased to approximately 3.0, the equilibria will consist of approximately a 50/50 mixture of the anionic trisulfonate as well as the protonated (neutralized) trisulfonate. Therefore, less ionic species would have been available to react with the ion-pairing reagent, thus resulting in lower recoveries. This hypothesis would assume that the protonated (neutralized) trisulfonate is insoluble in MeOH-modified CO_2; however, this assumption could not be experimentally confirmed. Due to the trisulfonate's low pKa, it would be extremely difficult to obtain experimental conditions (pH ~ 1) where it is assumed that the trisulfonate exists solely in the protonated (neutralized) form. Also, the use of an acid would be needed and thereby the analyte-matrix would be modified. Therefore, if enhanced extractability was observed, one could not simply attribute this response to charge neutralization. Decreased analyte-matrix (sand) interactions must also be considered.

The fourth parameter shown to influence the recoveries of TPPTS from a spiked-sand surface was the in-cell methanol spike volume. The purpose of the methanol was threefold. First, the methanol served as a way of introducing a known amount of the ion-pairing reagent to the matrix. Second, since ionic compounds are known to exhibit little solubility in CO_2, the methanol offers a medium for the analyte and ion-pairing reagent to mix and form the ion-pair. Third, the methanol could serve as a modifier, thus increasing the solvating power of the fluid towards more polar compounds. For these reasons, the recoveries were enhanced on the average of 17% when the volume of methanol was increased from 75 μL to 200 μL.

As previously stated, when examining TPPTS recoveries versus the various experiments, one particular method (Method 13) resulted in 103% recovery (6.0% RSD). If one compares the parameters for this method with the identified influential parameters, it is found that tetrahexylammonium hydrogen sulfate was used as the ion-pairing reagent and was added at a 15:1 molar excess. Also, no static extraction time was employed, and

200 μL of methanol was present in the extraction vessel. Experiment 13 offers proof that the influential parameters identified in this screening study were indeed optimal.

4.4 SUMMARY

A screening study consisting of a fractional-factorial design was performed to identify the influential parameters that significantly affected the recovery of triphenylphosphinetrisulfonate, sodium salt, from a spiked-sand surface with ion-pairing additives. The four influential parameters were ion-pairing reagent composition, mole ratio of ion-pairing reagent to TPPTS, static extraction time, and in-cell methanol spike volume. First, the recoveries of the anionic species were shown to be enhanced when in the presence of an ion-pairing additive. Of the two quaternaryalkylammonium salts investigated, the more lipophilic reagent, tetrahexylammonium hydrogen sulfate, was the only ion-pairing reagent that statistically enhanced the recoveries. The increased extractability with the ion-pairing reagent in the non-polar fluid, CO_2, was attributed to reduced analyte polarity. Second, the amount of ion-pairing reagent added was also shown to be influential. By adding an excess of ion-pairing reagent, the equilibrium was shifted towards full and complete ion-pair formation, and an enhancement was observed. Third, static time was shown to negatively affect the recoveries. Over time and in the presence of moisture, it was believed that a mixture of both the neutral and charged trisulfonate species were present, therefore, less ionic species were present to form the ion-pair complex thus lower recoveries were observed. Lastly, the recoveries of the polar compound were shown to be enhanced by increasing the polarity of the fluid by a simple increase in the in-cell methanol spike volume.

CHAPTER V

MODIFIER AND ADDITIVE EFFECTS IN THE SUPERCRITICAL FLUID EXTRACTION OF PSEUDOEPHEDRINE HYDROCHLORIDE FROM SPIKED-SAND AND SUPHEDRINE TABLETS

5.1 INTRODUCTION

In this chapter, the feasibility of extracting a cationic species with carbon dioxide was examined as well as determining what factors may improve its extractability and why. Pseudoephedrine hydrochloride (**Figure 5.1**) was chosen as our test analyte. Pseudoephedrine hydrochloride is an active ingredient in many pharmaceutical cold formulations and serves as a nasal decongestant.

Hedrick et al. investigated the feasibility of extracting nitrogenous bases including triprolidine, sulfamethazine, caffeine, 2,5-lutidine, and pseudoephedrine from aqueous solutions using direct SFE. [1] As expected, the greater the lipophilic characteristics of the basic molecule, the more readily it was extracted with the non-polar fluid carbon dioxide from the polar matrix (i.e. water), therefore, the extraction of the larger organic bases, triprolidine and pseudoephedrine from water was successful. The hydrochloride salts of triprolidine and pseudoephedrine were said to be extracted from the water, however, tetramethylammonium hydroxide (TMAOH) was added to the solution to neutralize the salts thus forming the free bases. No quantitation was reported, and the so-called extracted hydrochlorides were not absolutely identified.

Another amine hydrochloride, 3-chloro-*p*-toluidine hydrochloride (PTH), was successfully extracted from spiked avian feed with 10% methanol-modified carbon dioxide. [2] A two-level, two-factor (2^2) factorial design was used to examine the effect of

[1] *J.L. Hedrick, L.T. Taylor,* J. High Resolut. Chromatogr., **15** (1992)151.
[2] *M.L. Bicking,* J. Chromatogr. Sci., **30** (1992) 358.

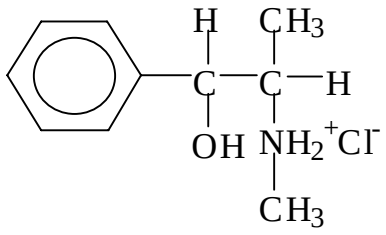


Figure 5.1. Chemical Structure of Pseudoephedrine Hydrochloride

pressure and temperature upon the extraction recoveries. Low temperature (45 °C) and high pressure (320 atm) conditions were shown to result in the highest average recovery (96%). As in the case of Hedrick's work, the extracted amine species was not identified.

More recently, Morrison et al. reported on the supercritical fluid extraction of the free base and hydrochloride salt of cocaine and benzoylecgonine from teflon wool, filter paper, and drug-fortified and drug-user hair with a triethylamine (TEA)/water modifier mixture. [3] It was proposed that the drugs were adsorbed strongly to the hair matrix because of a combination of hydrophobic interactions, hydrogen bonding, and ionic interactions, therefore it was expected that the ability of CO_2 alone to remove the strongly held drug from the hair matrix would be unsuccessful. Successful extraction was achieved with CO_2 modified with 100 μL of TEA/H_2O (85:15 (v/v) for the following reasons. First, the H_2O served to swell the hair making the fluid more accessible to the analyte-matrix sites. Second, it was believed that some of the triethylamine modifier existed as the triethylammonium cation due to acidic conditions in the presence of CO_2 and water. Therefore the triethylammonium cation may have displaced the cocaine from the negatively charged hair binding sites through an ion-exchange mechanism. Third, if the TEA existed as neutral in the bulk CO_2, it may serve as a buffer thereby accepting the proton from the displaced cocaine salt so that the cocaine was extractable as the free base.

For these three reported extractions of hydrochloride salts, the strategies of ion-suppression and ion-exchange were shown to be most successful. In these studies ion-exchange represented displacement of an ionic species from any exposed active matrix site with a "stronger" modifier. Ion-suppression involved charge neutralization by the addition of an appropriate acid or base. The strategy of ion-pairing wherein two species of opposite charge electrostatically interact with one another to form an ion-pair-analyte complex was not investigated in any of these studies. Ionic compounds are generally believed to have low solubility and poor extractability in carbon dioxide due to their high polarity; however it was shown in Chapter IV that at least one ionic compound can indeed

[3] *J.F. Morrison, S.N. Chesler, W.J. Yoo, C.M. Selavka,* Anal. Chem., **70** (1998) 163.

be extracted. Recall that triphenylphosphinetrisulfonate (TPPTS) was extracted from a spiked-sand surface with supercritical carbon dioxide. Extraction recoveries were shown to be enhanced in the presence of a methanol solution of tetrahexylammonium hydrogen sulfate. It was reasoned that through electrostatic interactions there was a reduction in the polarity of the analyte which increased solubility and thus extractability in carbon dioxide. Other parameters identified as influential to the extraction process were: ion-pair reagent concentration, static extraction time, and methanol-modifier volume. When this work was begun there were no reports on the use of ion-pairing additives as a means of increasing the extractability of cationic compounds with CO_2. Therefore, it was our objective to investigate whether the extraction of pseudoephedrine hydrochloride in the presence of an ion-pairing reagent was indeed feasible (Phase I).

Since the publication of our feasibility study (Phase I), there have been several reports describing the simultaneous ion-pair/supercritical fluid extraction of cationic species. [4-6] Jiminez-Carmona investigated the extraction of clenbuterol from spiked diatomaceous earth and food matrices (feedstuff, lyophilized milk and liver). [4] Three ion-pairing reagents (0.1M in methanol) were investigated: (1S)-(+)-10-camphorsulfonic acid, (1R)-(-)-10-camphorsulfonic acid, and (1R)-(-)-10-camphorsulfonic acid, ammonium salt. In the presence of 0.5 mL of a methanol solution of the ion-pairing reagent, clenbuterol was extracted from spiked diatomaceous earth with CO_2 at 30% and 70% recoveries using camphorsulfonic acid and its ammonium salt respectively. It was believed that camphorsulfonic acid itself could not form the ion-pair, however the ammonium salt could. The influence of the amount of ion-pairing reagent (e.g. ammonium salt) added was also ascertained. Surprisingly, no increase in recovery was observed when the concentration of the ion-pairing reagent was increased from 0.1M to 0.3M. The influence of the extraction fluid was also studied. The recoveries were compared between pure CO_2

[4] *M.M. Jimenez-Carmona, M.T. Tena, M.D. Luque de Castro*, J. Chromatogr. A, **711** (1995) 269.
[5] *P. Fernandez, A.C. Alder, M.J.F. Suter, W. Giger*, Anal. Chem., **68** (1996) 921.
[6] *J.T.B. Strode, L. Karlsson, M. Berglin*, "Ion-Pair Supercritical Fluid Extraction of Melagatrane from Gelatine Capsules", personal communication.

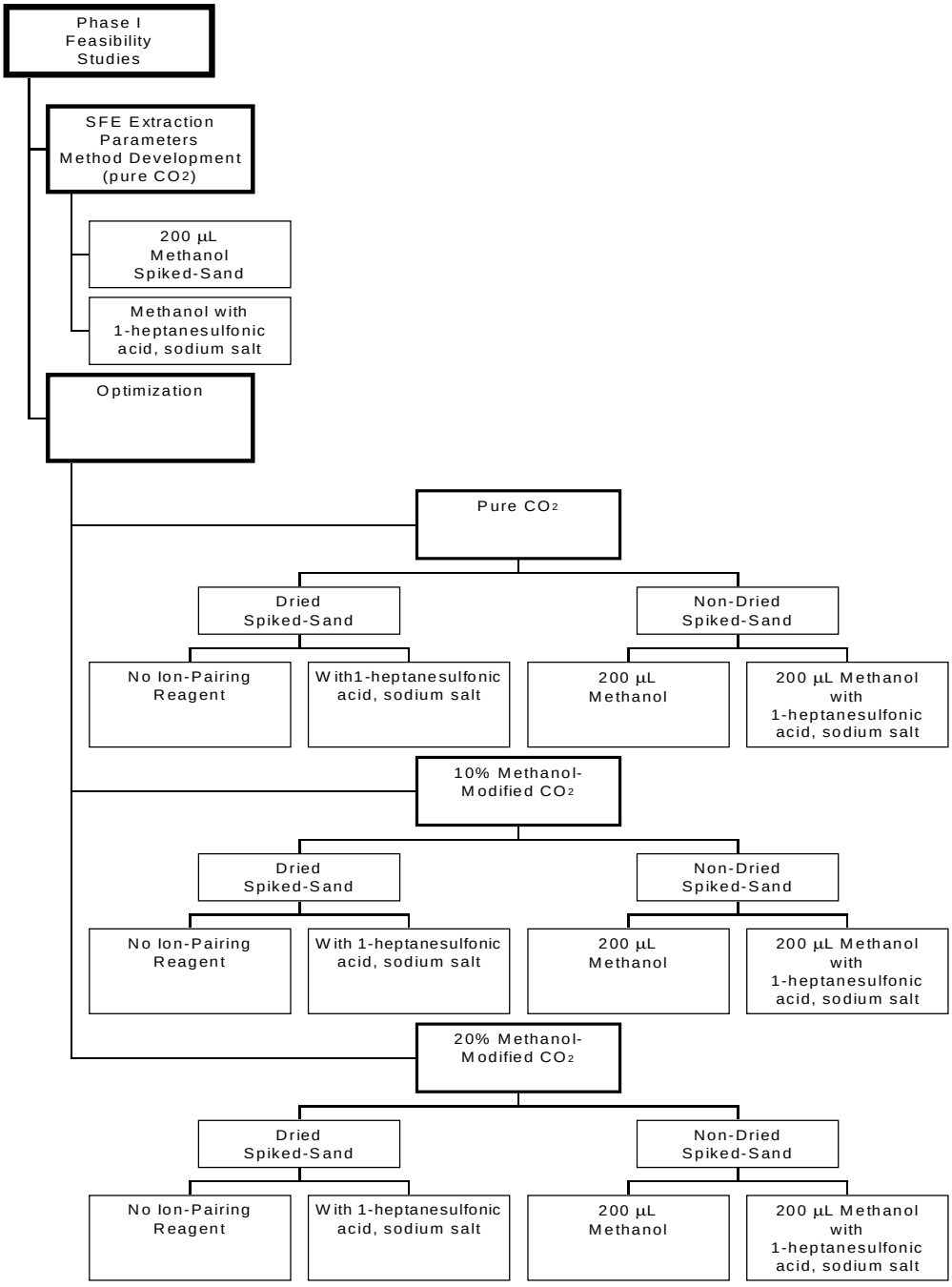
and methanol-modified CO_2 with static addition of the ion-pairing reagent in both cases. Methanol-modified CO_2 had a negative impact on the recoveries (e.g. 80% (pure CO_2), 60% (methanol-modified CO_2)). Once again, decreased recoveries with the methanol-modified fluid were attributed to poor analyte trapping. The influence of the mode of introduction of the ion-pairing reagent was investigated: 1) addition directly to the matrix (static addition), 2) continuous addition by means of in-line methanol-modified CO_2 (dynamic addition), and 3) combination of addition directly to the matrix (static addition) and continuous addition by means of the in-line modified fluid (dynamic addition). Since the extraction system plugged using strategy 2 and 3, only ion-pairing reagent introduction through static addition was explored. Extractions in the presence of the ion-pairing reagent were next performed on the food samples. Recoveries (20 μg clenbuterol) were only 12%, 47%, and 59% for the lyophilized liver, feedstuff, and lyophilized feedstuff respectively. No reasons were given for the observed low recoveries from the food samples. Recovery from the food matrices in the absence of camphorsulfonic acid, ammonium salt was not reported.

Later in 1996 Fernandez et al. used SFE to remove the quaternary ammonium surfactant ditallowdimethylammonium from sewage sludges and marine sediments. [5] Using a similar approach to that discussed above, 1 mL of toluene-4-sulfonic acid (TSA) in methanol at several concentrations was added to the matrix. The extraction was then performed with pure CO_2. Regardless of ion-pair reagent concentration, the extraction recoveries of the cationic surfactant from the sludge with a methanol-modified matrix alone versus a methanol-modified matrix in the presence of TSA were similar. Quantitative extraction recoveries from the sewage sludge were achieved with 30% methanol-modified CO_2. The usefulness of TSA was more apparent when extracting the marine sediment with 10% methanol-modified CO_2. The presence of the ion-pairing reagent was shown to be beneficial where an enhancement of approximately 35% was observed. However, identical recoveries for the marine sediment were achieved with both 30% methanol-modified CO_2 and 10% methanol-modified CO_2 in the presence of the ion-

pairing reagent. Although smaller amounts of modifier could be used with the ion-pairing reagent, 30% methanol-modified CO_2 was chosen as the extraction fluid because it would have been necessary to remove the ion-pairing reagent prior to HPLC.

Finally, melagatrane was quantitatively recovered from an aqueous emulsion (phosphate buffer) of a dissolved gelatine capsule spiked onto Celite by SFE. [6] Initial drug spike studies on Celite with methanol-modified CO_2 and methanol-modified CO_2 with trifluoroacetic acid were performed. Recoveries were similar. Octylsulfonic acid, sodium salt (OSA) was then added to the modified CO_2 (no trifluoroacetic acid). Again, no enhancement was observed. It was proposed that ion-pair formation with the analyte could not occur with the sodium salt. Consequently, trifluoracetic acid (trifluoroacetic acid) was added to the ion-pair/methanol modifier system to serve as a proton source. Quantitative recoveries were then observed. Next, extractions were performed on the gelatine capsules. A two-step extraction procedure was employed. First, the gelatine lipids were selectively removed from the emulsion with (1:1:98 (v/v/v) trifluoroacetic acid:OSA:methanol) modifier (4% (v/v)). Second, the drug was subsequently extracted with (1:1:98 (v/v/v) trifluoroacetic acid:OSA:methanol) modifier (25% (v/v)). Although quantitative recoveries were observed, the presence of the trifluoroacetic acid interfered in the analysis of the two degradates of melagatrane, therefore phosphoric acid was used. Recoveries in this case (96%) were achieved by first removing the lipids with (1:1:98 (v/v/v) phosphoric acid:OSA:methanol) modifier (4% (v/v)) followed by extraction of melagatrane with (1:1:98 (v/v/v) phosphoric acid:OSA:methanol) modifier (20% (v/v)).

The main goals of our work were to demonstrate that cationic species can be extracted via SFE, and to investigate the effects of modifiers and ion-pairing additives on their recovery. This chapter is divided into four phases. Phase I (**Scheme 5.1**) describes the feasibility of extracting an ionic species, pseudoephedrine hydrochloride, from a relatively simple matrix, Ottawa cement testing sand. An initial screening study was performed to qualitatively estimate the effect of CO_2 density, static extraction time, and the addition of an ion-pairing reagent to the spiked-sand surface on recovery. Then under

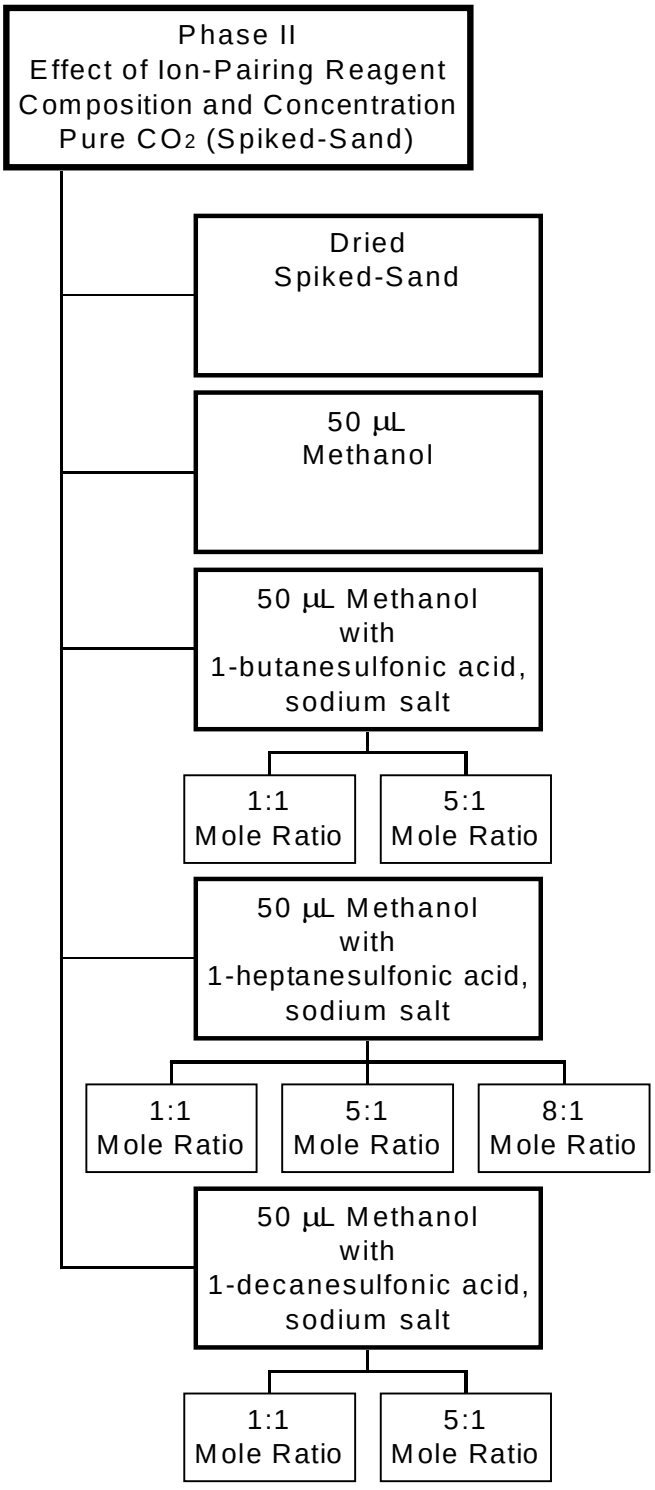


Scheme 5.1. Phase I General Experimental Scheme

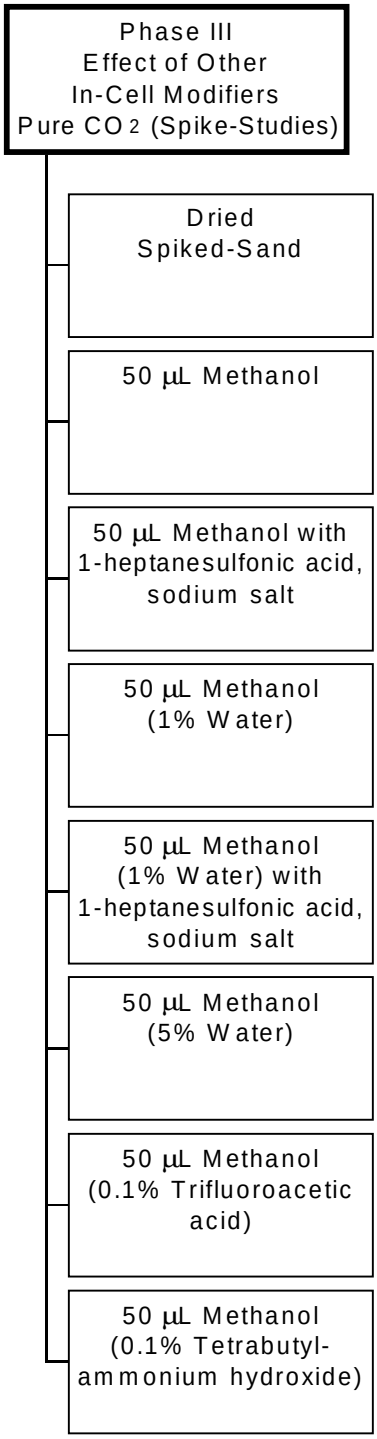
constant SFE conditions, the extraction of pseudoephedrine hydrochloride in the presence and absence of 1-heptanesulfonic acid, sodium salt (from both a previously dried sand surface and from one that had been pre-spiked with methanol) with pure-, 10% methanol-modified-, and 20% methanol-modified-carbon dioxide will be compared. As reported in Chapter IV, the composition and concentration of ion-pairing reagent relative to analyte concentration as well as the amount of modifier in the extraction vessel were shown to influence the extraction process. Therefore in Phase II (**Scheme 5.2**), several alkylsulfonic acid, sodium salts varying in lipophilicity and concentration were investigated. Phase III (**Scheme 5.3**) will consider the influence of acids and bases and other modifier compositions such as a methanol/water mixture on the recovery of pseudoephedrine hydrochloride. Finally the extraction of pseudoephedrine hydrochloride from a commercially available formulation, Suphedrine tablets, will be performed in Phase IV (**Scheme 5.4**). Similar to the sand-spike studies, the effect of the addition of the ion-pairing reagent and modifier composition will be examined. Also, attempts will be made to identify the composition of the extracted analyte.

5.2 EXPERIMENTAL

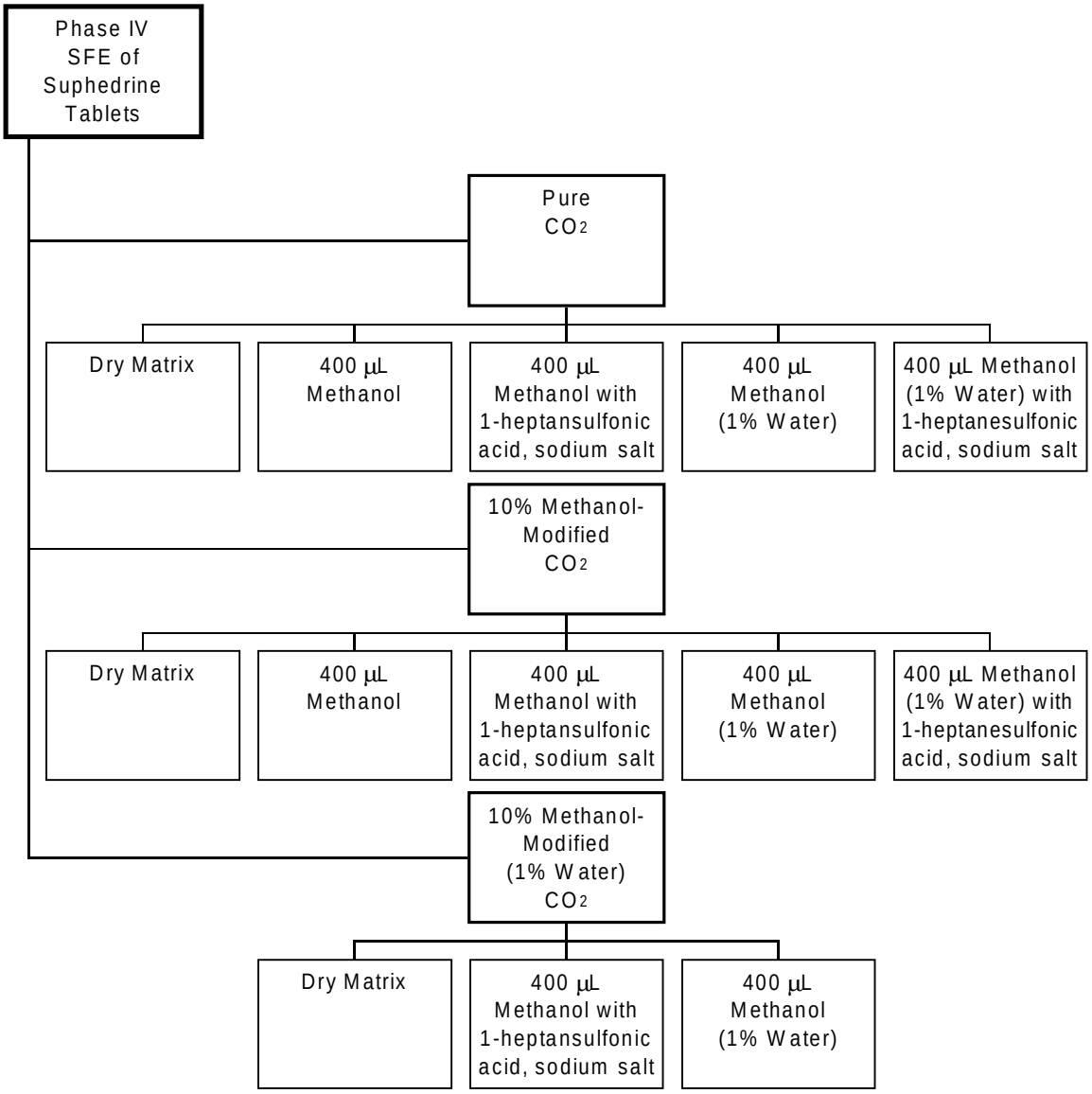
All extractions were performed on the Isco/Suprex Prepmaster (Lincoln, NE) consisting of a dual reciprocating pump, temperature-controlled oven, variable automatic restrictor, Accutrap solid-phase trap collection and rinsing device, and an in-line HPLC micro pump for modifier introduction. Carbon dioxide (SFE/SFC grade) with helium headspace was donated by Air Products and Chemicals, Inc. (Allentown, PA). Pseudoephedrine hydrochloride, 1-butane-, 1-heptane-, 1-decanesulfonic acid, sodium salt and caffeine were obtained from Sigma-Aldrich (St. Louis, MO) respectively. Suphedrine tablets (American Fare, Troy, MI) were purchased at Kmart. HPLC grade methanol and water were obtained from EM Science (Gibbstown, NJ) and Mallickrodt (Paris, KY) respectively. Trifluoroacetic acid and tetrabutylammonium hydroxide (25% in methanol)



Scheme 5.2. Phase II General Experimental Scheme



Scheme 5.3. Phase III General Experimental Scheme



Scheme 5.4. Phase IV General Experimental Scheme

were purchased from Fisher Scientific (Norcross, GA) and JT Baker (Phillisburg, NJ) respectively.

Extract Analysis

Upon completion of the extraction, caffeine was added to the solid-phase trap rinse and tandem-liquid trap as an internal standard. Analysis of the extract solutions was performed by HPLC. Values corresponding to 100% recovery were obtained by direct comparison to the 100% pseudoephedrine hydrochloride standard.

A Hitachi (Danbury, CT) Liquid Chromatograph consisting of a L-7100 pump, a D-7000 interface, a L-7250 autosampler, a L-7400 UV detector, and a D-7000 HPLC system manager was used for all extract analyses in Phase I. All other analyses were performed with a Hewlett Packard (Wilmington, DE) Series 1050 Liquid Chromatograph and a Hewlett Packard 3396 Series II integrator. All separations were isocratically performed on a Deltabond Cyanopropyl (25 cm x 4.6 mm i.d.) column (Keystone Scientific, Bellefonte, PA) with a mobile phase consisting of 89% water, 2% methanol, 9% acetonitrile, and 1.5 grams of 1-heptanesulfonic acid, sodium salt. The pH of the mobile phase was adjusted to 3.0 with phosphoric acid. A flow rate of 1.0 mL/min was used. The injection volume was 10 μL. Detection was by UV at 205 nm.

Extract Trapping

A solid-phase/tandem-liquid trap was used for analyte collection. The solid-phase consisted of a 50/50 (w/w) mixture of Porapak Q and glass beads. A vial containing 5 mL methanol immediately following the solid-phase trap was used to ensure quantitative trapping. During the extraction, the solid-phase trap was maintained at either 0 °C (pure CO_2) or 70 °C (methanol-modified CO_2). The tandem liquid trap was maintained at room temperature. Upon completion of the extraction, the solid-phase trap temperature was

raised to 25 °C for trap rinsing with 3.2 mL of methanol (Phase I) at a flow rate of 1.0 mL/min. into an empty collection vial. For Phases II-III the solid-phase trap was rinsed twice following the dynamic extraction with 2.0 mL of methanol into an empty collection vial. For Phase IV the solid-phase trap was rinsed with 2.0 mL methanol between dynamic extraction mini-steps directly into the tandem-liquid trap. In this case, the tandem-liquid trap was replaced following each dynamic extraction step.

Procedure - Phase I

Solutions of pseudoephedrine hydrochloride and 1-heptanesulfonic acid, sodium salt were prepared in methanol. A 100-μL aliquot of pseudoephedrine hydrochloride solution (100 μg) was spiked into a 3.5 mL Keystone Scientific (Bellefonte, PA) extraction vessel approximately 90% filled with Ottawa Cement Testing sand (Fisher Scientific, Raleigh, NC). Either 100 μL of pure methanol or 100 μL of 1-heptanesulfonic acid, sodium salt methanol solution (900 μg) was added to the drug-spiked sand surface. The mole ratio of ion-pairing reagent to pseudoephedrine hydrochloride was 9:1. The total spike volume of methanol added to the matrix, therefore, was always 200 μL (e.g. 100 μL (pseudoephedrine solution) plus 100 μL (pure methanol or ion-pair methanol solution)). The sand was either extracted immediately or after air drying overnight with pure-, 10%-, or 20%-methanol modified-carbon dioxide. Extraction conditions are found in **Table 5.1**.

Procedure - Phases II-III

Solutions of pseudoephedrine hydrochloride and 1-butane-, 1-heptane-, and 1-decane sulfonic acid, sodium salt were prepared in methanol. A 100-μL aliquot of pseudoephedrine hydrochloride solution (300 μg) was spiked into a 3.5 mL Keystone Scientific (Bellefonte, PA) extraction vessel approximately 90% filled with Ottawa

Cement Testing sand (Fisher Scientific, Raleigh, NC), after which the spiked-sand was allowed to dry prior to extraction. Either pure methanol (Phase II-III), methanol with 1% or 5% (v/v) water (Phase III), methanol with 0.1% (v/v) trifluoroacetic acid (Phase III), methanol with 0.1% (v/v) tetrabutylammonium hydroxide (Phase III), or the appropriate ion-pairing reagent in a methanol or methanol-modified solution (Phase II-III) was added to the drug-spiked sand surface. In the case of the addition of the ion-pairing reagent, the reagent was added at either a 1:1 (Phase II) or a 5:1 (Phase II-III) drug to reagent mole ratio unless stated otherwise. The total spike volume of methanol added to the matrix was always 50 μL (pure methanol, modified-methanol, or ion-pair methanol solution) unless otherwise noted. The sand was extracted immediately with pure carbon dioxide and was not allowed to dry. The extraction conditions used in Phases II-III are found in **Table 5.2**.

Procedure - Phase IV

The extraction of Suphedrine tablets was performed in Phase IV. The mass of pseudoephedrine hydrochloride per tablet was assumed to be 30 mg. Each Suphedrine tablet was placed on top of a piece of weighing paper which was sitting in a mortar cup. A pestle was placed on top of the tablet, and pressure was applied until the tablet particles appeared evenly dispersed as a powder. The weighing paper was carefully removed and the complete crushed tablet was poured into the extraction vessel filled approximately 3/4 with a cotton ball. The weighing paper, mortar, and pestle were wiped clean with an additional small piece of cotton. This particular piece of cotton was then placed on top of the other cotton ball inside the extraction vessel. More cotton was added to fill approximately 90% of the vessel volume. Then either 400 μL of pure methanol, methanol with 1% (v/v) water, or 1-heptanesulfonic acid, sodium salt in either methanol or methanol with 1% (v/v) water was spiked on top of the cotton ball. A 2:1 mole ratio of drug to reagent was employed. The extraction vessel was then sealed, and the extractions were performed with either pure, 10%-, 20%- methanol-modified CO_2, or 10% (1% (v/v)

Table 5.1. Phase I - Conditions for Ion-Pair/Supercritical Fluid Extraction Screening Study With Pure CO_2

Method #	Pressure (atm)	Oven Temp (°C)	CO_2 Density (g/mL)	Liquid Flow Rate (mL/min.)	Variable Restrictor Temp (°C)	Static Time* (min.)	Dynamic Time** (min.)
1	300	80	0.75	1.0	60	5	15
2	300	80	0.75	1.0	60	15	15
3	450	80	0.85	1.0	60	15	15
4	400	35	0.97	1.0	60	15	15

In-cell spike volume - 200 µL (i.e. 100 µL pseudoephedrine solution + 100 µL pure methanol or ion-pairing reagent in methanol)

* time allotted for equilibration between spiked-sand surface and supercritical fluid (SF)

** time allotted for SF to pass through extraction vessel at indicated flow rate

Table 5.2. Extraction Conditions Used in Phases II-III

SFE Conditions	
CO_2 Pressure	450 atm
Temperature	35 °C
Liquid Flow Rate	1.0 mL/min.
Restrictor Temperature	50 °C
Dynamic Mass	25 grams CO_2
Modifier	Variable
Ion-Pairing Reagent	Variable

water) methanol-modified CO_2. The extraction conditions and profile used in Phase IV are found in **Table 5.3**.

5.3 RESULTS AND DISCUSSION

The goal of this chapter was to extract a cationic compound, pseudoephedrine hydrochloride, via SFE. Due to its ionic and polar characteristics, it is expected that it will exhibit low solubility and poor extractability with a non-polar fluid such as carbon dioxide. Therefore, other means must be devised in order to improve the extractability of pseudoephedrine. Several strategies will be investigated including the addition of modifiers (i.e. methanol and methanol/water) to the matrix, addition of ion-pairing modifiers (alkylsulfonic acid, sodium salts) to the matrix, and modification of the CO_2 extraction fluid (i.e. methanol-, methanol/water-modified CO_2). The first strategy investigated was ion-pairing wherein the extraction from a relatively simple sand matrix was performed with and without an ion-pairing additive. Once this strategy had been shown to be successful, the influence of the ion-pairing reagent composition and concentration was investigated in Phase II. Although ion-pairing seemed beneficial, the extraction recoveries could also have been enhanced by simply modifying the polarity of the in-cell modifier thus favoring the extraction of the polar compound. Therefore, in Phase III, the effect of the in-cell modifier composition (methanol versus methanol/water) was examined. Since ion-suppression may prove worthwhile, a solution of an acid or base was added to the extraction vessel in hopes of shifting the equilibrium between the charged and neutral forms of pseudoephedrine. Correlations between the expected composition (neutral vs charged) and their subsequent recoveries were made. Finally in Phase IV, the extraction of pseudoephedrine from a more complicated matrix, Suphedrine tablets was performed. The effect of the addition of an ion-pairing reagent, the effect of various in-cell modifiers, and the effect of the composition of the fluid (pure vs modified CO_2) were examined to see if similar trends were observed as in the spike-studies. An

Table 5.3. Extraction Conditions and CO_2 Profile Used in Phase IV

SFE Conditions		
CO_2 Pressure	450 atm	
Temperature	35 °C	
Liquid Flow Rate	2.0 mL/min.	
Restrictor Temperature	60 °C	
Modifier	Variable	
Ion-Pairing Reagent	1-heptanesulfonic acid, sodium salt	
Extraction Profile		
Step	Mode	Time or Mass of CO_2 Used
1	Static	5 min.
2	Dynamic	10 grams
3	Dynamic	10 grams
4	Dynamic	20 grams
5	Dynamic	20 grams
6	Dynamic	20 grams

optimum strategy (i.e. in-cell modifier composition, fluid composition) for the extraction of the cationic species was identified. Finally, the extracted analyte will be identified.

Phase I - Feasibility Studies

The main objective of Phase I was to examine the feasibility of extracting pseudoephedrine hydrochloride with super/subcritical pure and modified-carbon dioxide. The extraction efficiencies were compared with and in the absence of an ion-pairing additive, 1-heptanesulfonic acid, sodium salt. The effect of CO_2 density, static extraction time, and temperature were first examined. Second, the extraction was optimized for quantitative recovery. Third, the extraction efficiencies in the presence of the ion-pairing reagent in methanol from a spiked-sand surface containing a fixed volume of the methanol solution versus a spiked-sand surface that was allowed to dry after it was spiked with the ion-pairing reagent solution were examined. Do the reagent and the drug need to mix in the pressurized CO_2 fluid prior to extraction or can a successful extraction be achieved if the ion-pair spiked-sand surface was dry prior to extraction?

SFE Method Development

A screening study was initially performed to qualitatively estimate the effect of various extraction parameters on pseudoephedrine hydrochloride recovery from a non-dried spiked-sand surface. **Table 5.1** lists the SFE extraction conditions. **Table 5.4** lists the recoveries (n=1) of pseudoephedrine hydrochloride at various temperatures, pressures, and static extraction times from a methanol-spiked sand surface with and without the addition of 1-heptanesulfonic acid, sodium salt. In most cases, methanol containing a small amount (approximately 0.1%) of water was added to the extraction vessel to serve as an in-cell modifier. A number of reactions could take place upon addition of this methanol. For example the hydrochloride salt could dissociate into its ionic components.

Table 5.4. Percent Recovery (n=1) From Screening Study of Pseudoephedrine Hydrochloride Employing SFE - Phase I
Methods 1-4 with and without Ion-Pairing Agent

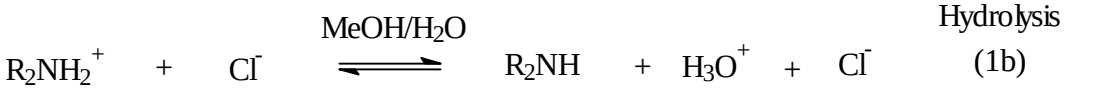
Method #	Pseudoephedrine HCl	Pseudoephedrine HCl w/ 1-heptanesulfonic acid, sodium salt
1	7.5	20.5
2	ND*	27.3
3	22.4	45.1
4	17.6	55.2

*ND indicates none detected

Secondly an equilibrium could exist between the ionized drug species and its corresponding free base through hydrolysis. (**Equation 1a, 1b**) Since it is anticipated that ionic species would not be extracted under these conditions, all extracted analyte will be assumed to be present in the free base form in the absence of the ion-pairing reagent. Attempts to identify the composition of the extracted analyte will be made in Phase IV. For the sake of terminology, the extracted analyte in this Chapter will be referred to as pseudoephedrine. Under the conditions listed in Method 1, low density and high temperature, only 7.5% (7.5 ug) of the spiked pseudoephedrine (100 ug) was recovered with pure carbon dioxide from the methanol-spiked sand surface (200 μL total spike volume).

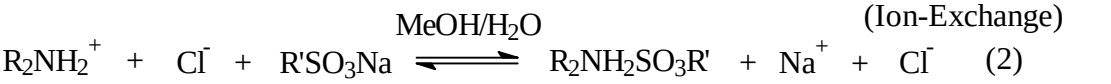
To examine whether the addition of an ionic additive could enhance recovery of the ionic pseudoephedrine species, an ion-pairing reagent was added to the vessel prior to extraction. Through ion-pairing the pseudoephedrine-sulfonate ion-pair would hopefully form and give enhanced non-polar characteristics to the compound. (**Equation 2**) Under the same SFE conditions and methanol-spike volume, the addition of the ion-pairing reagent, 1-heptanesulfonic acid, sodium salt on the extraction efficiency was then examined. As expected, the extraction recovery increased with the addition of the ion-pairing reagent to 20.5%.

Dissociation (1a)



Hydrolysis (1b)

Ion-Pair Formation (Ion-Exchange)



(2)

In an attempt to increase the extraction efficiency further, the time allotted for equilibration between the supercritical fluid and the drug-spiked sand surface was increased from 5 to 15 minutes (Method 2) while the density, oven temperature, and dynamic extraction time were held constant as in Method 1. It was believed that a higher recovery would result if more time was allotted for ion-pair formation and for equilibration between the pseudoephedrine-sulfonate ion-pair and the supercritical fluid. Very little pseudoephedrine was extracted without ion-pairing reagent. However the extraction recovery greatly increased to 27.3% with the addition of ion-pairing reagent. If one compares the pseudoephedrine-sulfonate ion-pair extraction recovery via Methods 1 and 2, it appears that the increased static time slightly improved extraction recovery. The true significance of static time cannot be accurately ascertained since each method was run only once.

The next SFE parameter investigated was density. It may be advantageous to increase the density of the fluid in order to increase its solvating power toward more polar analytes. An increase in extraction recovery of pseudoephedrine without the ion-pairing reagent from zero (Method 2) to 22.4% was observed when the CO_2 density was increased from 0.75 g/mL to 0.85 g/mL (Method 3). The added usefulness of the ion-pairing reagent can also be observed at the increased density. The recovery dramatically increased from 22.4% to 45.1% with the addition of 1-heptanesulfonic acid, sodium salt to the matrix.

Due to pump pressure limitations, the carbon dioxide density could only be increased to 0.97 g/mL albeit by decreasing the extraction temperature (Method 4). The recovery of pseudoephedrine with the addition of the ion-pairing reagent was slightly enhanced from 45.1% to 55.2% with this further increase in density; however, the extraction recovery of pseudoephedrine without the ion-pairing reagent appeared to level off or decrease at the greater density.

Ion-Pair/SFE Optimization

The main objectives in part II of Phase I were: 1) investigate the feasibility of increasing the solubility and extractability of pseudoephedrine via charge neutralization with 1-heptanesulfonic acid, sodium salt from a spiked-sand surface; and 2) determine the optimal extraction conditions for full recovery of pseudoephedrine from the spiked-sand surface. High density conditions (0.99 g/mL CO_2, 450 atm, 35 °C) were chosen for part II of this phase. To ensure an exhaustive extraction, the dynamic time was increased to 25 minutes; while the static time was decreased to 5 minutes.

The average percent recoveries of pseudoephedrine with and without the addition of 1-heptanesulfonic acid, sodium salt from a spiked-sand surface (e.g. spiking performed directly into the extraction vessel) with pure-, 10%-, and 20%-methanol-modified carbon dioxide are found in **Figure 5.2.** Although it was previously thought that ionic compounds and salts could not be extracted by SFE, 67.3% (67.3 μg/100 μg) of pseudoephedrine was recovered with pure CO_2 from the methanol enriched spiked-sand surface with no ion-pairing reagent. The in-cell methanol-spike apparently modified the CO_2 making it more polar thus leading to pseudoephedrine's greater solubility in CO_2. Disruption of analyte-matrix interactions may also be another possible explanation for the observed increase in extractability. In-line 10%- and 20%-methanol-modified CO_2 were then utilized to further enhance the extraction of pseudoephedrine from a methanol enriched spiked-sand surface. No significant increase was observed when employing 10%-methanol-modified CO_2 (61.6%); however, a significant increase to 89.3% was observed with 20%-methanol-modified CO_2.

It was believed that the addition of 1-heptanesulfonic acid, sodium salt would further increase the solubility and extractability of pseudoephedrine. This hypothesis was realized with 100% CO_2 **(Figure 5.2)** in that the recovery from the methanol enriched spiked-sand surface was increased from 67.3% (without IP) to 76.5 % (with IP). Another possible explanation for the observed enhancement with the ion-pairing reagent is

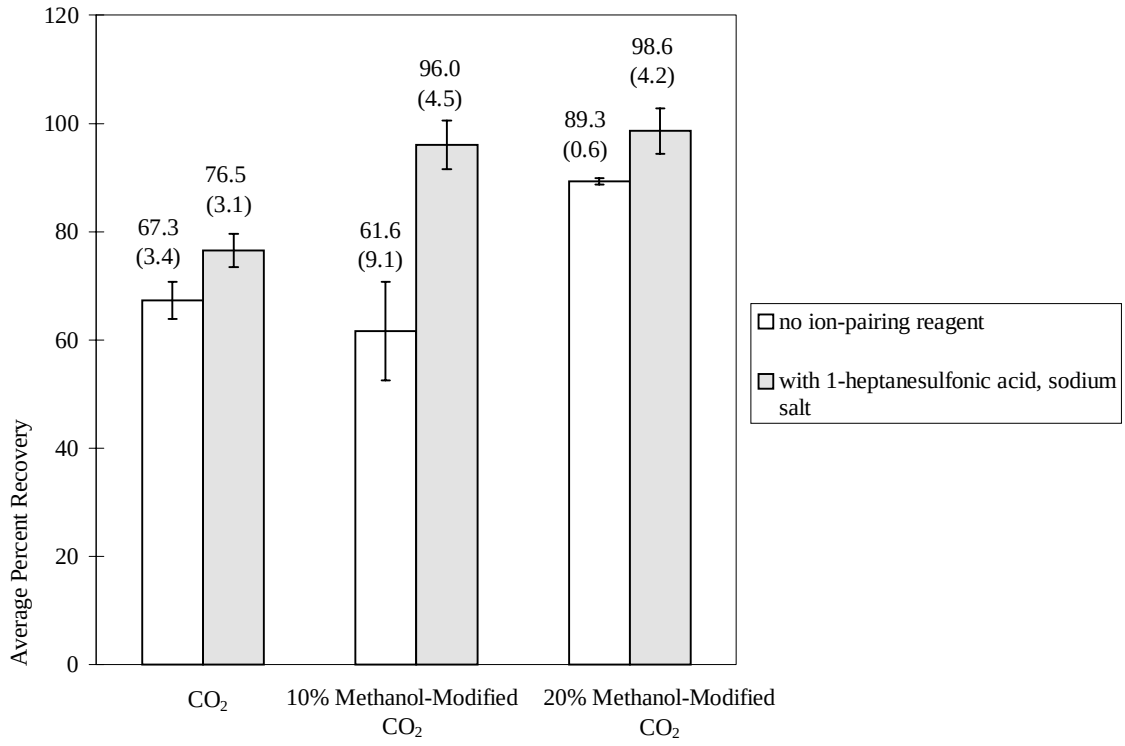


Figure 5.2. Average Percent Recoveries (n=3) of □ Pseudoephedrine and ▨ Pseudoephedrine with 1-Heptanesulfonic Acid, Sodium Salt from a Non-Dried Drug Spiked-Sand Surface. Bars and Numbers in Parentheses Represent One Standard Deviation.
SFE Conditions: 0.99 g/mL CO_2, 450 atm, 35 °C, 1.0 mL/min., 5 min. static time, 25 min. dynamic time. In-cell modifier volume - 200 µL methanol or ion-pairing reagent in methanol.

displacement of the pseudoephedrine from the sand surface by the ion-pairing reagent. Although it was expected that methanol alone should have been able to reduce these interactions, the ion-pairing reagent itself may act as an ionic displacer preferentially adsorbing to these sites thus freeing the ionic pseudoephedrine. Once released into the bulk methanol-modified CO_2 fluid, an equilibrium between the ionic analyte and the free base may ensue such that the free base may be further extracted. This mechanism would presume no electrostatic interaction between the analyte and the ion-pairing reagent existed.

The extraction of pseudoephedrine from the spiked-sand surface in the presence of ion-pairing reagent with CO_2 was shown to be even more successful with in-line-methanol-modified CO_2. Recoveries of 96.0% and 98.6% were achieved with 10%- and 20%-methanol-modified CO_2 respectively relative to 61.6% and 89.3% with no ion-pairing reagent. Paired t-tests were performed to test if the means of the extraction recoveries with the use of the ion-pairing reagent were greater than the recoveries without the ion-pairing reagent with pure-, 10%-, and 20%-methanol-modified CO_2 at a 95% confidence interval (alpha=0.05, T-critical=2.9 (one-tailed)). The extraction recoveries of the pseudoephedrine-sulfonate ion-pair as compared to pseudoephedrine alone were shown to be statistically greater (T-experimental > T-critical) in the presence of 1-heptanesulfonic acid, sodium salt with pure- (T-exp.=3.6), 10%- (T-exp.=5.9) , and 20%-methanol-modified CO_2 (T-exp.=3.8).

The need for the presence of methanol inside the extraction vessel was next examined. For example, if the spiked-sand surface (100-μL pseudoephedrine solution + 100-μL pure methanol or ion-pair solution) was allowed to dry overnight prior to extraction, would the recoveries of pseudoephedrine be enhanced by addition of 1-heptanesulfonic acid, sodium salt relative to the situation without ion-pairing reagent with pure-, 10%-, and 20%-methanol-modified CO_2? The percent recoveries of pseudoephedrine with and without the addition of 1-heptanesulfonic acid, sodium salt from a previously dried spiked-sand surface are shown in **Figure 5.3**. The recoveries of

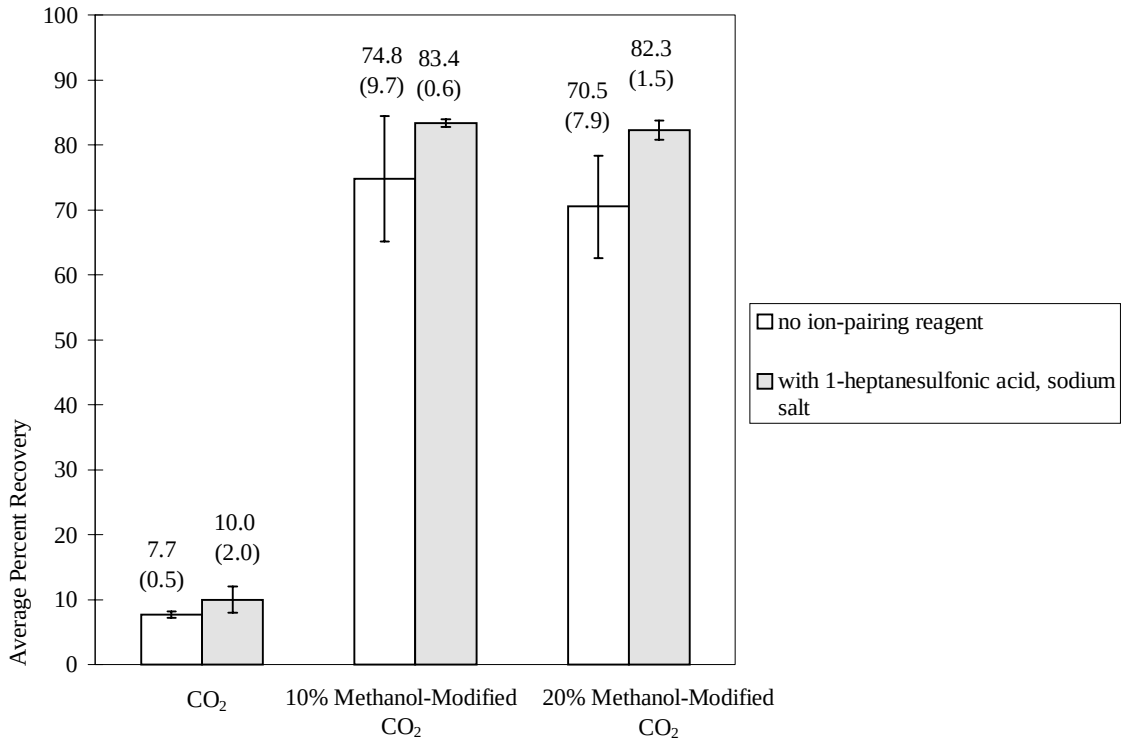


Figure 5.3. Average Percent Recoveries (n=3) of □ Pseudoephedrine and ▨ Pseudoephedrine with 1-Heptanesulfonic Acid, Sodium Salt from a Dried Drug Spiked-Sand Surface. Bars and Numbers in Parentheses Represent One Standard Deviation.
SFE Conditions: 0.99 g/mL CO_2, 450 atm, 35 °C, 1.0 mL/min., 5 min. static time, 25 min. dynamic time.

pseudoephedrine in the absence of the ion-pairing reagent with pure-, 10%-, and 20%-methanol-modified CO_2 were first compared. With pure carbon dioxide, only 7.7% of the spiked pseudoephedrine was recovered. Methanol-modified CO_2 was then utilized to increase the solvating power of the medium. The recovery of pseudoephedrine from the previously dried spiked-surface increased dramatically from 7.7% to 74.8% with 10%-methanol-modified carbon dioxide. This favorable result was expected as the addition of methanol should increase the solvating power of the fluid for the polar compound. Methanol-modified CO_2 at 20% (v/v) was then utilized in an attempt to further increase the extraction efficiency in the absence of the ion-pairing reagent; however, no significant increase (70.5%) was observed.

The usefulness of the addition of the 1-heptanesulfonic acid, sodium salt to the pseudoephedrine hydrochloride spiked-sand surface that was previously dried was then examined with pure- and methanol-modified carbon dioxide. The recoveries obtained in the presence of the ion-pairing reagent as compared to the situation without the ion-pairing reagent were statistically comparable with pure- (7.7% without vs 10.0% with), 10% methanol- (74.8% without vs 83.4% with), and 20% methanol-modified CO_2 (70.5% without vs 82.3% with). In other words, the ion-pairing reagent apparently had no significant effect on extraction efficiency under these conditions.

The presence of in-cell methanol has, therefore, been shown to play a vital role in the extraction process. If indeed an ion-pair complex is being created, the ion-pairing reagent must be allowed to form the pseudoephedrine-sulfonate ion-pair in the pressurized CO_2 fluid during the equilibration period prior to the dynamic extraction. Methanol appears to serve two purposes: 1) increases the solubility of the formed ion-pair in pure-, and methanol-modified carbon dioxide, and 2) support the pseudoephedrine-sulfonate formation.

Phase II - Investigation of Ion-Pairing Reagent Composition and Concentration on the SFE of Pseudoephedrine From A Spiked-Sand Surface

From Phase I it was shown that the extraction of pseudoephedrine from a non-dried spiked-sand surface was feasible with pure and methanol-modified carbon dioxide, and that the recoveries were statistically enhanced when the matrix was in the presence of a methanol solution of 1-heptanesulfonic acid, sodium salt versus methanol alone. It was observed in Chapter IV that an increase in the lipophilicity of the ion-pairing reagent as well as an increase in its concentration relative to the analyte enhanced the extraction efficiencies. Therefore, it was the objective of Phase II to determine if the same trend would hold true when extracting a cationic species. In this case several alkylsulfonic acids varying in chainlength were investigated including 1-butane-, 1-heptane-, and 1-decanesulfonic acid, sodium salt. It was hypothesized that if ion-pair formation between the analyte and reagent occurred, recovery of the pseudoephedrine should increase. Therefore, recoveries of pseudoephedrine in the presence of 1-decane vs 1-butanesulfonic acid, sodium salt should be greater. Also, the amount of ion-pairing reagent added was investigated (i.e. a 1:1 mole ratio and a 5:1 mole ratio of ion-pairing reagent to analyte). Attempts to increase the recovery of cationic analytes by ion-pairing reagent compositional changes have not been found in the literature.

The spike mass of pseudoephedrine hydrochloride was increased from 100 μg to 300 μg; while, the spike volume of methanol with and without the ion-pairing additive was reduced from 200 μL to 50 μL. The spike mass was increased and the methanol solution volume was decreased in order that changes in recovery due to the ion-pairing reagent could be observed in greater detail. Therefore, the purpose of this study was not necessarily to achieve quantitative recoveries but to examine the extraction process in more fundamental detail. **Figure 5.4** shows the recoveries of pseudoephedrine in the presence and absence of various ion-pairing reagents at variable concentrations. Error bars represent one standard deviation. For baseline purposes, extraction of pseudoephedrine hydrochloride from a previously dried spiked-sand surface were

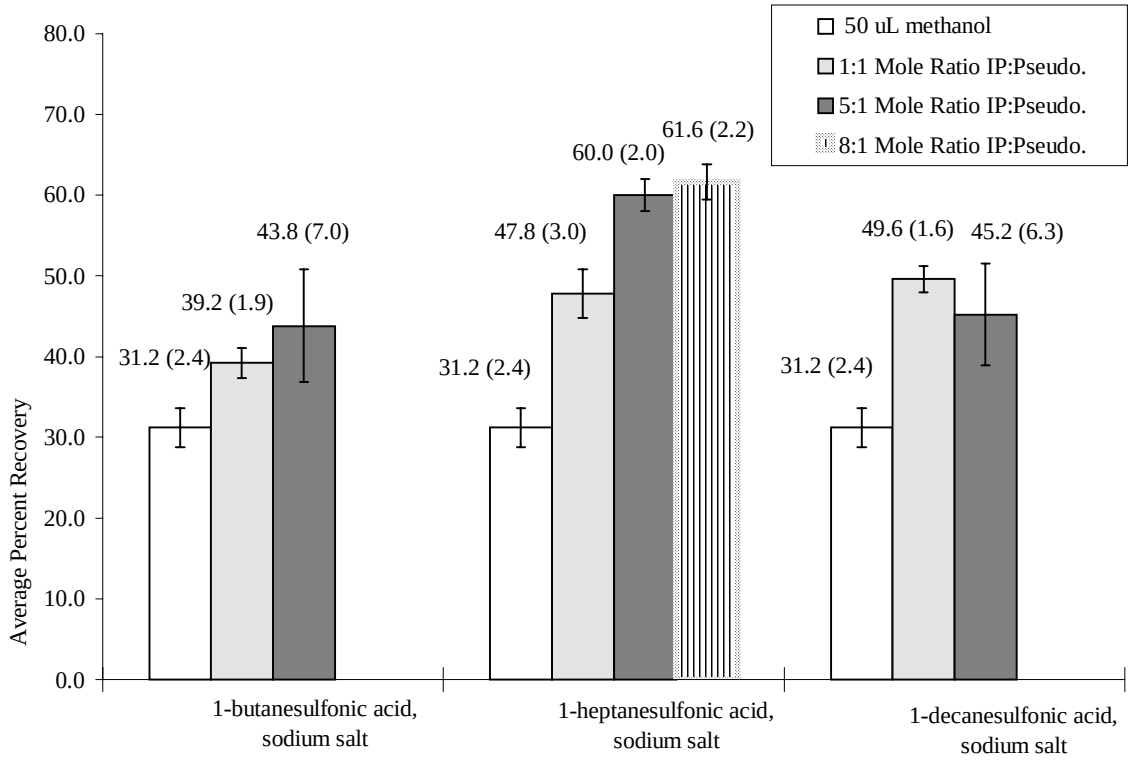


Figure 5.4. Average Percent Recoveries (n=3) of Pseudoephedrine with and without Various Ion-Pairing Additives at Various Concentrations from a Drug Spiked-Sand Surface. Error Bars and Values in Parentheses Represent One Standard Deviation. SFE Conditions: 0.99 g/mL CO_2, 450 atm, 35 °C, 1.0 mL/min., 5 min. static time, 40 grams CO_2. In-cell modifier volume - 50 µL methanol or ion-pairing reagent in methanol.

performed with pure CO_2, and only 10% (+/- 4.0%) of 300 μg pseudoephedrine was recovered. The ion-pairing reagent must be introduced as a solution of methanol. In order to examine the effectiveness of the ion-pairing reagent, first the sand surface was spiked with only 50 μL of pure methanol. Recovery was enhanced from 10% (no methanol) to 31% (50 μL methanol). Methanol may have served two purposes: 1) disruption of analyte-sand interaction and 2) increased the solvating power of the fluid.

Next the recoveries as a function of ion-pairing reagent composition (1:1 mole ratio of pseudoephedrine:ion-pairing reagent) were examined (**Figure 5.4**). In the presence of 1-butanesulfonic acid, sodium salt 1-heptanesulfonic acid, sodium salt and 1-decanesulfonic acid, sodium salt the recoveries were shown to increase by approximately 8%, 16%, and 18% respectively. Paired t-tests (95% confidence interval, T-critical = 2.9 (one-tailed)) were performed to determine if the recoveries in the presence of the ion-pairing reagents were indeed greater. The T-experimental values were determined to be: 4.5 (no reagent vs 1-butanesulfonic acid, sodium salt), 7.5 (none vs 1-heptanesulfonic acid, sodium salt), and 11.1 (none vs 1-decanesulfonic acid, sodium salt). In all three cases, a statistical enhancement was observed as compared to a purely methanol enriched spiked-surface. When comparing the composition of the ion-pairing reagent, it can be seen that the recoveries in the presence of 1-heptanesulfonic acid, sodium salt were greater than with 1-butanesulfonic acid, sodium salt. As the formed ion-pair became less polar, its solubility in the CO_2 should increase. Surprisingly, a further increase in recovery in the presence of 1-decanesulfonic acid, sodium salt versus 1-heptanesulfonic acid, sodium salt was not observed. The preparation of the 1-decanesulfonic acid, sodium salt spike-solution in methanol was problematic because sonication with heating was needed. It is believed that when in the presence of a less polar medium (CO_2/methanol) versus methanol, precipitation of 1-decanesulfonic acid, sodium salt occurred. Therefore, less ion-pairing reagent would be present to form the ion-pair thus resulting in no further enhancement. Let it be noted that the dissolution of the other ion-pairing reagents in methanol was immediate.

The pseudoephedrine recoveries in the presence of an excess of the various ion-pairing reagents were next compared. As in Chapter IV, it was believed that an excess of ion-pairing reagent would be advantageous because the equilibrium would be shifted thus favoring more complete ion-pair formation. At the lower concentration (5:1), significant increases were observed where 43%, 60%, and 45% pseudoephedrine was recovered with 1-butanesulfonic acid, sodium salt, 1-heptanesulfonic acid, sodium salt, and 1-decanesulfonic acid, sodium salt as compared to 31% with methanol alone (**Figure 5.4**). Of the three ion-pairing reagents investigated, only the addition of an excess of 1-heptanesulfonic acid, sodium salt further increased recovery. An enhancement of 13% was seen with the 5:1 mole ratio versus the 1:1 mole ratio. This was confirmed by a paired t-test (T-exp.=5.9). Next, it was of interest to increase the amount of 1-heptanesulfonic acid, sodium salt added from 5:1 to 8:1 to see if the recoveries could be further increased. However this strategy was not beneficial. In the case of 1-butanesulfonic acid, sodium salt, it was believed that complete ion-pair formation occurred at a 1:1 mole ratio. Also, no further increase was observed when adding an excess of 1-decanesulfonic acid, sodium salt. As stated before, there were problems dissolving 1-decanesulfonic acid, sodium salt in methanol. If the solubility of 1-decanesulfonic acid, sodium salt in the fluid played a role thus causing precipitation, this effect would no doubt be further worsened at a higher concentration.

As described in Phase I, the effectiveness of the ion-pairing reagent may be due to displacement. Here, when added in excess, the reagent may cover more active sites than at the 1:1 mole ratio thus freeing the analyte from the sand to a greater extent.

Phase III - Effect of Various In-Cell Modifiers on the SFE of Pseudoephedrine From A Spiked-Sand Surface

It was shown in Phase II that the extraction efficiencies of pseudoephedrine from spiked-sand with pure CO_2 were increased in the presence of various methanol solutions of ion-pairing additives. Recovery was shown to be a function of ion-pairing reagent

composition and concentration. The most successful recovery of 60% was achieved in the presence of 1-heptanesulfonic acid, sodium salt in methanol at a 5:1 mole ratio. When compared to the purely methanol enriched sand-surface, a 30% enhancement was observed with 1-heptanesulfonic acid, sodium salt.

The objective of Phase III was to examine the effect of other types of in-cell modifiers such as a methanol-water mixture, an acid, and a base. By modifying the methanol-spike with water, an overall increase in the solvating power of the fluid should occur. Thus, the extractability of the polar pseudoephedrine in the presence of methanol/water may increase as compared to methanol alone. Hydrogen bonding interactions between the fluid and the analyte should also increase. Second, the water-methanol mixture would be expected to interact with the sand through hydrogen bonding interactions to a greater extent than methanol alone thus freeing any bound pseudoephedrine. **Figure 5.5** shows the recovery of pseudoephedrine obtained in the presence of several in-cell modifiers. As stated previously, when the spiked-sand surface was allowed to dry prior to extraction and extracted with pure CO_2, 10% pseudoephedrine was recovered. By simply modifying the matrix with 50 μL of methanol, 31% pseudoephedrine was recovered. Next the spiked-sand surface was spiked with 50 μL of methanol containing 1.0% (v/v) water. As expected the pseudoephedrine recovery was further increased by 23% to 59% versus methanol alone. A paired t-test (95% confidence interval, T-critical=2.9) was performed, and a statistical increase was confirmed (T-exp.= 7.6). Also, the recovery with the methanol/water mixture alone versus the recovery with 1-heptanesulfonic acid, sodium salt in methanol was compared, and it was shown that the methanol/water mixture was just as effective as pure methanol containing 1-heptanesulfonic acid, sodium salt. Next a solution of 1-heptanesulfonic acid, sodium salt (5:1 mole ratio) in methanol (1% (v/v) water) was spiked onto the matrix. Surprisingly, the recovery with methanol/H_2O alone versus recovery with 1-heptanesulfonic acid, sodium salt in methanol/H_2O was comparable (**Figure 5.5**).

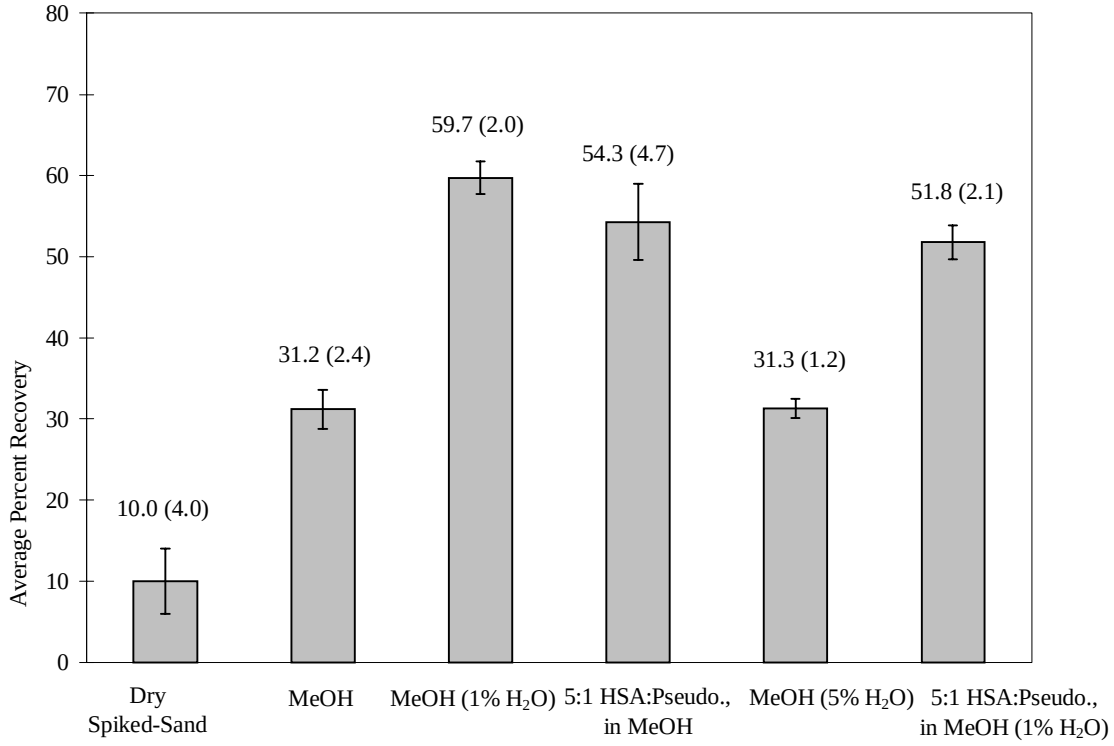


Figure 5.5. Average Percent Recoveries (n=3) of Pseudoephedrine with Various Modifiers and Ion-Pairing Additives from a Drug Spiked-Sand Surface. Error Bars and Values in Parentheses Represent One Standard Deviation.
SFE Conditions: 0.99 g/mL CO_2, 450 atm, 35 °C, 1.0 mL/min., 5 min. static time, 40 grams CO_2, 50 µL in-cell modifier.

Pseudo. - Pseudoephedrine
HSA - 1-heptanesulfonic acid, sodium salt

The effect of water concentration was next examined. For example, the percentage of water in methanol was increased from 1% (v/v) to 5% (v/v), however this strategy did not prove worthwhile. The pseudoephedrine recovery decreased from 59% (1% H_2O) to 31% (5% H_2O) (**Figure 5.5**). In this case it was believed that the solubility of water in the CO_2 was exceeded, therefore, a two-phase system resulted. It would be expected then that a portion of the methanol-water dissolved in the bulk fluid while a plug of methanol-water remained on the sand surface. Consequently a percentage of pseudoephedrine would be solubilized into the bulk CO_2 fluid while a percentage would remain entrapped in the aqueous solution. Due to the high polarity of the aqueous solution, the ability of the bulk fluid to partition into the aqueous solution and thus dissolve and remove the analyte would be reduced.

The effect of acid and base on the pseudoephedrine recovery from a spiked-sand surface was next determined. As stated before, an equilibrium exists between the ionized pseudoephedrine and free base (**Equation 1a and 1b**). By simply adding an acid or a base, the equilibrium should shift thereby favoring the ionic or neutral species. It is expected that in the presence of an acid, more charged species would exist and, therefore, the pseudoephedrine recovery should decrease. Likewise, formation of the free base should be favored under basic conditions thus an increase in the recovery should be observed. Separate solutions of methanol containing 0.1% (v/v) trifluoroacetic acid and 0.1% (v/v) tetrabutylammonium hydroxide were prepared and spiked onto the drug-spiked surface at a volume of 50 μL. The effect of the addition of the acid and base upon the recovery of pseudoephedrine is shown in **Figure 5.6.** In the presence of trifluoroacetic acid, the recovery increased by approximately 13% from 31% (methanol alone) to 44% (methanol with trifluoroacetic acid). Again, a paired t-test verified a statistical increase (T-exp.=6.2). This increase was unanticipated because under acidic conditions the analyte should exist solely ionized. As an acid, some trifluoroacetic acid will ionize. Ion-pair formation between the ionic pseudoephedrine and the ionized trifluoroacetic acid may have occurred. Also, the negatively charged acid may through electrostatic interactions

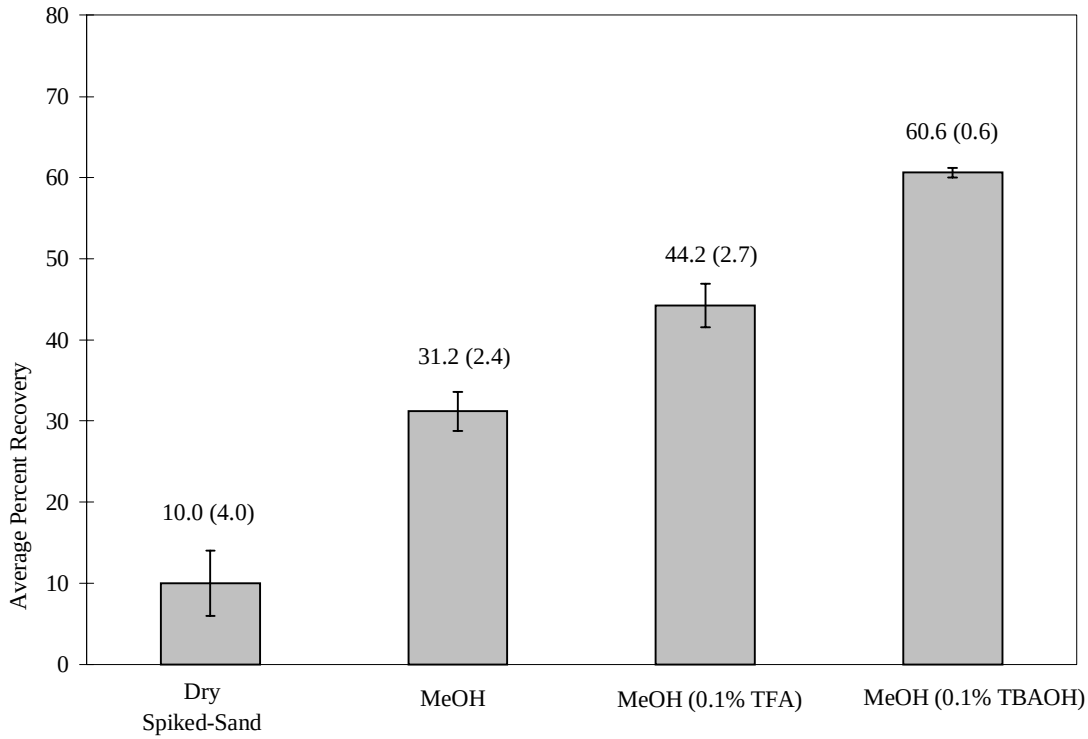


Figure 5.6. Average Percent Recoveries (n=3) of Pseudoephedrine from a Drug Spiked-Sand Surface. Effect of Addition of Acid and Base. Error Bars and Values in Parentheses Represent One Standard Deviation.
SFE Conditions: 0.99 g/mL CO_2, 450 atm, 35 °C, 1.0 mL/min., 5 min. static time, 40 grams CO_2, 50 µL in-cell modifier.

TFA - Trifluoroacetic acid
TBAOH - Tetrabutylammonium hydroxide

adsorb to any exposed positively charged sites on the sand surface thus displacing the ionic pseudoephedrine. Once released into the bulk fluid, a portion of the charged pseudoephedrine will be converted to free base and extracted. Likewise, through hydrophobic interactions, trifluoroacetic acid may adsorb to any neutral sites freeing the free base from the matrix.

The effect of the addition of tetrabutylammonium hydroxide was next examined. As expected, the pseudoephedrine recovery was statistically enhanced by 29% in the presence of tetrabutylammonium hydroxide from 31% (methanol alone) to 60% (methanol with tetrabutylammonium hydroxide) (T-exp.=10.3) (**Figure 5.6**). First, the base may serve as an analyte-matrix displacer. Second, under basic conditions, the pseudoephedrine existed predominantly as the free base thus favoring its extraction in CO_2 as compared to the ionic species.

Phase IV - Extraction Of Suphedrine Tablets

Pseudoephedrine recoveries (from non-dried drug spike studies) achieved with the addition of an excess of 1-heptanesulfonic acid, sodium salt in methanol, methanol (1% water), and methanol (0.1% tetrabutylammonium hydroxide) were shown to be comparable. Quantitative recovery from a spiked-sand was, however, not achieved. The effect of various modifiers and additives was nevertheless examined, and several enhancement strategies were discovered.

The extraction from a tablet matrix may be considered more difficult than extraction from a spiked-sand surface due simply to the tablet's complicated composition. Besides the active drug component, pseudoephedrine, Suphedrine tablets contain: carnauba wax, dicalcium phosphate, FD&C Red No. 40, hydroxypropyl methylcellulose, magnesium stearate, microcrystalline cellulose, polyethylene glycol, polysorbate 80, silicon dioxide, and titanium dioxide. Strong interactions between the pseudoephedrine and the matrix were expected.

It was observed in Phase III that pseudoephedrine recovery from spiked-sand can be enhanced by a) adding an ion-pairing additive, 2) varying the composition of the in-cell modifier, and 3) favoring free base formation. It was therefore our objective to apply these same strategies to the extraction of pseudoephedrine from a more complicated matrix, Suphedrine tablets. Besides pure CO_2, modified-carbon dioxide was investigated in this part of the study because the amount of pseudoephedrine in the tablet was 30 mg. It was expected that CO_2 alone would not be able to extract such a large amount in a timely manner regardless of the presence of an in-cell modifier.

100% CO_2

As described in the Experimental, one Suphedrine tablet was crushed and placed into an extraction vessel that contained approximately 50% cotton. More cotton was placed on top of the crushed tablet powder so as to eliminate possible clogging of the extraction vessel frits with tiny tablet particles and to reduce the void volume in the extraction vessel. Then either the extraction was performed immediately or a modifier solution was added directly to the cotton matrix. Extractions were then performed with pure CO_2. The amount of modifier spiked into the vessel was increased from 50 μL (spike-studies) to 400 μL. This spike volume (400 μL) was chosen for the following reasons. From Phase II, it was shown that pseudoephedrine recovery was enhanced if there was an excess of 1-heptanesulfonic acid, sodium salt. In order to achieve this situation, large amounts of methanol dissolved with 1-heptanesulfonic acid, sodium salt must be added to the matrix therein. The addition of 400 μL was the smallest volume that could be added to ensure that the mole ratio of 1-heptanesulfonic acid, sodium salt to pseudoephedrine inside the extraction vessel was 2:1. Larger in-cell modifier spike volumes could have been used, however, slight difference due to the effectiveness of the ion-pairing reagent may be more difficult to ascertain. Also, experimental difficulties such as restrictor plugging occurred at in-cell modifier spike volumes greater than 500 μL.

Other extraction parameters were also altered for the tablets. The CO_2 liquid flow rate was increased from 1.0 mL/min. to 2.0 mL/min. while the dynamic extraction time remained the same as in the spike studies (40 min.). Since much larger amounts of pseudoephedrine needed to be extracted, the amount of fluid was increased; however, a reasonably short extraction time was desired. As in the extraction of the MEVACOR® tablets, dynamic extraction mini-steps were employed so that: 1) the solid-phase trap could be rinsed in short intervals in case that the trapping capacity was exceeded, and 2) to increase the equilibration time between the fluid and the matrix in case of a diffusion limited process.

The extraction with pure CO_2 of pseudoephedrine from Suphedrine tablets with the addition of various modifiers to the matrix was performed (**Figure 5.7**). With pure CO_2 only 0.1% of 30 mg was extracted. This was expected due to the low anticipated solubility of pseudoephedrine in pure CO_2 as well as the possibility of very strong analyte-matrix binding. In order to decrease the analyte-matrix interactions as well as increase the solvating power of the fluid, methanol was added to the extraction vessel as an in-cell modifier. Pseudoephedrine recovery was 8%. Next 400 μL of 1-heptanesulfonic acid, sodium salt (2:1 mole ratio) in methanol was added to the tablet matrix. Heptanesulfonic acid, sodium salt addition with the pure CO_2 fluid was shown to be partially successful since the recovery was doubled to 16% (1-heptanesulfonic acid, sodium salt in methanol).

Next a methanol/water mixture as an in-cell modifier was investigated. The recovery of pseudoephedrine was 15% (methanol/water). As in the spike-studies, the recovery in the presence of the ion-pairing reagent, 1-heptanesulfonic acid, sodium salt in methanol, was comparable to the recovery with only the methanol/water spike and no 1-heptanesulfonic acid, sodium salt. Next, 1-heptanesulfonic acid, sodium salt was added to the methanol/water spike solution but only 16% pseudoephedrine was again recovered. A similar trend was observed in the spike studies where no further enhancement was observed when 1-heptanesulfonic acid, sodium salt was added to the methanol/water spike solution.

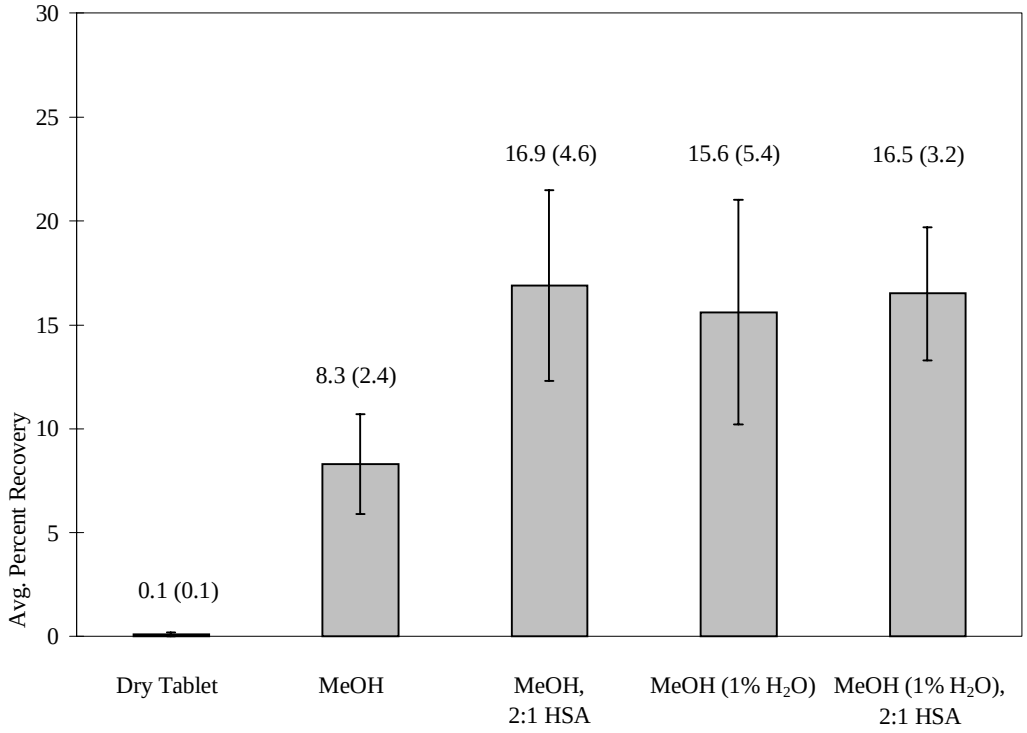


Figure 5.7. Average Percent Recovery (n=3) of Pseudoephedrine from Suphedrine Tablets as a Function of In-Cell Modifier Composition with Pure CO_2. Error Bars and Numbers in Parentheses Represent One Standard Deviation.

SFE Conditions: 0.99 g/mL CO_2, 450 atm, 35 °C, 2.0 mL/min., 5 min. static time, 80 grams CO_2 (5 dynamic mini-steps), 2 min. static time during trap rinsing between dynamic mini-steps, 400 μL in-cell modifier volume.

HSA - 1-heptanesulfonic acid, sodium salt

10% Methanol-Modified CO_2

The extraction efficiencies of pseudoephedrine from Suphedrine tablets were less than desirable with pure CO_2 regardless of in-cell modifier composition. The addition of modifier directly to the CO_2 via a HPLC pump was next investigated as a means of further increasing recovery. **Figure 5.8** examines the effect of various in-cell modifiers with the fluid being methanol-modified CO_2. First a Suphedrine tablet without an in-cell modifier was extracted with 10% methanol-modified CO_2. An increase from 0.1% (pure CO_2) (**Figure 5.7**) to 44% was observed. The addition of an in-line modifier no doubt increased the solvating power of the extraction fluid thus favoring the extraction of the polar pseudoephedrine from the tablet. This was expected because of methanol's ability to hydrogen bond with the analyte. Likewise the methanol could be displacing the pseudoephedrine from the tablet powder.

The addition of methanol directly to the matrix was next investigated. Pseudoephedrine recovery in the presence of 400 μL methanol in-cell modifier increased from 44% to 51%. Attempts were made to further increase recovery by increasing the percentage of in-line methanol from 10% to 20%. It was expected that a subsequent increase in the solvating power of the fluid would result at a higher modifier percentage therefore more pseudoephedrine should be recovered. Surprisingly only 52% pseudoephedrine was recovered from the tablet with 20% methanol-modified CO_2. Since no further increase was observed, it was believed that the extraction of pseudoephedrine was not solubility limited but diffusion limited.

Subsequently the addition of the ion-pairing reagent, 1-heptanesulfonic acid, sodium salt in a 2:1 mole ratio was considered. As expected and previously observed via spike studies, a pseudoephedrine recovery from 51% (methanol-spike alone) to 72% (with 1-heptanesulfonic acid, sodium salt) was observed with 10% methanol-modified CO_2. Next it was of interest to examine if quantitative recovery could be obtained if 1-heptanesulfonic acid, sodium salt in methanol was used as an

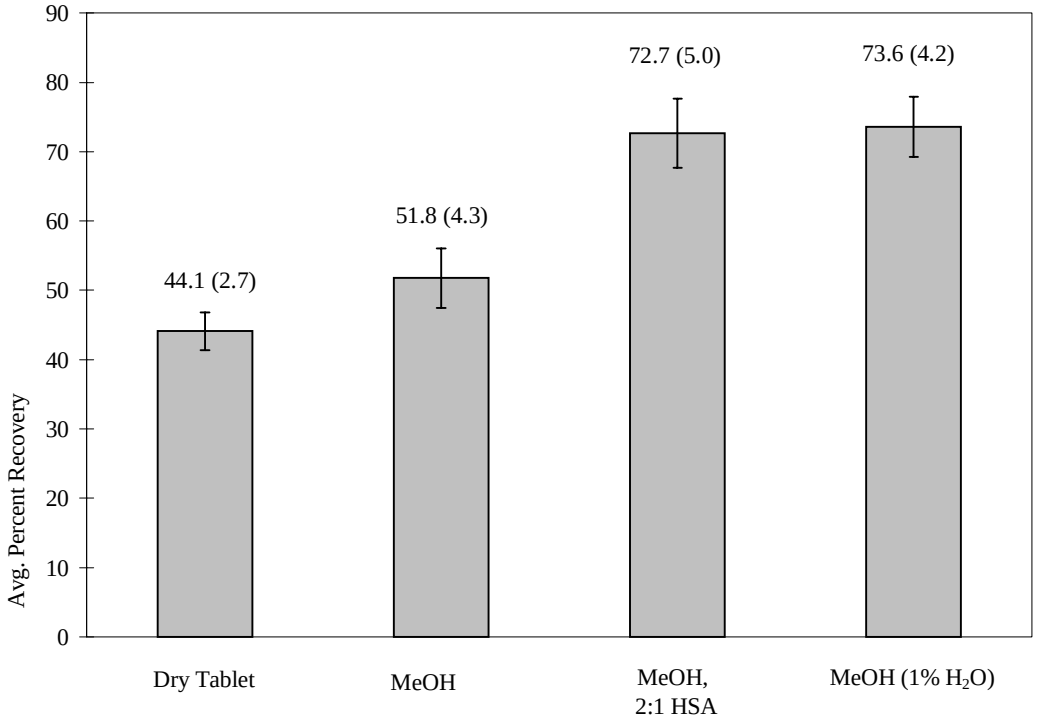


Figure 5.8. Average Percent Recovery (n=3) of Pseudoephedrine from Suphedrine Tablets as a Function of In-Cell Modifier Composition with 10% Methanol-Modified CO_2. Error Bars and Numbers in Parentheses Represent One Standard Deviation.

SFE Conditions: 0.99 g/mL CO_2, 450 atm, 35 °C, 2.0 mL/min., 5 min. static time, 80 grams CO_2 (5 dynamic mini-steps), 2 min. static time during trap rinsing between dynamic mini-steps, 400 µL in-cell modifier volume

HSA - 1-heptanesulfonic acid, sodium salt

in-line modifier. It was expected that further ion-pair formation would occur if the ion-pairing reagent was added continuously. Unfortunately this strategy could not be considered due to experimental difficulties. Problems including modifier pump seal and check valve damage as well as line clogging resulted, therefore, this option was no longer explored.

Finally, a methanol/water mixture was investigated as an in-cell modifier with 10% methanol-modified CO_2. Recoveries increased from 51% (methanol spike alone) to 73% (methanol/water). Once again the methanol/water in-cell modifier proved just as useful the ion-pairing reagent in pure methanol.

Although the addition of tetrabutylammonium hydroxide in methanol to the cell was shown to be beneficial in the extraction of pseudoephedrine from spiked-sand, its role as an in-line modifier was not examined for the tablet extractions. Tetrabutylammonium hydroxide was not explored because: 1) under highly basic conditions, tetrabutylammonium hydroxide may react with the pseudoephedrine causing degradation, 2) immediate flushing of the extractor lines would be needed so as to eliminate possible precipitation, and 3) a methanol/water modifier would be much easier to work with assuming equal effectiveness.

10% (1% H_2O) Methanol-Modified CO_2

The benefits of using the methanol/water mixture as an in-cell modifier were substantial. Next, its ability to serve as an in-line modifier was explored. Extractions were performed with 10% (1% H_2O) methanol-modified CO_2 while the effect of the addition of the ion-pairing reagent and the methanol/water in-cell spike was also examined (**Figure 5.9**). When extracting the tablet with 10% (1% H_2O) methanol in the absence of any in-cell modifier, 51% pseudoephedrine was extracted. When compared to 10% methanol-modified CO_2, a slight enhancement of 7% with the methanol/water in-line modifier was observed.

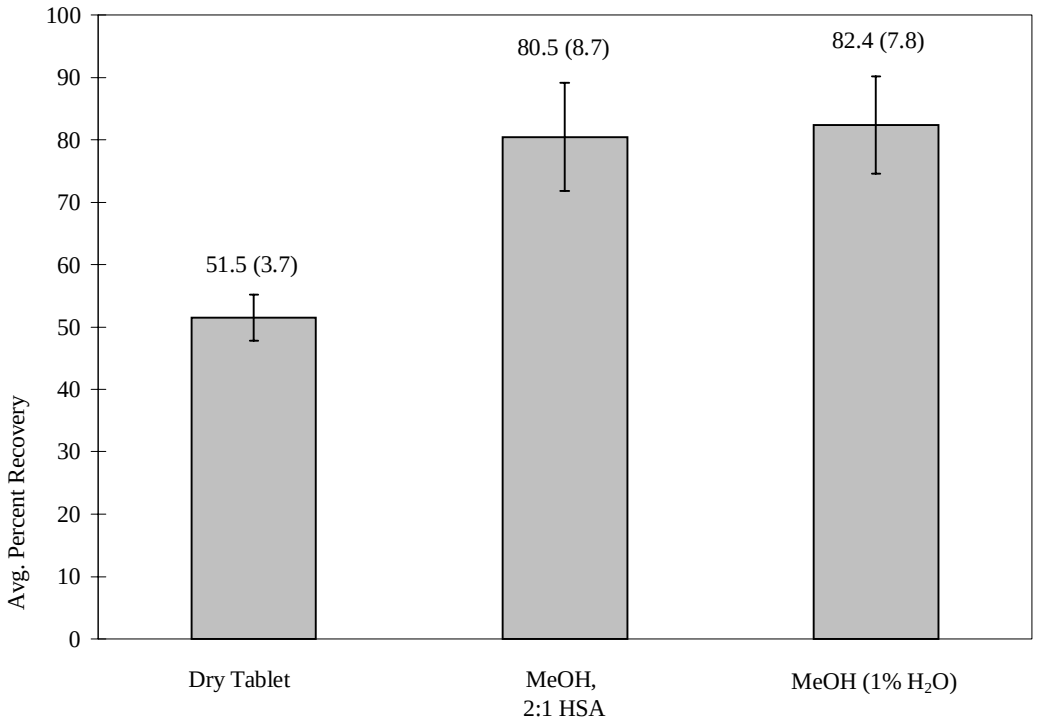


Figure 5.9. Average Percent Recovery (n=3) of Pseudoephedrine from Suphedrine Tablets as a Function of In-Cell Modifier Composition with 10% (1% H_2O) Methanol-Modified CO_2. Error Bars and Numbers in Parentheses Represent One Standard Deviation.

SFE Conditions: 0.99 g/mL CO_2, 450 atm, 35 °C, 2.0 mL/min., 5 min. static time, 80 grams CO_2 (5 dynamic mini-steps), 2 min. static time during trap rinsing between dynamic mini-steps, 400 µL in-cell modifier volume

HSA - 1-heptanesulfonic acid, sodium salt

The ability of the ion-pairing reagent to serve an extraction aide was also explored using this fluid. Heptanesulfonic acid, sodium salt (2:1 mole ratio) was dissolved in methanol, and the tablet was spiked at a volume of 400 μL. As expected a significant increase versus the dry tablet was observed where 80% was extracted. Extractions in the presence of a methanol in-cell modifier were not performed with this fluid. In-line modifier composition comparisons were then made between the two modifiers, 10% methanol and 10% (1% H_2O) in the presence of 1-heptanesulfonic acid, sodium salt where 72% and 80% were reported respectively. A paired t-test was performed, and the pseudoephedrine recoveries in the presence of a 1-heptanesulfonic acid, sodium salt methanol solution versus the two in-line modifiers were comparable.

Next tablet extractions with 10% (1% H_2O) methanol-modified CO_2 were performed in the presence of a methanol/water in-cell modifier and 82% was recovered. Recoveries in the presence of either methanol/water or 1-heptanesulfonic acid, sodium salt in methanol were similar.

Recovery as a function of in-line modifier composition (methanol, methanol/water) in the presence of the methanol/water spike were then compared where 73% (10% methanol) and 82% (10% methanol (1% H_2O) pseudoephedrine was recovered. A paired t-test was performed, and it was shown that regardless of in-line modifier composition, similar recoveries were obtained.

Regardless of the composition of the in-cell modifier, the results obtained were similar. It was expected that the recoveries with the methanol/water in-line modifier should be greater as compared to methanol alone because the solubility of pseudoephedrine in a more polar fluid should increase. Once again it is postulated that the extraction of pseudoephedrine from the tablet matrix regardless of the in-line modified fluid was not solubility limited but diffusion limited.

Of the various strategies investigated, the extractions with 10% methanol (1% H_2O) in the presence of a 400 μL spike of methanol (1% H_2O) and 1-heptanesulfonic acid, sodium salt in methanol were shown to be equally successful. Of the two in-cell modifiers,

methanol/water would be suggested for further investigation due to the simplicity of its use. When using the ion-pairing reagent in-cell modifier restrictor plugging was observed during the first dynamic mini-step most probably due to the large amounts of both extracted analyte and ion-pairing reagent. However, this occurrence was not observed with the methanol/water in-cell modifier.

Identification of the Extracted Analyte

Previous studies have made attempts to extract amine hydrochlorides with pure and modified carbon dioxide. [1-3] Of the three reports, the composition of the extracted analyte was not identified. In all three cases, it was believed that the free base was extracted because salts should not be extractable by SFE due to their ionic characteristics. Therefore it was of interest to identify the form of pseudoephedrine that was being extracted in the absence of any in-cell modifier. Suphedrine tablet extractions were performed with 10% methanol- and 10% (1% H_2O) methanol-modified CO_2. Silver chloride tests were conducted to confirm the presence of chloride in the extracts. Approximately 1-2 mL of 5% (w/v) silver nitrate in water was added to pure methanol and to the methanol extract solutions. When silver nitrate was added to pure methanol, the solution remained clear and colorless. Therefore, it was assured that the amount of chloride in methanol was negligible. Then $AgNO_3$ was added to the extract solutions (methanol and methanol/water in-line modified), and a white precipitate was observed. Therefore it was concluded that chloride was present in the extracts.

Next infrared spectra of the extracts were obtained with a Perkin Elmer (Norwich, CT) Series 1600 FT-IR spectrophotometer. First a pseudoephedrine hydrochloride solution containing the expected extractant concentration was prepared in methanol. This will be referred to as the pseudoephedrine hydrochloride standard. Via a nitrogen purge, the extract solutions were evaporated to approximately 1.0 mL. Using a disposable pipette, the extract solution was spiked onto a NaCl salt plate and allowed to dry. This

process was repeated until a visual film was observed. Then an IR spectrum of the spiked-salt plate was obtained. The IR of the pseudoephedrine hydrochloride standard is shown in **Figure 5.10**. IR studies of pseudoephedrine hydrochloride from a KBr pellet were previously performed by Benezra et al. [7] Several spectral assignments were made: 1) OH stretch (3270 cm^{-1}), 2) asymmetric C-H stretch (3010 cm^{-1}), 3) symmetric C-H stretch (2930 cm^{-1}), 4) $^{+}$NH stretch (2700 cm^{-1}), 5) C=C aromatic stretch (1587, 1490 cm^{-1}), OH bend, secondary alcohol (1430 cm^{-1}), and 6) C-H bend, monosubst. benzene (762, 702 cm^{-1}). The spectra of the pseudoephedrine hydrochloride standard and the literature reference were compared and similarities were noted. Bands corresponding to the OH stretch, asym. and sym. C-H stretch, C=C aromatic stretch, OH bend, secondary alcohol, and C-H bend, monosubst. benzene were observed at approximately +/- one wavenumber as compared to the literature reference. The $^{+}$NH stretch for the standard appeared at approximately 2725 cm^{-1} versus 2700 cm^{-1} as referenced; however this difference was felt to be insignificant. Suphedrine tablets were then extracted with 10% methanol- and 10% (1% H_2O) methanol-modified CO_2, and the infrared spectra were obtained (**Figures 5.11 - 5.12**). The spectra of the extracts and the pseudoephedrine hydrochloride standard were compared and almost identical spectra were observed regardless of modifier composition. Bands were observed at approximately 3270 (OH bend), 3010 (asym. C-H stretch), 1588 (C=C aromatic stretch), 1427 (OH bend, secondary alcohol), 761, and 702 cm^{-1} (C-H bend, monosubst. benzene). The appearance of the band corresponding to the $^{+}$NH stretch at 2725 cm^{-1} offered the most proof that the extracted pseudoephedrine existed as the hydrochloride salt versus the free base. Secondary amine salts absorb strongly in the 3000-2700 cm^{-1} with multiple bands extending to 2273 cm^{-1}. [8] While secondary amines show a single weak band in the 3350-3310 cm^{-1} corresponding to the asym. and sym. N-H stretching, and medium-to-weak bands in the 1250-1020 cm^{-1} region corresponding to

[7] *K. Florey*, ed., Analytical Profiles of Drug Substances, Volume 8, Academic Press, New York (1979) 489.

[8] *R.M. Silverstein, G.C. Bassler, T.C. Morrill*, Spectrometric Identification of Organic Compounds, Fourth Edition, John Wiley and Sons, New York (1981) 125-129.

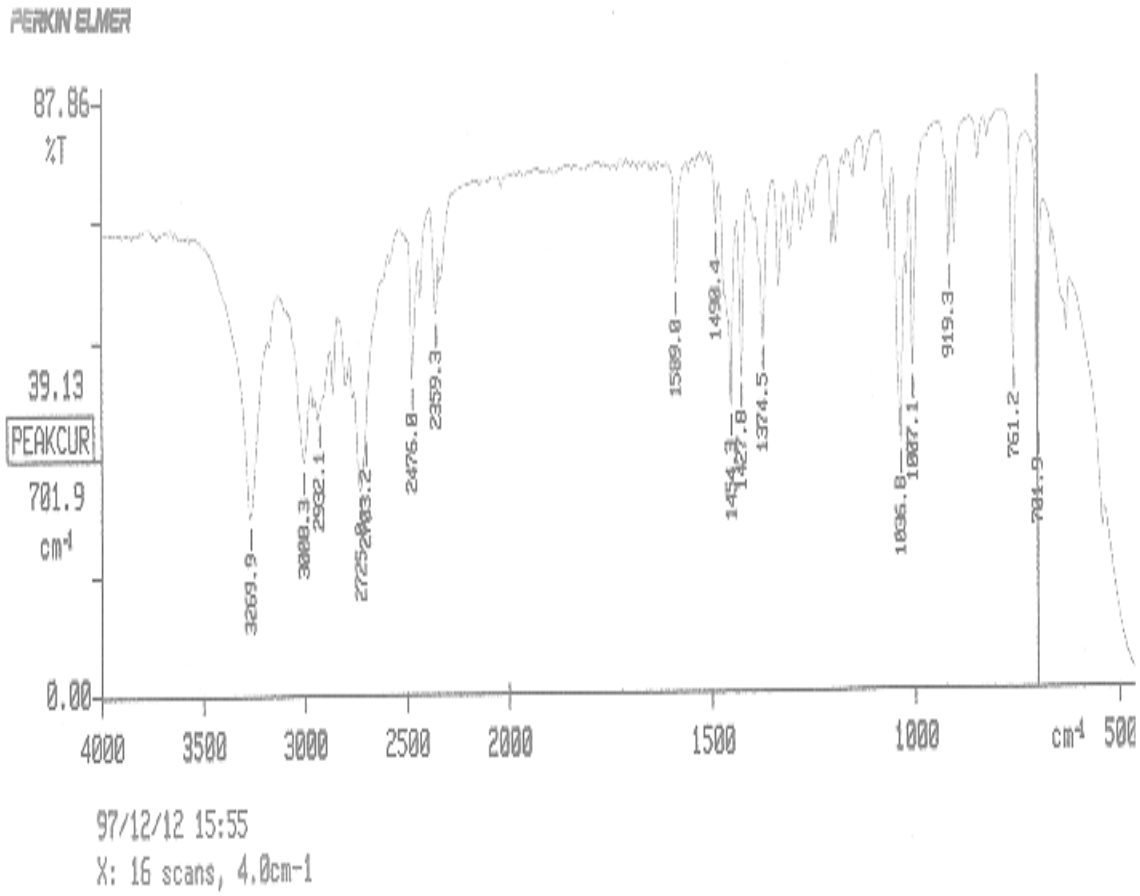


Figure 5.10. Infrared Spectrum of Pseudoephedrine Hydrochloride Standard. Solution spotted on a NaCl plate

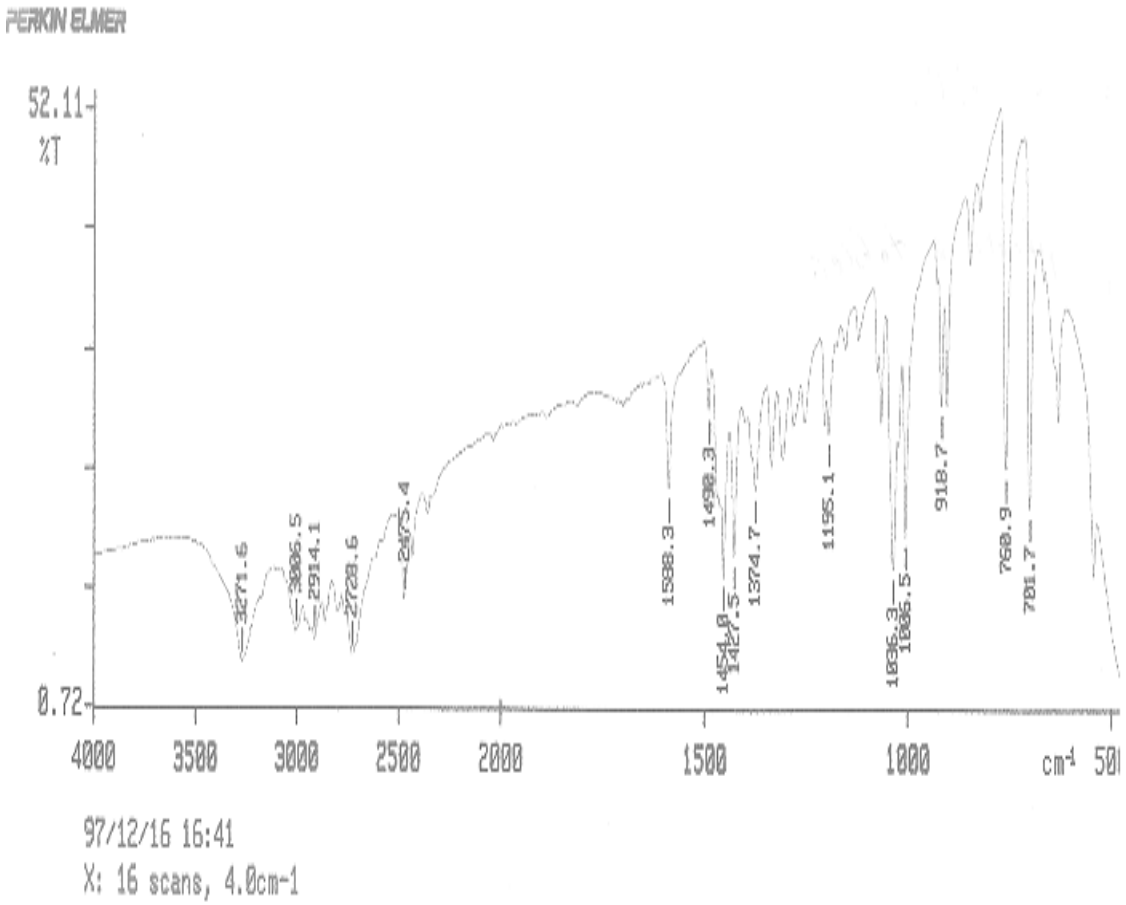


Figure 5.11. Infrared Spectrum of a Suphedrine Tablet Extract With 10% Methanol-Modified CO_2. Extract spotted on a NaCl plate.
SFE Conditions: 0.99 g/mL CO_2, 450 atm, 35 °C, 2.0 mL/min., 5 min. static time, 80 grams CO_2 (5 dynamic mini-steps), 2 min. static time during trap rinsing between dynamic mini-steps

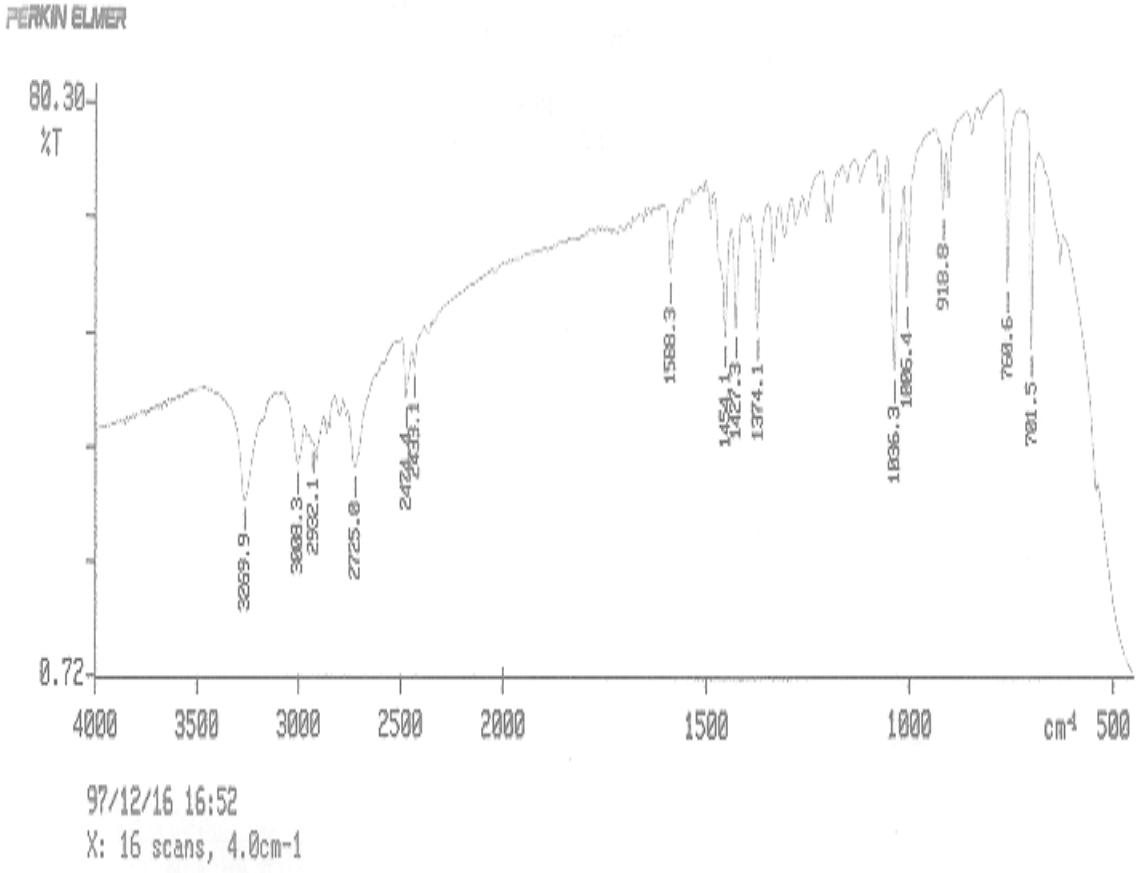


Figure 5.12. Infrared Spectrum of a Suphedrine Tablet Extract With 10% (1% H_2O) Methanol-Modified CO_2. Extract spotted on a NaCl plate.
SFE Conditions: 0.99 g/mL CO_2, 450 atm, 35 °C, 2.0 mL/min., 5 min. static time, 80 grams CO_2 (5 dynamic mini-steps), 2 min. static time during trap rinsing between dynamic mini-steps

C-N vibrations. From these experiments, it has been shown that hydrochloride salts can indeed be extracted with modified CO_2.

5.4 SUMMARY

The effect of modifier and additive composition upon pseudoephedrine recovery from spiked-sand and Suphedrine tablets was examined. First, the feasibility of enhancing the extractability through the use of an ion-pairing reagent was investigated. Recovery was shown to be dependent on CO_2 density as well as the addition of 1-heptanesulfonic acid, sodium salt to the spiked-sand surface. The presence of methanol in the extraction vessel prior to SFE was shown play a significant role in the ion-pair extraction process. The addition of ion-pairing reagent in methanol significantly increased the recovery of pseudoephedrine from a spiked-sand surface with pure-, 10%-, and 20%-methanol-modified CO_2. Quantitative pseudoephedrine recoveries of greater than 95% were shown to be achievable with 1-heptanesulfonic acid, sodium salt with 10%-methanol-modified carbon dioxide.

Second, the effect of the composition and concentration of the ion-pairing reagent was investigated. Recovery was shown to be a function of ion-pairing reagent composition and concentration. The most successful recovery was obtained in the presence of 1-heptanesulfonic acid, sodium salt in methanol at a 5:1 mole ratio. The increased extractability in the presence of ion-pairing reagent was attributed to reduced analyte polarity and analyte-matrix displacement.

Third, the effectiveness of other in-cell modifiers such as a methanol/water mixture, an acid, and a base were compared for the extraction of pseudoephedrine from spiked-sand. The recoveries obtained with an in-cell modifier of methanol/water and 1-heptanesulfonic acid, sodium salt in methanol were comparable. Subsequently no further enhancement was observed with 1-heptanesulfonic acid, sodium salt in methanol/water. It was believed that the solvating power of the extraction fluid was increased in the presence

of the methanol/water in-cell modifier, and analyte-matrix displacement from the spiked-sand was greater than in the presence of methanol alone.

The addition of an acid and a base as in-cell modifiers as a function of analyte recovery was also examined. Recovery was shown to be enhanced in the presence of trifluoroacetic acid. Although it was expected that the equilibrium between the ionic and free base species would be shifted favoring the ionic species, a slight enhancement was observed. In this case it was postulated that through hydrophobic and electrostatic interactions, trifluoroacetic acid was preferentially adsorbing to exposed active sites thus displacing the pseudoephedrine from the spiked-sand. Also, ion-pair formation between trifluoroacetic acid and pseudoephedrine may have occurred. The extractability in the presence of tetrabutylammonium hydroxide was significantly enhanced as compared to methanol alone. Under basic conditions, free base formation was favored, therefore, the recovery of a neutral species in the relatively non-polar fluid was obtained to a greater extent. Likewise, recovery in the presence of tetrabutylammonium hydroxide was shown to be statistically equal to those achieved in the presence of 1-heptanesulfonic acid, sodium salt in methanol and in the presence of the methanol/water in-cell modifiers.

Third several in-cell and in-line modifiers were examined for the extraction of pseudoephedrine from Suphedrine tablets. As observed in the sand spike-studies, equivalent recoveries in the presence of the in-cell modifiers, 1-heptanesulfonic acid, sodium salt in methanol and methanol/water were obtained and were shown to be significantly greater than in the presence of methanol alone with pure and methanol-modified CO_2. The effectiveness of a methanol/water as an in-line modifier versus methanol alone was also examined. Although it was expected that recovery with the more polar modifier (methanol/water) should be greater, no significant differences were noted. Therefore it was hypothesized that the extraction process of the pseudoephedrine from the tablets was not necessarily solubility limited in the extraction fluid but diffusion limited. The greatest pseudoephedrine recovery from Suphedrine tablets of 82% (7.0%) was

achieved with 10% (1% H_2O) methanol-modified CO_2 in the presence of 400 μL of methanol (1% H_2O).

Finally AgCl tests and infrared analyses were performed on two tablet extracts. It was confirmed that in the absence of any in-cell modifier, pseudoephedrine hydrochloride was extracted thus disproving the overall assumption that salts cannot be extracted via SFE with a carbon dioxide based fluid.

CHAPTER VI

METHOD DEVELOPMENT FOR THE SEPARATION OF PHOSPHOLIPIDS BY SUBCRITICAL FLUID CHROMATOGRAPHY

6.1 INTRODUCTION

Super/subcritical Fluid Chromatography (SFC) has been used successfully for the analysis of a variety of compounds including foods, polymers, and pharmaceuticals. [1-7] Supercritical carbon dioxide exhibits solvating power comparable to hexane and has been primarily utilized as a fluid for the separation of relatively non-polar compounds. [8] For this reason, many SFC methods have been developed as substitutes for normal-phase HPLC methods. Commonly used normal-phase HPLC mobile phases consist generally of chlorinated solvents such as chloroform and methylene chloride. Traces of water in these mobile phases cause retention times to fluctuate in the normal-phase mode. These solvents also present an environmental hazard, which can be eliminated in part by using a mobile phase of carbon dioxide which may or may not have been modified with a small percentage of organic solvent. Additionally, the quantity of solvent required and the high disposal costs commonly encountered with many normal-phase HPLC solvents may be dramatically reduced by employing carbon dioxide based mobile phases.

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[1] *J.W. King*, J. Chromatogr. Sci., **28** (1990) 9.
[2] *K.S. Nam, J.W. King*, J. High Resolut. Chromatogr., **17** (1994) 577.
[3] *K. Fudor-Csorba*, J. Chromatogr., **624** (1992) 353.
[4] *F.P. Schmitz, E. Klesper*, J. Supercrit. Fluid, **3** (1990) 29.
[5] *M.W. Raynor, K.D. Bartle, K. Davies, A. Williams, A.A. Clifford, J.M. Chalmers, B.W. Cook*, Anal. Chem., **60** (1988) 427.
[6] *P. Macaudiere, M. Claude, R. Rosset, A. Tambute*, J. Chromatogr. Sci., **27** (1989) 383.
[7] *J.T.B. Strode III, L.T. Taylor, A.L. Howard, M.A. Brooks, D. Ip*, J. Pharm. Biomed. Anal., **12** (1994) 1003.
[8] *C.R. Yonker, R.D. Smith*, "Supercritical Fluid Extraction and Chromatography", B.A. Charpentier, M.R. Sevenants (Ed.), ACS Symposium Series, Vol. 406, American Chemical Society, Washington, DC (1989) 52.

More polar analyte separations can be achieved by adding polar solvents such as methanol directly to the CO_2. [9] In many cases, high modifier percentages are needed to elute highly polar and/or high molecular weight compounds from a packed column in a timely manner. When using modified fluids, the critical pressure (P_c) and temperature (T_c) of the mixture lies between the P_c and the T_c of the modifier and CO_2. [10] Consequently, the combined mobile phase mixture may no longer be supercritical at a stated condition after incorporation of the modifier. Supercritical and subcritical fluids as well as enhanced-fluidity liquid mixtures (i.e. common HPLC eluents that have been pressurized with high pressures of CO_2) exhibit several advantageous properties compared with normal liquids including higher diffusivities and lower viscosities. The lowered viscosity, as compared to HPLC mobile phases, allows the use of higher flow rates, due to a decreased pressure drop along the length of the column. Numerous reports have illustrated that carbon dioxide based mobile phase separations can be achieved in significantly less time without a subsequent compromise in efficiency than via HPLC. [11-17]

[9] *M.L. Lee, K.E. Markides (Ed.),* "Analytical Supercritical Fluid Chromatography and Extraction", Chromatography Conferences, Inc., Provo, UT (1990) 100.
[10] *R.C. Reed, T.K. Sherwood*, Properties of Gases and Liquids, 2nd ed., McGraw-Hill, NY (1966).
[11] *E. Stahl, K.W. Quirin, D. Gerard*, "Dense Gases for Extraction and Refining", translated by M.R.F. Ashworth, Springer-Verlag, New York (1988) 176.
[12] *L.G. Randall (Ed.),* "Ultrahigh Resolution Chromatography", ACS Symp. Ser. 250, American Chemical Society, Washington, DC (1984) 135.
[13] *D.R. Gere, R. Board, D. McManigill,* Anal. Chem., **54** (1985) 736.
[14] *Y. Cui, S.V. Olesik,* Anal. Chem., **63** (1991) 1812.
[15] *S.T. Lee, S.V. Olesik,* Anal. Chem., **66** (1994) 4498.
[16] *S.T. Lee, S.V. Olesik,* J. Chromatogr. A**, 707** (1995) 217.
[17] *Y. Cui, S.V. Olesik,* J. Chromatogr. A**, 691** (1995) 151.

Phospholipids are commonly found in plant and animal tissue and serve as structural components in membranes in addition to playing a role in enzyme activation. [18] For this reason, both their biochemical and functional activities as related to their molecular structure are explored widely. Most commonly, phospholipid mixtures are used as emulsifying additives, thus, they have found many uses in the foods, cosmetics, and pharmaceutical industries. [19]

Phospholipid separations are most commonly performed by thin-layer chromatography (TLC) and normal-phase high-performance liquid chromatography (HPLC). TLC is traditionally used to qualitatively separate classes of phospholipids, but the quantitative separation of individual species is a common problem. [20] Recently, however, several reports have been published which describe more successful separations of individual phospholipid species by HPLC. [21-24] Most separations although are limited by the mode of detection. For example, when UV is utilized, low wavelengths (~200 nm) must be chosen or analyte derivatization is necessary. In a few instances, evaporative light scattering detection (ELSD) has been utilized. Specifically, Olsson et al. separated via HPLC phosphatidylethanolamine, phosphatidylinositiol, phosphatidic acid, and phosphatidylcholine from extracted brain tissues on a Nucleosil Diol packed column with a linear mobile phase gradient consisting of a mixture of hexane:2-propanol:*n*-butanol:tetrahydrofuran:isooctane:water and a second mixture of 2-propanol:*n*-butanol:tetrahydrofuran:isooctane:water. [21]

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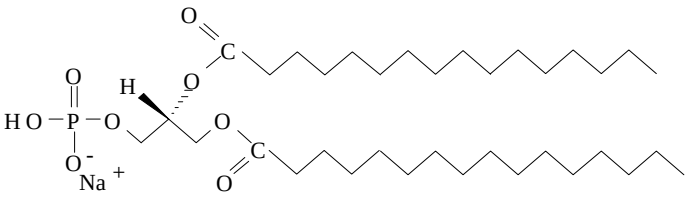
[18] *T.M. Devin*, "Textbook of Biochemistry With Clinical Correlations", 3rd ed., Wiley-Liss, New York (1992) 427.
[19] *B.F. Szuhaj (Ed.)*, "Lechithins: Sources of Manufacture and Uses", Am. Oil Chem. Soc., Champaign, 1989.
[20] *W.W. Christie*, "Lipid Analysis", 2nd ed., Permagon Press, New York (1992) 107.
[21] *N.U. Olsson, A.J. Harding, C. Harper, N. Salem Jr.*, J. Chromatogr. B, **681** (1996) 213.
[22] *A. Sakamoto, M. Novotny*, J. Microcol. Sep., **8** (1996) 397.
[23] *R. Szücs, K. Verleysen, G. Duchateau, P. Sandra, B. Vandeginste*, J. Chromatogr. A, **738** (1996) 25.
[24] *J. Becart, C. Chevalier, J.P. Biesse*, J. High Resolut. Chromatogr., **13** (1990) 126.

In 1992, Lafosse et al. reported on the use of the evaporative light scattering detection for the analysis of pharmaceuticals by both liquid and supercritical fluid chromatography. [25] In particular, the first analysis of phospholipids by SFC with ELSD was described briefly. The separation of phosphatidylcholine, phosphatidic acid, phosphatidylinositol, and phosphatidylethanolamine from soya lecithin was isocratically achieved in 22 minutes on a Zorbax Silica column (4.6 mm x 25 cm, d_p = 5 μm) with a mobile phase consisting of carbon dioxide modified with a mixture of methanol:water:triethylamine (95:4.95:0.05) in a 78.4:21.6 (w/w) ratio at a column outlet pressure of 278 bar, 45 °C, and a mobile phase flow rate of 4.3 mL/min. The only SFC parameter specifically discussed was column temperature. Increased detector sensitivity and reduced analysis time were achieved by working at a low temperature, 30 °C. No SFC vs HPLC comparisons were made.

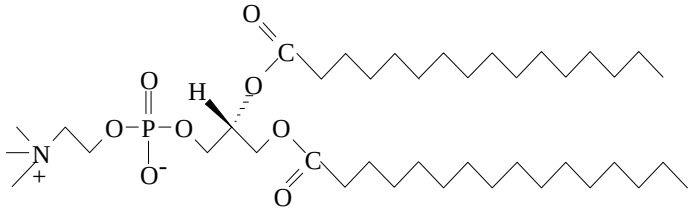
The main goal of this Chapter was to demonstrate further the applicability of SFC to phospholipid analysis. Specifically, the objective was to develop a qualitative analytical SFC method for the separation of five phospholipids varying in polarity and ionic nature. Structures for these compounds can be found in **Figure 6.1**. This work differs from the work described by Lafosse in that a systematic method development approach was undertaken to examine the effect of several SFC parameters on peak resolution and peak shape of five phospholipids. The parameters investigated were: stationary phase composition, acidic modifier additive concentration, modifier ramp rate, and column outlet pressure. In contrast to the Lafosse work, the fatty acid substituents of each phospholipid were well characterized in this study. For example, phosphatidyl choline, as assayed by Lafosse, contains mostly palmitic acid (16:0) or stearic acid (18:0) in the *sn*-1 position, and unsaturated C_{18} fatty acids such as oleic, linoleic, or linolenic

[25] *M. Lafosse, C. Elfakir, L. Morin-Allory, M. Dreux*, J. High Resolut. Chromatogr. **15** (1992) 312.

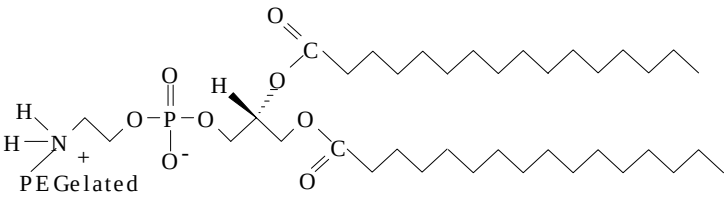
1,2-Dipalmitoyl-*sn*-Glycero-3-Phosphate (DPPA)



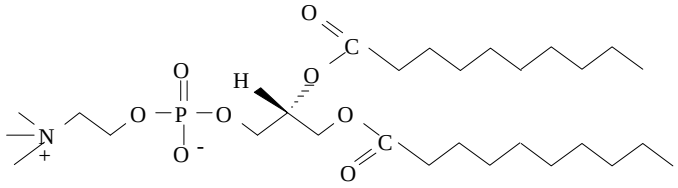
1,2-Dipalmitoyl-*sn*-Glycero-3-Phosphocholine (DPPC)



1,2-Dipalmitoyl-*sn*-Glycero-3-Phosphoethanolamine-N-[Poly(ethylene glycol)] (DPPE-PEG)



1,2-Dicaproyl-*sn*-Glycero-3-Phosphocholine (DCPC)



1,2-Dipalmitoyl-*sn*-Glycero-3-[Phospho-rac-(1-glycerol)] (DPPG)

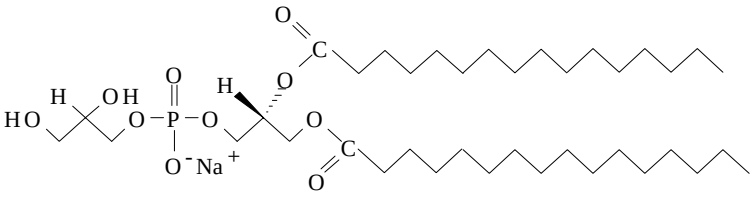


Figure 6.1. Chemical Structures of Phospholipids Investigated

acid in the *sn*-2 position. [18] In our separation, the fatty acid substituents of the choline substituted phospholipid were known to be palmitic acid in both the *sn*-1 and *sn*-2 positions.

6.2 EXPERIMENTAL

A Gilson SFC system (Middleton, WI) consisting of a model 308 liquid CO_2 pump, a 306 modifier pump, a 233 XL injector, a 811 C dynamic mixer, a 831 column oven, and a 821 pressure regulator were used for all separations. An Alltech Varex Mark II ELSD (Deerfield, IL) modified earlier for SFC was used as the detector unless stated otherwise. [26] All data were collected and analyzed using Gilson Unipoint software. All phospholipid solutions were prepared at 1.0 mg/mL in methanol. The injection volume in all cases was 25 μL. The column effluent was split between a variable pressure controlling restrictor and the ELSD using a three-way union. The effluent was introduced to the ELSD using a silica capillary (2-3 m, 50 μm i.d.). The ELSD conditions were as follows: CO_2 gas flow rate entering detector = 0.3 L/min., nitrogen flow rate = 0.7 mL/min., and drift tube temperature = 70 °C. The split ratio (ELSD:Variable restrictor) was 1:3. The columns investigated in this study were: a) Valuepak Amino, 4.6 mm X 15 cm, 5 μm (Keystone Scientific, Bellefonte, PA), b) Deltabond Cyano, 4.6 mm X 25 cm, 5 μm (Keystone Scientific), c) Hypersil Silica, 4.6 mm X 25 cm, 5 μm (Keystone Scientific), and d) Luna Octyl, 4.6 mm X 25 cm, 5 μm (Phenomenex, Torrance, CA). All chromatographic conditions can be found in the Figure captions.

SFE/SFC grade carbon dioxide without helium headspace was obtained from Air Products and Chemicals Inc. (Allentown, PA). HPLC grade methanol, ethanol, and trifluoroacetic acid were purchased from EM Science (Gibbstown, NJ), Aaper Alcohol (Shelbyville, KY) , and Sigma Aldrich (St. Louis, MO) respectively. All phospholipids

[26] *J.T.B Strode, L.T. Taylor,* J. Chromatogr. Sci. **34** (1996) 261.

were donated by the DuPont Merck Pharmaceutical Company but they were originally obtained from Avanti Polar Lipids (Alabaster, AL). No further purification was performed.

6.3 RESULTS AND DISCUSSION

The objective of this study was to develop a separation of five phospholipids that vary in polarity and ionic nature. Several chromatographic parameters including stationary phase composition, modifier additive, modifier additive concentration, modifier gradients, and column outlet pressure were investigated in an attempt to produce a baseline resolved separation in under 20 minutes. Due to the poorly UV absorbing nature of these analytes, Evaporative Light Scattering Detection (ELSD) was employed.

Effect of Stationary Phase

Since SFC best emulates normal-phase chromatography, several normal-phase packed columns were investigated. They were: a) Valuepak Amino, b) Deltabond Cyano, and c) Hypersil Silica. The SFC conditions were: 230 bar CO_2, 40 °C, 2.0 mL/min. liquid CO_2, 40% (v/v) methanol-modified CO_2. No peaks were observed for each singly injected phospholipid after a run time of 60 min., thus indicating that 40% methanol-modified CO_2 did not have sufficient solvent strength to solubilize the phospholipids or to break the hydrogen bonding interactions that may exist between the hydrophilic portions of the phospholipids and the highly active stationary phases. To remove the possibility that light scattering detection may have been inadequate, the column was removed, and each phospholipid was injected separately under the same conditions. A peak was observed for each of the phospholipids by ELSD, therefore, the detector was assumed to be operating properly concluding that each phospholipid did have sufficient solubility in 40% methanol-modified CO_2.

Due to the lipophilicity of the phospholipids, the separation was next attempted on a reversed-phase column (5 μm Luna Octyl). A modifier gradient was utilized due to the varying chemical characteristics of the phospholipids investigated as well as to ensure elution of all 5 analytes. The mobile phase consisted of a mixture of CO_2 and 50/50 (v/v) (0.01% trifluoroacetic acid (TFA)) ethanol/methanol. Use of these conditions resulted in a fairly successful separation wherein 4 chromatographic peaks were observed (**Figure 6.2**). Sufficient resolution was observed between DCPC and DPPA [R_s = 1.4], however, DCPC tailed greatly. Under these conditions, DPPC and DPPG were unresolvable. A sharp peak for DPPE-PEG was observed, however, an unidentified impurity peak eluted just prior to DPPE-PEG. Although a totally successful separation was not achieved at this point, we were satisfied that all five phospholipids were eluted from the column.

Effect of Modifier Additive

Secondary interactions between basic and acidic compounds with acidic silanols on the stationary phase surface may result in strong analyte retention and peak tailing in HPLC. [27] The addition of acidic and basic additives directly to the CO_2 modifier has been investigated in SFC in order to increase apparent solvent strength as well as to reduce unfavorable secondary interactions. [28-32] For example, Berger et al. reported on the separation of benzene polycarboxylic acids by packed column SFC using methanol-modified CO_2 mixtures that contained a small amount of a very polar additive. Using 5.7% (v/v) (0.5% trifluoroacetic acid) methanol-modified CO_2, 1,2-, 1-3-, and 1,4-

[27] *L.R. Snyder, J.L. Glajch, J.J. Kirkland*, Practical HPLC Method Development, John Wiley and Sons, New York (1988).
[28] *T. Berger, J. Deye*, J. Chromatogr. Sci., **29** (1991) 26
[29] *M. Ashraf-Khorassani, M.G. Fessahaie, L.T. Taylor, T.A. Berger, J.F. Deye*, J. High Resolut. Chromatogr., **11** (1988) 352.
[30] *L.J. Mulcahey, L.T. Taylor*, J. High Resolut. Chromatogr., **13** (1990) 393.
[31] *T.A. Berger, W.H. Wilson*, J. Chromatogr. Sci., **31** (1993) 127.
[32] *T.A. Berger, J.F. Deye*, J. Chromatogr. Sci., **29** (1991) 141.

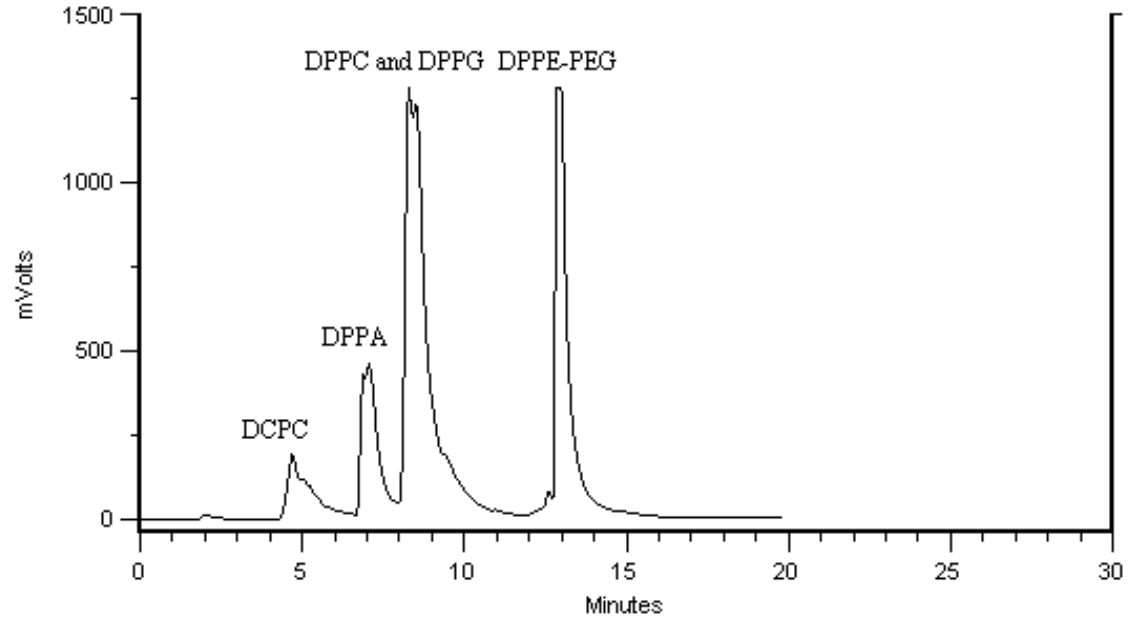


Figure 6.2. Chromatogram A of 5 phospholipids. Order of elution: DCPC, DPPA, DPPC, DPPG, DPPE-PEG. Mobile Phase, 9% (50/50 (v/v) ethanol/methanol (0.01% (v/v) trifluoroacetic acid)) for 2 min., ramp to 25% in 10 min. (2.0%/min.), 40% at 10.1 min.; oven temperature, 70 °C; outlet pressure, 125 bar, flow; 2.0 mL/min.; Column, 4.6 mm x 25 cm, 5 μm Luna Octyl. 25 μg of each phospholipid in methanol injected.

DCPC - 1,2-Dicaproyl-*sn*-Glycero-3-Phosphocholine
DPPA - 1,2-Dipalmitoyl-*sn*-Glycero-3-Phosphate
DPPC - 1,2-Dipalmitoyl-*sn*-Glycero-3-Phosphocholine
DPPG - 1,2-Dipalmitoyl-*sn*-Glycero-3-[Phospho-rac-(1-glycerol)]
DPPE-PEG - 1,2-Dipalmitoyl-*sn*-Glycero-3-Phosphoethanolamine-N-[Poly(ethylene glycol)]

benzenecdicarboxylic acids and 2-, 3-, 4-hydroxybenzoic acids were eluted from a Diol packed column. It was reported that without the use of the very polar additive, elution of all analytes was not achievable even at higher methanol modifier concentrations (9 and 11%).

Trifluoroacetic acid (TFA) was, therefore, added to the ethanol/methanol modifier (0.01% (v/v)) in hopes of neutralizing the charged phosphate group on DPPA as well as to reduce secondary interactions between the polar functionality on the phospholipid and any acidic silanols on the stationary phase. Only a solution of DPPA, DPPC, and DPPE-PEG was injected since DCPC and DPPA at this point coeluted with DPPA and DPPC respectively. Three visual observations can be made. First, only DPPC and DPPE-PEG were initially thought to be observed **(Figure 6.3)**. Second, the peak shape of DPPC appeared to be the same with and without the modifier additive. Third, in absence of the additive, detrimental effects on the peak shape of DPPE-PEG (i.e. decreased peak height, broadening) were observed. Clearly, when the acidic additive was present, the secondary interactions were lessened thereby resulting in a favorable peak shape for DPPE-PEG. A mechanism that may be used to explain the additive's advantageous effect on the peak shape of DPPE-PEG is stationary phase modification. It is well known that complete coverage of the silanol sites with the octyl groups is impossible. For instance, the polar ethanolamine functionality on the phospholipid may hydrogen bond to the residual silanol groups on the stationary phase surface. When introduced, the additive may preferentially absorb to these exposed silanol groups thus reducing any interactions with the phospholipids. [28]

In order to decipher whether coelution of DPPA and DPPC was occurring or whether DPPA was not entirely eluting from the column, DPPA was singly injected. By overlaying the chromatograms of the phospholipid mixture and DPPA alone **(Figure 6.4)** without the additive, the coelution of DPPA and DPPC was obvious. The peak shape of DPPA in the absence of TFA was also not desirable due to its excessive peak width. The addition of trifluoroacetic acid to the modifier allowed the efficient elution of DPPA from

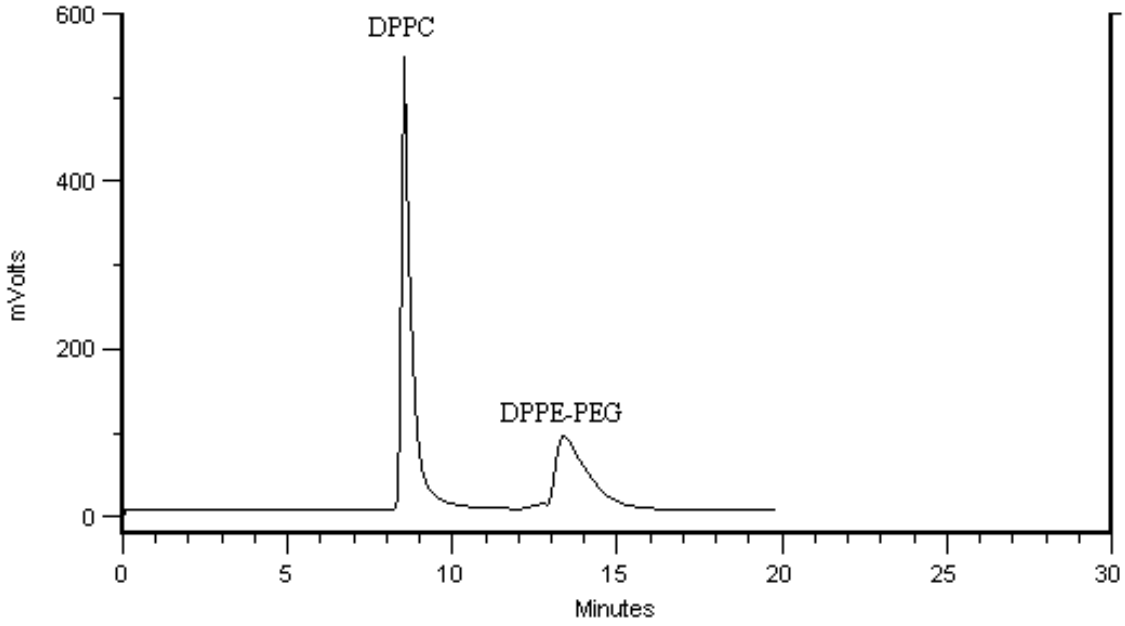


Figure 6.3. Chromatogram B of 3 phospholipids. Order of elution: DPPC, DPPE-PEG. Mobile Phase, 9% (50/50 (v/v) ethanol/methanol for 2 min., ramp to 25% in 10 min. (2.0%/min.), 40% at 10.1 min.; oven temperature, 70 °C; outlet pressure, 125 bar, flow, 2.0 mL/min.; Column, 4.6 mm x 25 cm, 5 µm Luna Octyl. 25 µg of each phospholipid in methanol injected.

DPPC - 1,2-Dipalmitoyl-*sn*-Glycero-3-Phosphocholine
DPPE-PEG - 1,2-Dipalmitoyl-*sn*-Glycero-3-Phosphoethanolamine-N-[Poly(ethylene glycol)]

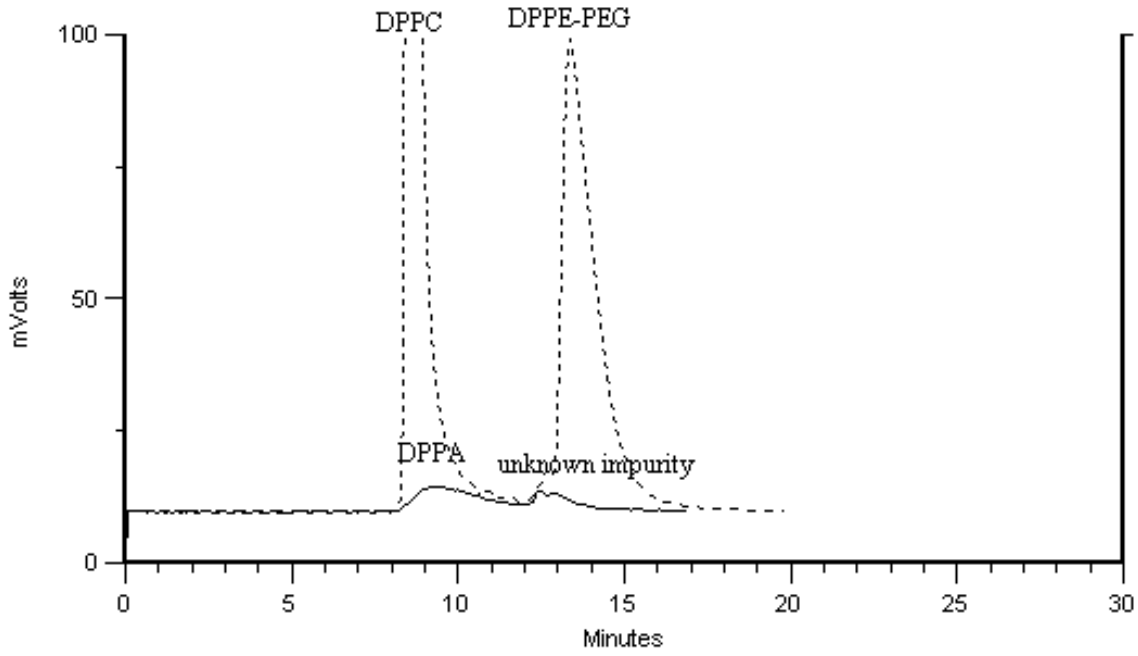


Figure 6.4. Chromatogram C of 3 phospholipids. Order of elution: DPPA = DPPC, DPPE-PEG.
—— DPPA alone ····· Phospholipid mixture

Mobile Phase, 9% (50/50 (v/v) ethanol/methanol for 2 min., ramp to 25% in 10 min. (2.0%/min.); 40% at 10.1 min.; oven temperature, 70 °C; outlet pressure, 125 bar; flow, 2.0 mL/min.; Column, 4.6 mm x 25 cm, 5 µm Luna Octyl. 25 µg of each phospholipid in methanol injected.

DPPA - 1,2-Dipalmitoyl-*sn*-Glycero-3-Phosphate
DPPC - 1,2-Dipalmitoyl-*sn*-Glycero-3-Phosphocholine
DPPE-PEG - 1,2-Dipalmitoyl-*sn*-Glycero-3-Phosphoethanolamine-N-[Poly(ethylene glycol)]

the column with improved peak shape and its complete separation from DPPC. The additive's usefulness can be attributed to neutralization of the DPPA as well as to stationary phase modification. Since charged species are believed to not exhibit significant solubility in pure CO_2, some of the charged DPPA may have precipitated out on the column; therefore, giving rise to a low detector response. Alternatively, it is possible that a mixture of both charged and neutral DPPA existed in solution, thereby giving rise to a large peak width and subsequent tailing.

Effect of Modifier Additive Concentration

Previously, the addition of trifluoroacetic acid to the modifier was shown to significantly improve the peak shape of DPPA and DPPE-PEG and to alter the selectivity of DPPA and DPPC. The modifier additive concentration was increased from 0.01% (v/v) to 0.1% (v/v) with a modifier ramp rate of 2.0%/min (same conditions as in **Figure 6.2**). Upon comparing the chromatograms with 0.01% (**Figure 6.2**) and 0.1% (v/v) TFA (**Figure 6.5**), the higher additive concentration was a definite improvement. Six peaks were observed in this chromatogram including the solvent peak, DCPC, DPPA, DPPG, DPPC, and DPPE-PEG (**Figure 6.5**). Additionally, the peak shape/height of DPPA was further improved in the presence of a higher concentration of TFA. The selectivity of the separation was also altered where the elution order of DPPC and DPPG reversed. To explain these phenomena, the structures of DPPC and DPPG (**Figures 6.1**) were compared in terms of polarity. DPPG is the more polar of the two due to it's glycerol-functionality versus the choline-functionality. In a reversed-phase process, the more polar compounds are expected to elute from the non-polar stationary phase first which indeed was observed in the separation with the higher additive concentration. The reversal in the elution order between DPPC and DPPG can be explained by the following reasoning. By increasing the additive concentration from 0.01% (v/v) to 0.1% (v/v), the stationary phase surface became less active. Consequently, partitioning of the nonpolar

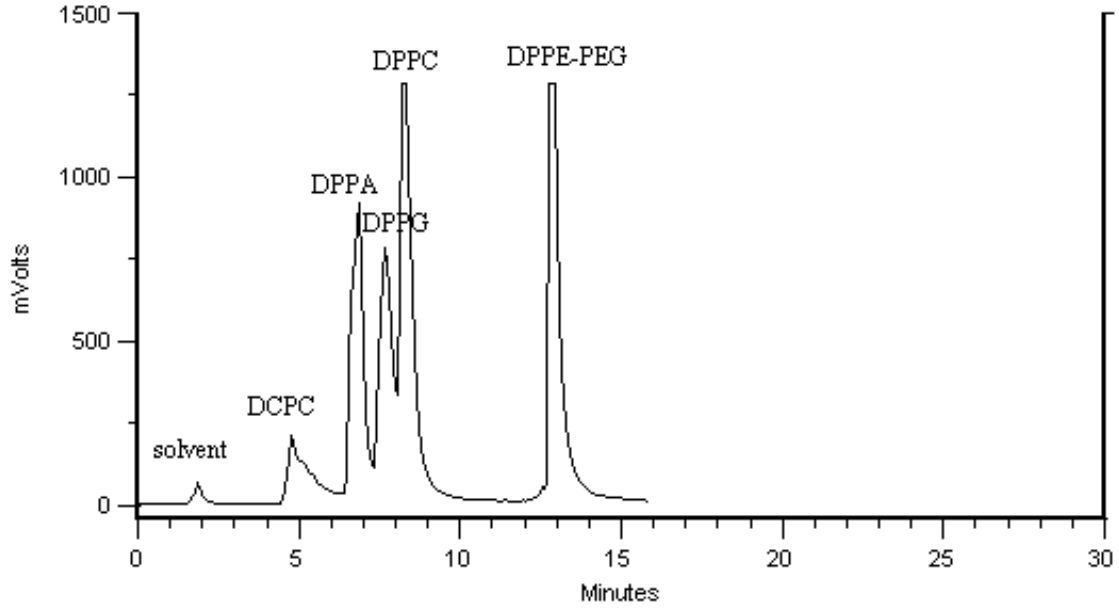


Figure 6.5. Chromatogram D of 5 phospholipids. Order of elution: DCPC, DPPA, DPPG, DPPC, DPPE-PEG. Mobile Phase, 9% (50/50 (v/v) ethanol/methanol (0.10% (v/v) trifluoroacetic acid)) for 2 min., ramp to 25% in 10 min. (2.0%/min.), 40% at 10.1 min.; oven temperature, 70 °C; outlet pressure, 125 bar, flow; 2.0 mL/min.; Column, 4.6 mm x 25 cm, 5 µm Luna Octyl. 25 µg of each phospholipid in methanol injected.

DCPC - 1,2-Dicaproyl-*sn*-Glycero-3-Phosphocholine
DPPA - 1,2-Dipalmitoyl-*sn*-Glycero-3-Phosphate
DPPC - 1,2-Dipalmitoyl-*sn*-Glycero-3-Phosphocholine
DPPG - 1,2-Dipalmitoyl-*sn*-Glycero-3-[Phospho-rac-(1-glycerol)]
DPPE-PEG - 1,2-Dipalmitoyl-*sn*-Glycero-3-Phosphoethanolamine-N-[Poly(ethylene glycol)]

functionality of the DPPG with the octyl stationary phase was believed to have increased at the higher additive concentration due to less interaction of analyte with the silanol groups. For these reasons, reversal in the elution order and better peak shape as well as improved separation of DPPG and DPPC was observed.

Effect of Modifier Ramp Rate

Similar to reversed-phase HPLC, mobile phase gradients are commonly employed in packed-column super/subcritical fluid chromatography. During the gradient, the stronger solvent, methanol, is increased in concentration at a certain rate. Initially, the separation of DPPC and DPPG was not achieved. Although an improved separation was observed at the higher modifier additive concentration, the resolution between DPPG and DPPC remained unsatisfactory. In hopes of improving the separation, the modifier ramp rate was reduced from 2.0%/min. to 0.5%/min. (**Figure 6.6**). Simply by lowering the modifier ramp rate, or allowing more time for the analytes to partition with the lipophilic stationary phase, baseline resolution between DPPG and DPPC became apparent. However, the peak shape of DPPA and DPPC became worse.

Effect of Pressure

At this point while the resolution between all of five phospholipids was satisfactory, the peak shapes were less than desirable. The pressure was increased from 125 bar to 135 bar, while the modifier ramp rate was held at 0.5%/min. (**Figure 6.7**). By simply increasing the pressure by 10 bar, the peak shape and response of all analytes was improved without a significant loss in resolution, thereby resulting in the optimized separation of all five phospholipids. The resolution values (R_s) between the following adjacent peaks were: 1.6 (DCPC and DPPA); 1.6 (DPPA and DPPG); 1.1 (DPPG and DPPC); and 10.1 (DPPC and DPPE-PEG). Other peak parameters including peak width

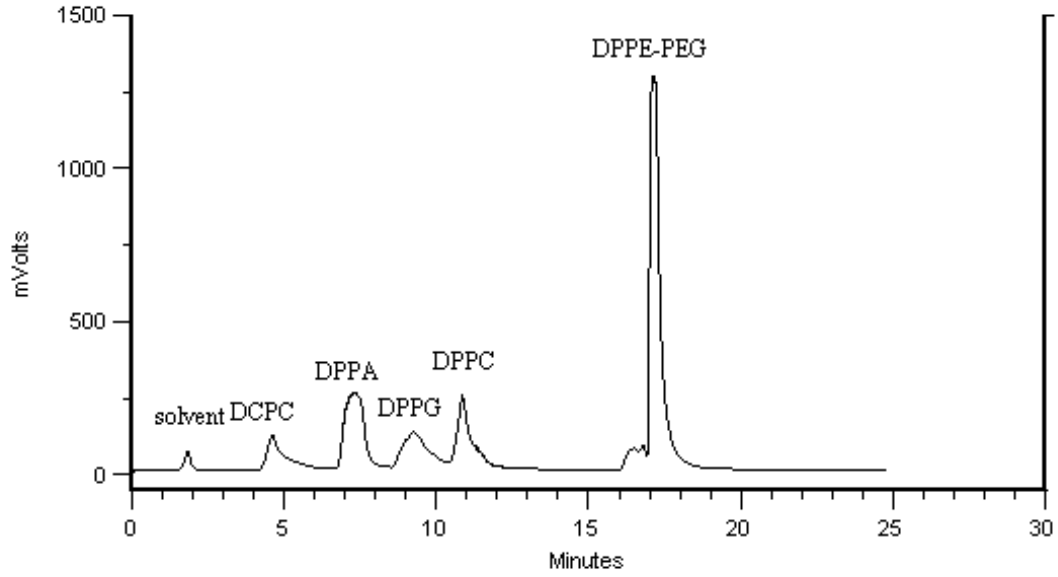


Figure 6.6. Chromatogram E of 5 phospholipids. Order of elution: DCPC, DPPA, DPPG, DPPC, DPPE-PEG. Mobile Phase, 9% (50/50 (v/v) ethanol/methanol (0.10% (v/v) trifluoroacetic acid)) for 2 min., ramp to 15% in 14 min. (0.5%/min.), 40% at 14.1 min.; oven temperature, 70 °C; outlet pressure, 125 bar, flow; 2.0 mL/min.; Column, 4.6 mm x 25 cm, 5 µm Luna Octyl. 25 µg of each phospholipid in methanol injected.

DCPC - 1,2-Dicaproyl-*sn*-Glycero-3-Phosphocholine
DPPA - 1,2-Dipalmitoyl-*sn*-Glycero-3-Phosphate
DPPC - 1,2-Dipalmitoyl-*sn*-Glycero-3-Phosphocholine
DPPG - 1,2-Dipalmitoyl-*sn*-Glycero-3-[Phospho-rac-(1-glycerol)]
DPPE-PEG - 1,2-Dipalmitoyl-*sn*-Glycero-3-Phosphoethanolamine-N-[Poly(ethylene glycol)]

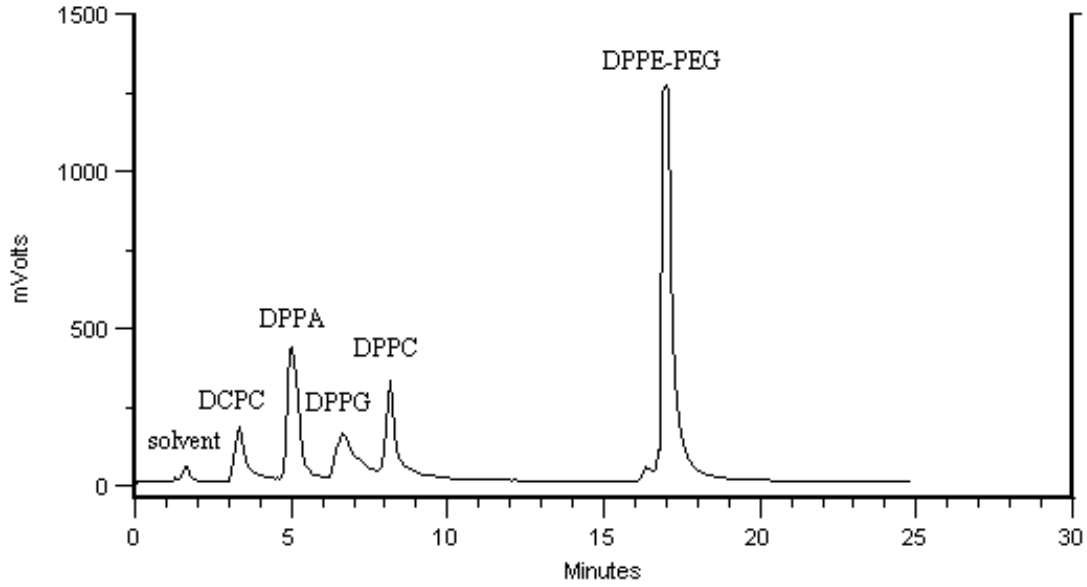


Figure 6.7. Chromatogram F of 5 phospholipids. Order of elution: DCPC, DPPA, DPPG, DPPC, DPPE-PEG. Mobile Phase, 9% (50/50 (v/v) ethanol/methanol (0.10% (v/v) trifluoroacetic acid)) for 2 min., ramp to 15% in 14 min. (0.5%/min.), 40% at 14.1 min.; oven temperature, 70 °C; outlet pressure, 135 bar, flow; 2.0 mL/min.; Column, 4.6 mm x 25 cm, 5 µm Luna Octyl. 25 µg of each phospholipid in methanol injected.

DCPC - 1,2-Dicaproyl-*sn*-Glycero-3-Phosphocholine
DPPA - 1,2-Dipalmitoyl-*sn*-Glycero-3-Phosphate
DPPC - 1,2-Dipalmitoyl-*sn*-Glycero-3-Phosphocholine
DPPG - 1,2-Dipalmitoyl-*sn*-Glycero-3-[Phospho-rac-(1-glycerol)]
DPPE-PEG - 1,2-Dipalmitoyl-*sn*-Glycero-3-Phosphoethanolamine-N-[Poly(ethylene glycol)]

at half height ($w_{1/2}$), capacity factor (k´), and selectivity factor (α), for the optimized separation can be found in **Table 6.1.** Specifically, the capacity factor (k') is a measurement that describes how long the component is retained on the stationary phase versus how long it resides in the mobile phase. Generally, k' values should be greater than 1 so that there is sufficient separation from the injection solvent and no greater than 10 where the analysis time would be too great. [33] As can be seen from the calculated capacity factors, all k' values for the phospholipid peaks ranged between 1-10. Another value that is used to evaluate the chromatographic separation is the selectivity factor (α). This value can be used to compare how close two adjacent peaks are to one another. Obviously, an α of greater than 1 must be achieved to ensure separation. Once again, all α values were at least 1.3. The third parameter is peak resolution (R_s). Resolution expresses how well two adjacent peaks are separated from one another. Adequate peak separation must be ensured for quantitative purposes. Baseline separation is represented by a R_s value of 1.5 while a R_s of 1.0 represents that the separation between the two peaks is 90% complete. [33] The separation of 4 of the 5 phospholipids resulted in baseline resolution (R_s >1.5), while the resolution between DPPG and DPPC was 1.1. Although this method was developed to serve qualitative purposes, it may prove to be a successful quantitative method due to good retention and separation in a timely manner.

Comparison of the Reproducibility of Two ELSDs

It was of great interest to examine whether this method could be transferred to another ELSD detector. Three phospholipids, DPPA, DPPC, and DPPE-PEG, each exhibiting a low, medium, and high ELSD response, were chosen for this evaluation. For this study, two ELSD detectors were compared in terms of area count reproducibility for

[33] *R.W. Yost, L.S. Ettre, R.D. Conlon*, Practical Liquid Chromatography, An Introduction, Perkin-Elmer, Norwalk, CT (1980).

Table 6.1. Various Chromatographic Peak Parameters
Mobile Phase, 9% (50/50 (v/v) ethanol/methanol (0.1% (v/v) TFA)) for 2 min., ramp to 15% in 14 min. (0.5%/min.), 40% at 14.1 min.; oven temperature, 70 °C; outlet pressure, 135 bar, flow, 2.0 mL/min.; Column, 4.6 mm x 25 cm, 5 μm Luna Octyl. 25 μg of each phospholipid in methanol injected.

	DCPC	DPPA	DPPG	DPPC	DPPE-PEG
Retention Time (min.)	3.33	5.00	6.67	8.16	16.97
T_r' (min.)	1.68	3.35	5.02	6.51	15.32
$w_{1/2}$ (min.)	0.33	0.40	0.62	0.29	0.32
k´	1.0	2.0	3.0	3.9	9.3
α		2.0	1.5	1.3	2.4
R_s		1.6	1.6	1.1	10.1

DCPC - 1,2-Dicaproyl-*sn*-Glycero-3-Phosphocholine
DPPA - 1,2-Dipalmitoyl-*sn*-Glycero-3-Phosphate
DPPC - 1,2-Dipalmitoyl-*sn*-Glycero-3-Phosphocholine
DPPG - 1,2-Dipalmitoyl-*sn*-Glycero-3-[Phospho-rac-(1-glycerol)]
DPPE-PEG - 1,2-Dipalmitoyl-*sn*-Glycero-3-Phosphoethanolamine-N-[Poly(ethylene glycol)]

a series of injections. The detectors compared were the Alltech Varex Mark II ELSD (Deerfield, IL) and the Sedere ELSD (Alfortville Cedex, France). ELSD and SFC conditions as well as area count comparisons (RSDs) are found in **Table 6.2**. Chromatographic comparisons can be visualized in **Figure 6.8**. Area count precisions for both detectors were found to be excellent. Regardless of detector, for two peaks, the RSDs were under 3% while, one peak resulted in a RSD of under 8 %. However, when comparing response (areas and peak height) as well as the RSDs, variances were observed amongst the two detectors. First, when comparing the response resulting for DPPA, the Varex detector response was 1.9 times greater than the Sedere detector, however, the precision of the area counts for the successive injections was comparable at approximately 3%. Visually, the peak width and peak shape produced with the two detectors was comparable. Second, the reverse trend was seen for DPPC. Although the peak height was greater with the Varex detector, the peak tailing of DPPC was more severe with the Sedere detector. Consequently, the reproducibility was greater, thus resulting in a 7% RSD vs 2% RSD obtained with the Varex. Third, once again the peak height and response was greater for DPPE-PEG with the Varex detector, however, greater peak tailing was observed. Therefore, the higher RSD of 7.4% resulted versus 1.9% for the Sedere detector. Overall, the reproducibilities were comparable and satisfactory regardless of detector manufacturer.

Table 6.2. Reproducibility Comparisons of Two ELSDs
Alltech Varex Mark II ELSD
CO_2 gas flow entering detector: 0.3 L/min.
N_2 flow rate = 0.7 mL/min.
Drift tube temperature = 60 °C
Sedere ELSD
N_2 pressure = 0.3 bar
Drift tube temperature = 70 °C
Gain = 7
Mobile Phase, 9% (50/50 (v/v) ethanol/methanol (0.1% (v/v) TFA)) for 2 min., ramp to 15% in 14 min. (0.5%/min.), 40% at 14.1 min.; oven temperature, 70 °C; outlet pressure, 135 bar, flow, 2.0 mL/min.; Column, 4.6 mm x 25 cm, 5 μm Luna Octyl. 25 μg of each phospholipid in methanol injected.

Injection #	Varex Area Counts (DPPA)	Sedere Area Counts (DPPA)	Varex Area Counts (DPPC)	Sedere Area Counts (DPPC)	Varex Area Counts (DPPE-PEG)	Sedere Area Counts (DPPE-PEG)
1	5912372		7437424		18527042	
2	5441115		7361325		18606146	
3	5735241	2982946	7432577	10940631	19400954	15549362
4	5659742	3057047	7203082	10921601	21010416	15542923
5	5720216	2836616	7496150	10131608	19438068	14988550
6	5626522	3041959	7206636	10013061	22657764	14952279
7	5811993	2932888	7436769	9111561	20689258	15418418
RSD	2.6	3.0	1.6	7.4	7.4	1.9

DPPA - 1,2-Dipalmitoyl-*sn*-Glycero-3-Phosphate
DPPC - 1,2-Dipalmitoyl-*sn*-Glycero-3-Phosphocholine
DPPE-PEG - 1,2-Dipalmitoyl-*sn*-Glycero-3-Phosphoethanolamine-N-[Poly(ethylene glycol)]

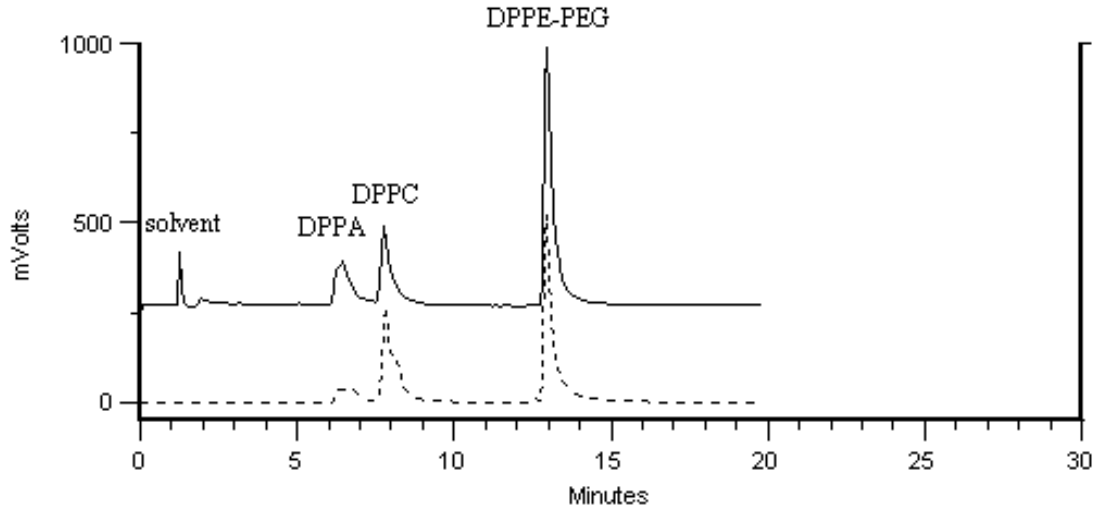


Figure 6.8. Reproducibility comparison of 2 detectors. Order of elution: DCPC, DPPA, DPPG, DPPC, DPPE-PEG.
—— Varex ELSD - - - Sedere ELSD

Mobile Phase, 9% (50/50 (v/v) ethanol/methanol (0.10% (v/v) trifluoroacetic acid)) for 2 min., ramp to 15% in 14 min. (0.5%/min.), 40% at 14.1 min.; oven temperature, 70 °C; outlet pressure, 135 bar, flow; 2.0 mL/min.; Column, 4.6 mm x 25 cm, 5 µm Luna Octyl. 25 µg of each phospholipid in methanol injected.

DPPA - 1,2-Dipalmitoyl-*sn*-Glycero-3-Phosphate
DPPC - 1,2-Dipalmitoyl-*sn*-Glycero-3-Phosphocholine
DPPE-PEG - 1,2-Dipalmitoyl-*sn*-Glycero-3-Phosphoethanolamine-N-[Poly(ethylene glycol)]

6.4 SUMMARY

Subcritical fluid chromatography is a viable technique for the separation of phospholipids. A normal stationary phase was not effective due to high adsorption of the polar functionality of the phospholipids onto the polar stationary phase, however, a reversed stationary phase proved useful. Retention was attributed to partitioning of the lipholiphilic portions of the phospholipids with the non-polar octyl phase. Elution of each analyte and its peak shape was improved by the addition of an acidic modifier additive, trifluoroacetic acid. Secondary interactions between the phospholipids and the exposed silanol sites on the stationary phase were further reduced by an increase in additive concentration thus altering the column selectivity and improving resolution. The separation of all five phospholipids was achieved by optimizing the modifier gradient and pressure. Several chromatographic parameters used to describe the separation were compared, and good retention and resolution was achieved in a timely matter. Finally, two ELSD detectors were compared in terms of peak response (peak height, area) and peak shape. No significant differences were observed regardless of detector manufacturer.

CHAPTER VII

CONCLUSIONS

The focus of this research was to examine the effects of primary and secondary modifiers on the extraction and separation of neutral and ionic pharmaceutical compounds with pure and modified carbon dioxide. Before one can examine the effects of various extraction parameters, one must achieve quantitative analyte trapping. The first part of this research examined several solid-phase traps in hopes of identifying one that could: 1) retain high amounts of analyte, and 2) have the capability of trapping a wide variety of analytes. Therefore, the trapping capacities of three solid-phase traps was determined for a mixture of components varying in polarity and volatility. Three solid-phase traps were investigated including: 1) a 50/50 (w/w) mixture of octadecyl silica (ODS) and glass beads, 2) glass beads, and 3) 50/50 (w/w) mixture of Porapak Q and glass beads. It was seen that analyte breakthrough from the solid-phase varied with trap and analyte composition. The trapping capacity for the ODS/glass beads trap was determined to be less than 8 mg/g of ODS for four analytes and less than 4 mg/g of ODS for naphthalene. The glass beads trap was the least successful for the trapping of the three more volatile analytes resulting in a capacity of less than 0.5 mg/1.5 g. Consequently, the retention of acetophenone, dimethylaniline, and naphthalene on the ODS trap was due to adsorption on the ODS material. However, the glass beads were shown to be an effective trapping material for the two less volatile, higher molecular weight components; 2-naphthol and *n*-tetracosane. A trapping capacity for these two analytes was comparable to the ODS/glass beads trap of > 2 mg/1.5 g of glass beads. In this case, their retention was attributed to cryogenic freezing on the glass beads surface. Finally, the most successful trapping material was shown to be the mixture of Porapak Q® and glass beads resulting in a trapping capacity of at least 10 mg/g of Porapak Q® per analyte.

Now that an optimum solid-phase trap was identified, the extraction of several neutral and ionic pharmaceuticals was investigated via SFE. Although the solubility of a polar analyte may be high in a modified fluid, successful extraction of the analyte from a complicated matrix such as a tablet may be problematic due to large analyte/matrix interactions. The addition of secondary modifiers (i.e. additives) for the extraction of a lovastatin from several complicated matrices such as in-house prepared tablet mixtures and MEVACOR® tablets was then investigated. In this particular case, the analyte being extracted was neutral and contained no ionizable functionalities. Although it was expected that ion-suppression would not play a role in this study, the effect of additive type (acidic, basic, neutral) on its ability to cover active matrix sites and thus displace the polar analyte from the complicated tablet matrix was investigated. In-line methanol-modifier percentage, additive type (acidic, basic, neutral) in the in-line methanol modifier, and the effect of additive concentration on the extraction efficiency were examined. Extractability was shown to be dependent upon modifier concentration and additive type. Due to the lactone ring contributing basic characteristics to lovastatin, isopropylamine was believed to be the most successful additive because of its ability to displace adsorbed lovastatin from the acidic tablet matrix sites, an effect not possible with in-line methanol-modified CO_2 alone. An optimized extraction method was developed, and lovastatin recoveries of 99.5% with a RSD of 1.2% from MEVACOR® tablets with 15% (v/v) (1.0% (v/v) isopropylamine) methanol-modified CO_2 were achieved.

The next goal of this research was to further examine the feasibility of extracting ionic compounds via SFE. Ionic compounds exhibit low solubility and extractability in carbon dioxide because of their high polarity. The recovery of ionic compounds with carbon dioxide may be increased if the analyte is in the presence of an ion-pairing reagent. Through electrostatic interactions, two species of opposite charge may form an ion-pair of reduced polarity, therefore, the extractability of the formed ion-pair complex would be expected to increase. In this part of the research, a screening study consisting of a fractional-factorial design was performed to identify the influential parameters that

significantly affected the recovery of triphenylphosphinetrisulfonate, sodium salt, (TPPTS) from a spiked-sand surface with ion-pairing additives. Several extraction parameters were examined, and four parameters were found to influence the recovery. They were: ion-pairing reagent composition, mole ratio of ion-pairing reagent to TPPTS, static extraction time, and in-cell methanol spike volume. First, the recoveries of the anionic species were shown to be enhanced when in the presence of an ion-pairing additive. Of the two quaternaryalkylammonium salts investigated, the more lipophilic reagent, tetrahexylammonium hydrogen sulfate, was the only ion-pairing reagent that statistically enhanced the recoveries. The increased extractability with the ion-pairing reagent in the non-polar fluid, CO_2, was attributed to reduced analyte polarity. Second, the amount of ion-pairing reagent added was also shown to be influential. By adding an excess of ion-pairing reagent, the equilibrium was shifted towards full and complete ion-pair formation, and an enhancement was observed. Third, static time was shown to negatively affect the recoveries. Over time and in the presence of moisture, it was believed that a mixture of both the neutral and charged trisulfonate species were present, therefore, less ionic species were present to form the ion-pair complex and lower recoveries were observed. Lastly, the recoveries of the polar compound were shown to be enhanced by increasing the polarity of the fluid by a simple increase in the in-cell methanol spike volume.

The extraction of an anionic compound was shown to be feasible with supercritical carbon dioxide, and recovery was enhanced in the presence of an ion-pairing additive. The main goals of this part of the research were to demonstrate that cationic species can be extracted via SFE, and to investigate the effects of modifiers and ion-pairing additives on the recovery of pseudoephedrine hydrochloride from spiked-sand and Suphedrine tablets. First, the feasibility of enhancing the extractability through the use of an ion-pairing reagent was investigated. Quantitative pseudoephedrine recoveries from spiked-sand were shown to be achievable with 1-heptanesulfonic acid, sodium salt, with 10%-methanol-modified carbon dioxide. Second, the effect of the composition and concentration of the ion-pairing reagent was investigated. Recovery was shown to be a

function of ion-pairing reagent composition and concentration. The most successful recovery was obtained in the presence of 1-heptanesulfonic acid, sodium salt, in methanol at a 5:1 mole ratio. The increased extractability in the presence of ion-pairing reagent was attributed to reduced analyte polarity and analyte-matrix displacement. Third, the effectiveness of other in-cell modifiers such as a methanol/water mixture, an acid, and a base were compared for the extraction of pseudoephedrine from spiked-sand. The recoveries obtained with an in-cell modifier of methanol/water and heptanesulfonic acid, sodium salt, in methanol were comparable. Subsequently no further enhancement was observed with heptanesulfonic acid, sodium salt, in methanol/water. It was believed that the solvating power of the extraction fluid was increased in the presence of the methanol/water in-cell modifier, and analyte-matrix displacement from the spiked-sand was greater than in the presence of methanol alone. The addition of an acid and a base as in-cell modifiers as a function of analyte recovery was also examined. The basic additive, tetrabutylammonium hydroxide, was shown to be more successful than the acidic additive, trifluoroacetic acid. Under basic conditions, free base formation was favored of pseudoephedrine, therefore, the recovery of a neutral species in the relatively non-polar fluid was obtained to a greater extent. Likewise, recovery in the presence of tetrabutylammonium hydroxide was shown to be statistically equal to those achieved in the presence of heptanesulfonic acid, sodium salt, in methanol and in the presence of the methanol/water in-cell modifiers. Third, several in-cell and in-line modifiers were examined for the extraction of pseudoephedrine from Suphedrine tablets. As observed in the sand spike-studies, equivalent recoveries in the presence of the in-cell modifiers, heptanesulfonic acid, sodium salt, in methanol and methanol/water, were obtained and were shown to be significantly greater than in the presence of methanol alone with pure and methanol-modified CO_2. The effectiveness of a methanol/water as an in-line modifier versus methanol alone was also examined. Although it was expected that recovery with the more polar modifier (methanol/water) should be greater, no significant differences were noted. Therefore it was hypothesized that the extraction process of the

pseudoephedrine from the tablets was not necessarily solubility limited in the extraction fluid but diffusion limited. The greatest pseudoephedrine recovery from Suphedrine tablets of 82% (7.0%) was achieved with 10% (1% H_2O) methanol-modified CO_2 in the presence of 400 μL of methanol (1% H_2O). Finally AgCl tests and infrared analyses were performed on two tablet extracts. It was confirmed that in the absence of any in-cell modifier, pseudoephedrine hydrochloride was extracted thus disproving the overall assumption that salts cannot be extracted via SFE with a carbon dioxide based fluid.

The last part of this research investigated the use of a carbon dioxide based mobile phase as means of separating a mixture of neutral and ionic phospholipids. Several chromatographic parameters were investigated including: 1) stationary phase composition, 2) addition of an acidic additive to the modified carbon dioxide and its concentration, 3) modifier ramp rate, and 4) column outlet pressure. As in the case of the extraction studies, a secondary modifier was investigated in order to possibly suppress analyte ionization as well as reduce secondary interactions between the analyte and the stationary phase. The results indicated that a normal stationary phase was not effective due to high adsorption of the polar functionality of the phospholipids onto the polar stationary phase, however, a reversed stationary phase proved useful. Retention was attributed to partitioning of the lipholiphilic portions of the phospholipids with the non-polar octyl phase. Elution of each analyte and its peak shape was improved by the addition of an acidic modifier additive, trifluoroacetic acid. Secondary interactions between the phospholipids and the exposed silanol sites on the stationary phase were further reduced by an increase in additive concentration thus altering the column selectivity and improving resolution. The separation of all five phospholipids was achieved by optimizing the modifier gradient and pressure. Several chromatographic parameters used to describe the separation were compared, and good retention and resolution was achieved in a timely matter. Finally, two ELSD detectors were compared in terms of peak response (peak height, area) and peak shape. No significant differences were observed regardless of detector manufacturer.

In summary, both neutral and ionic pharmaceuticals can be extracted and chromatographed with super/subcritical fluid based extraction solvents and mobile phases. Addition of secondary modifiers (i.e. additives) was shown to be critical in improving analyte recovery and separation.

VITA

Phyllis Ruth Eckard was born in Allentown, Pennsylvania, on July 29, 1970. She received her Bachelor of Science from Widener University in May of 1992. While attending Widener University, she completed two summer internships at the Foamex Co. in their Safety and Health Division, and the Esschem Co. in their Analytical Testing Division. After graduation, she was employed as a Contract Associate Scientist for the DuPont Merck Pharmaceutical Company in their Analytical Research and Development Division. In August of 1993, she began her graduate studies in Analytical Chemistry at Virginia Polytechnic Institute and State University, and later joined the research group of Dr. Larry T. Taylor in December of 1993. In the fall of 1995, she completed a semester internship at DuPont Merck in their Radiopharmaceuticals Division. She received her Ph.D. in analytical chemistry in February of 1998. Phyllis will join Allied Signal in their Polymers/Chemical Intermediates Division as a chemist in March of 1998.