EVALUATION OF CONDITIONS FOR QUANTITATIVE RECOVERY OF A DRUG FROM ANIMAL FEED USING SUPERCRITICAL FLUID CO_2 EXTRACTION

by

Dale C. Messer

This thesis submitted to the Graduate Faculty
of Virginia Polytechnic Institute and
State University
in partial fulfillment of the requirements
for the degree of

Masters of Science

in

Chemistry

APPROVED:

Larry T. Taylor, Chairman

Herve Marand

Mark R. Anderson

June 1991

Blacksburg, Virginia

This thesis is dedicated to

Ruth, Tim & Jess

with love.

ACKNOWLEDGMENTS

The author would like to take opportunity to thank those people who have made this time a Virginia Tech so fruitful. First a special thanks to Dr. Larry T. Taylor, I am grateful for the chance to work for and with you. I would like to thank you of your patience, prodding and support. I would also like to thank the members of my committee, Dr. Herve Marand and Dr. Mark Anderson, and Dr. Joseph Merola for his support of this project.

I would like to acknowledge all the members of our research group for allowing me the opportunity to work and grow with them. A many thanks to Leah Mulcahey for helping me to get started, and to Angela Howard for helping me to finish. I would also like to thank Angela for the special friendship she has shared with me.

Last, but certainly not least, I would like to thank my family, Ruth, Tim, and Jessica. Without their love and support, this would not have been possible.

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I. Introduction

In the pharmaceutical industry extraction of drugs from a variety of matrices is necessary as part of toxicological and dosage studies. Various means of extraction are used to facilitate this purpose. The focus of the following study is the initial evaluation of a supercritical fluid (SF) as an extraction solvent. This study will focus on the extraction of a hyperlipidemic drug (trade name xenalipin) from a rat feed matrix. Xenalipin has the chemical name of 4'-trifluoromethly-2-biphenylcarboxylic acid (1). Xenalipin is a chemically novel compound which has been found to significantly reduce serum cholesterol and triglycerides in two animal species, Spraque-Dawley rats and African green monkeys. Later studies found the drug to have significant side-effects, which caused further research on the xenalipin to cease. Partly because of the inactivity in other areas of xenalipin research, it was chosen for initial supercritical fluid extraction studies.

The current extraction method for xenalipin from rat food is as follows (2):

1. A 3g aliquot of feed is extracted with 25 mL methanol in a 50 mL centrifuge tube by mechanical shaking for 10 minutes.

2. Sample is allowed to settle or is slowly centrifuged.

3. A 2 mL aliquot of the supernatent is diluted to 25 mL with 80:20 methanol/water

4. This solution is filtered with a Millex SR filter (0.5 μm PTFE membrane) and quantitation is accomplished by high performance liquid chromatography.

Although this extraction method is quick and free of chlorinated organic solvent, xenalipin was chosen to be a test drug to explore the feasibility of supercritical fluid extraction. This information may then be generalized for extraction of other pharmaceutical compounds from rat feed matrix.

The goal of this study is to achieve quantitative extraction recovery (>95%) and a relative standard deviation of less than 5%. The preliminary study involves the extraction of pure drug from filter paper. These preliminary studies are then used to determine optimum extraction parameters. These optimized parameters are then used for the extraction of xenalipin from the rat feed matrix.

II. HISTORICAL ASPECTS OF SUPERCRITICAL FLUID EXTRACTION

A. INTRODUCTION

As stated by Anderson, (3) the goal of the analytical chemist is to acquire information about substances and the processing of substances in the hope that this knowledge will benefit our existence. As a result, the precision and sensitivity of analytical methods have consistently improved. Despite this continual improvement in analytical methodology, very little attention has been paid to improving sample preparation. Sample preparation is, in many cases, slow and difficult (4).

Sample preparation can take many forms such as grinding, preparative scale chromatography, derivatization and/or extraction. Several forms of extraction are possible. Liquid-liquid, (5) soxhlet type (6), and solid phase deposition (7) are all commonly used extraction techniques. The ideal extraction method must meet several criteria. First, an extraction method should be fast as well as selective and produce quantitative recovery of the analyte without degradation. Second, the extraction procedure should yield a solution that is ready for analysis without further need of cleanup or concentrating. Lastly, the extraction should be simple and inexpensive to perform.

One of the oldest and most commonly used extraction

procedures employs a Soxhlet apparatus. It has been the standard extraction procedure for nearly 90 years even though it fails to exhibit many of the ideal extraction criteria. The length of an average Soxhlet extraction ranges from 1 to 72 hours. The completed extraction produces a high volume, dilute solution which usually needs to be concentrated prior to analysis. In many cases, Soxhlet extraction is not selective, because interfering compounds may also be extracted which may further complicate the assay of the analyte(s) of interest. The choice of solvent is the only way to control the solvating power of the extraction. Perhaps the greatest disadvantage of using the Soxhlet method for extractions is its utilization of expensive, high purity organic solvents such as acetone and methylene chloride. The toxic effects of chlorinated solvents have already been well documented, and the proper disposal of these solvents is becoming rather costly (8).

B. Properties and Characteristics of Supercritical Fluids

A supercritical fluid is defined as any substance that is above both its critical temperature and its critical pressure. There are many compounds that may be used as a solvent in supercritical fluid extraction (SFE) (Table 1) (9). The ideal solvent should have mild critical parameters

Table 1. Physical Parameters for Supercritical Fluid Solvents

Fluid	Critical Temp. (C)	Critical Pressure (atm)	Dipole Moment (D)
Carbon dioxide	31.3	72.9	0
Nitrous oxide	36.5	72.5	0.51
Ammonia	132.5	112.5	1.65
Pentane	196.6	33.3	0
Sulfur hexafluoride	45.5	37.1	0
CCl_2F_2	111.8	40.7	0.17
Xenon	16.6	58.4	0

and a similar dipole moment. It should also be relatively inert, inexpensive, easily purified and non-toxic (4).

All of the solvents listed in Table 1 meet the requirement for critical parameters, but most fall short in other respects. Ammonia has by far the largest dipole moment, but it is a very active compound. Due to its toxicity and corrosiveness to pumping systems, it can not be used for routine analysis. Freon is a chlorofluorocarbon and is considered too harmful to the environment for use as a SFE solvent. Xenon is very expensive and has no dipole moment. Even though nitrous oxide supports combustion, it has been shown to be very useful in SFE, because of its permanent dipole moment.

Carbon dioxide is the most commonly used supercritical fluid for several reasons. The low critical parameters of carbon dioxide makes it easily employed in SFE. Carbon dioxide is also unreactive with most analytes, matrices, and instruments. In addition, carbon dioxide is safe and readily available at high levels of purity.

A supercritical fluid (SF) occupies the area shown in the phase diagram of Figure 1. Supercritical fluids have several unique physical properties which make them useful as extraction solvents. First, the solvating power of a supercritical fluid is related to its density which can be controlled by its pressure and temperature. It has been suggested by King et al. (10) that the solvating power of a

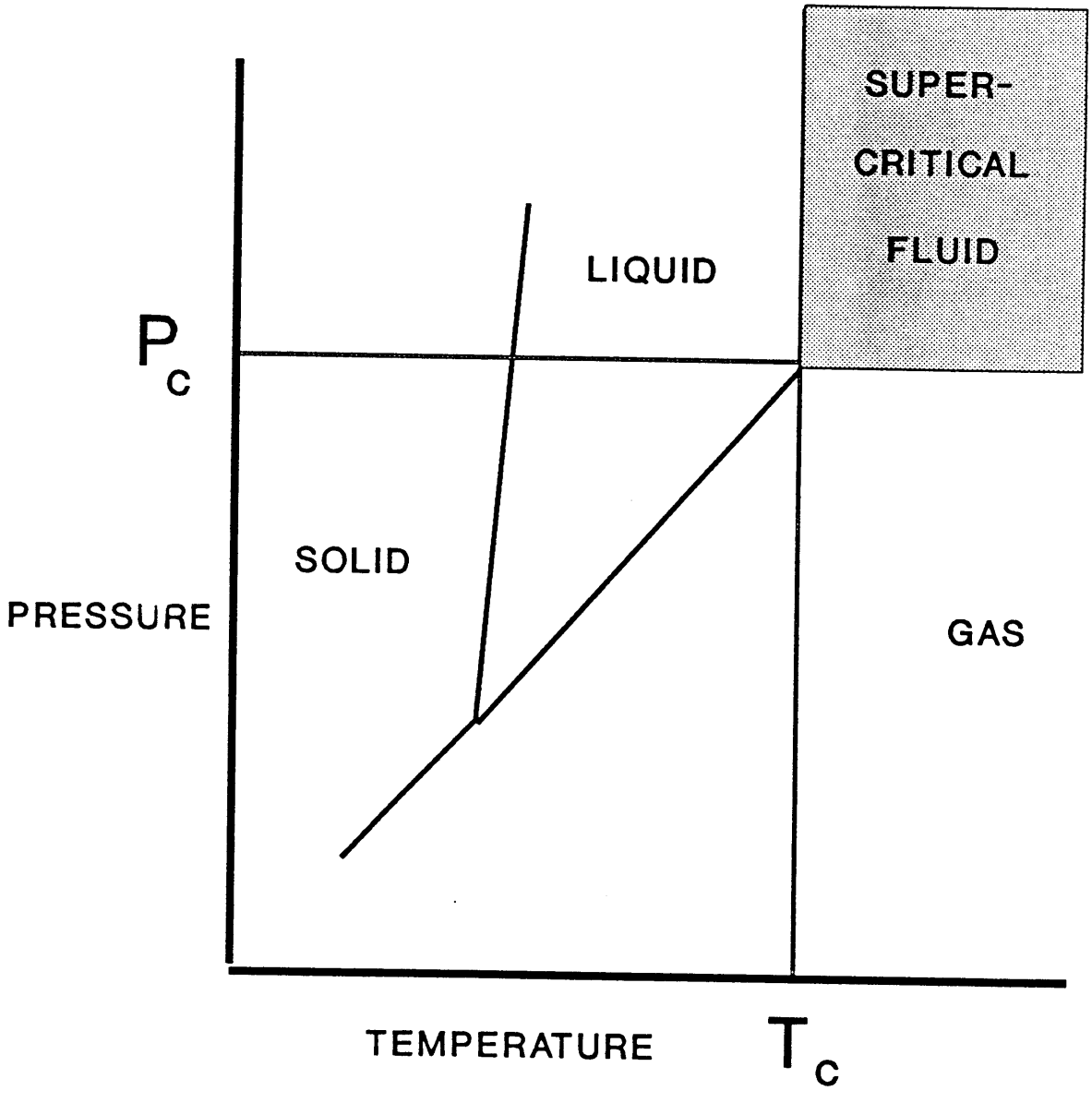

Figure 1. Phase diagram for pure compounds.

supercritical fluid can be predicted by the Hildebrand solubility parameter. The Hildebrand solubility parameter is defined as:

$$\delta = 1.25\ P_c^{1/2}\ (\rho\ /\rho_l).$$

where δ is the Hildebrand solubility parameter, P_c is the critical pressure, ρ is the density of the supercritical fluid and ρ_l is the density of the fluid in its liquid state.

These types of solubility parameters have been useful in comparing the solvating strength of one supercritical fluid to another, and/or to traditional solvents. As pointed out by Hawthorne (11) these solubility parameters have limited usefulness in predicting optimum extraction parameters. They are helpful when the analtye of interest is in high percentages and in bulk form and the solubility parameter of the analyte is available. In these cases maximum solubility is needed.

Hildebrand solubility parameters are less useful when a variety of analytes are to be extracted, or when the analytes are in trace amounts. For trace amounts, the analyte needs only to be soluble enough to be transported from the vessel. In these cases the solubility parameters are useful for setting initial conditions or a range of conditions to be explored, but the optimum extraction

conditions are usually found empirically. The first reason for this is the effect of the matrix on the extraction. During the extraction, the SF does not only solvate and transport the analyte but it also must compete for active sites on the matrix. The latter may become of primary importance in the extraction of the analyte.

In many extractions, several analytes are extracted and analyzed. This complicates the application of Hildebrand solubility parameter for the extraction and therefore limits their usefulness for these applications.

Anderson (3) explained that solubility in a SF is also a function of the volatility of the solute. When plotting pressure versus solubility, isotherms intersect at a pressure specific to a given solute. Below this pressures an increase in temperature decreases solubility and above this pressure the solubility increases. Therefore, at a given pressure, an increase in temperature will decrease the density of the fluid, while at the same time, this temperature increase exponentially raises the vapor pressure of the solute which may ultimately increase the solubility of a solute in a SF. To simplify the optimization of extraction parameters, many workers use density rather than pressure. At constant density, an increases in temperature increases the solubility. The possibility of achieving a selective extraction where the analyte is solvated and the

matrix is left behind may be achieved because the solvating power of the SF can be controlled in this manner.

Another beneficial attribute of a supercritical fluid is its high diffusivity and low viscosity. In general, supercritical fluids, have a diffusion coefficient approximately one order of magnitude higher than the corresponding liquid and a viscosity one order of magnitude lower (Table 2)(9). These properties give supercritical fluids gas-like mass transport properties and the ability to penetrate porous matrices easily.

A third advantage of most supercritical fluids is that they are gases at ambient conditions. As a result, removal of the analyte from the supercritical fluid is easily accomplished. The SF is simply allowed to decompress at room temperature which results in the precipitation of the analyte into a trap. This eliminates the time-consuming concentration steps involved in an extraction which utilizes organic solvents. A fourth advantage of a SF is its ability to extract thermally labile analytes. For example, the critical temperature of the most commonly used supercritical fluid, carbon dioxide, is 31°C. This low critical temperature enables SFE to be carried out at conditions amenable to thermally-labile compounds without degradation.

In summary, supercritical fluid extractions can be more selective and efficient than traditional Soxhlet-type

Phyiscal Properties of Carbon Dioxide

Mobile Phase	Density (g/mL)	Viscosity (poise)	Diffusivity (cm2/s)
Gas	0.001	.00005-.00035	.01-1.0
SCF	0.2-0.9	.0002-.001	.00001-.00033
Liquid	0.8-1.0	.003-.024	.000005-.00002

Table 2

extractions because the solvating power of the extraction solvent can be controlled. The need for solvent removal is eliminated and organic solvent use is largely reduced. Supercritical fluid extracts are generally more concentrated than those obtained from traditional extraction procedures making them more applicable to trace analysis. Finally, SFE can be used for sample preparation of thermally labile compounds.

C. Supercritical Fluid Extractions: Modes

Supercritical fluid extraction can be defined as the use of a supercritical fluid to remove an analyte from a solid or semi-solid matrix. Supercritical fluid extraction has shown itself to be a very versatile sample preparation method because of the advantages inherent in supercritical fluids outlined previously. Supercritical fluid extraction can be accomplished by using either a "static" or "dynamic" mode. A static extraction refers to one where a fixed amount of solvent is used to interact with the analyte/matrix. A static extraction may employ a recirculating pump which continuously passes the same supercritical fluid through the matrix. One of the experimental problems encountered with such an extraction is that it may not be exhaustive because the SF may become completely saturated with analyte. Although SFE has been

touted as being a selective extraction technique, there may be a problem with matrix mobility, i.e. the co-extraction of components of the matrix along with the analyte.

The alternative to a static extraction is a dynamic one. A dynamic extraction employs a continuous flow of fresh supercritical fluid which is continuously passed over and/or through the sample. A dynamic extraction can be more exhaustive than a static one because fresh SF is always in contact with the sample. However, impurities in the supercritical fluid become a concern when using larger amounts of fluid during an extraction. The contaminants in the supercritical fluid will ultimately arrive at the collection device and may interfere with the extract analysis. Other experimental problems with a dynamic extraction are enhanced matrix mobility and the possible loss of volatiles during the extraction.

D. Supercritical Fluid Extraction: On-line Analysis

A supercritical fluid extract can be analyzed either off-line or on-line. The on-line method of extraction uses the direct coupling of the SFE to the assay instrumentation. Supercritical fluid extraction has been successfully coupled to several spectroscopic and chromatographic analytical techniques. Several popular techniques include the coupling of SFE with a supercritical fluid chromatograph (SFC) or a

gas chromatograph (GC). The interface of SFE with either of these techniques has been accomplished with little or no loss of chromatographic efficiency (12). The interface between SFE and GC is achieved by simply allowing the analyte(s) to precipitate at the head of the column or into an injector by allowing the CO_2 to decompress. Supercritical fluid chromatography is interfaced to SFE by first trapping the extracted analyte and then using the same supercritical solvent at a higher density to purge the trap and to perform the chromatographic separation. The coupling is done by way of a six or ten port valve. To a lesser degree, it has been reported in the literature that on-line coupling of SFE with high performance liquid chromatography (HPLC) is possible (13,14). The interfacing of SFE with HPLC is made difficult because the gas generated by the expanded SF is trapped in the HPLC system which can interfere with the operation of the HPLC pump.

The advantages of on-line SFE as compared to off-line include providing the greatest extractant concentration for analysis, reducing volatile losses, and allowing for the use of very small sample sizes. On-line SFE can be performed both in the static and dynamic modes. On-line extraction also has fewer sample manipulations and is beneficial for the extraction and analysis of air and light sensitive analytes. One of the disadvantages of on-line SFE analysis is that the use of small extraction samples may not

represent the sample as a whole. The system can also be easily overloaded due to the direct coupling between the SFE and the chromatographic system. A third disadvantage is the necessity of having one chromatographic system dedicated to one SFE.

As reported by Hawthorne et al. (4,12) SFE has been successfully interfaced with GC. It was reported that the interface can be achieved by either cryogenically trapping the analyte on the head of the GC column or by trapping it into a split/splitless injector system. The effect of restrictor size and supercritical fluid flow rate on the recovery of an eucalyptus leaf extraction was investigated. Also included was an investigation of the effect that trapping temperature has on recovery. Hawthorne illustrated that loss of volatile analytes (pinene and 1,8-cineole) at a trapping temperature of 10°C was occurring, while at -30°C, the recovery of these two components was substantially increased. As the temperature was lowered further to -50°C a substantial loss of non-volatile components was noted. Hawthorne attributed the loss of these non-volatile compounds to the formation of a solid CO_2 plug inside the GC column. It was stated that the additional cooling produced during the expansion of the CO_2 allowed the system to reach the freezing point of CO_2 (-78°C). Hawthorne (4) reported a similar effect when trapping flavor and fragrance compounds

This type of trapping phenomenon has important implications in off-line SFE as well which will be discussed later.

Anderson et al. (3) have reported the effect of density and modifiers on on-line extraction, trapping and the subsequent supercritical fluid chromatographic analysis. The instrumental set-up is as follows. In general, a SFE system is connected to some type of trapping system. The trapping system employed is usually a chromatographic loop or a cryogenic trap. The trap can be filled by an absorbent or, in most cases, it is left empty. A valve system then allows the backflushing of the analyte from the trap onto the chromatographic column. Anderson (3) points out in his work that the effects of modifiers on trapping is not fully understood. The modifier probably changes the surface chemistry of the adsorptive traps. This implies that a trap which is adequate for an unmodified system may be inadequate when a modifier is added, or vice versa. It has been reported in the literature (15) that on-line SFE-SFC systems have been used for the analysis of a variety of biological, environmental, and polymeric samples.

E. Supercritical Fluid Extractions: Off-line Analysis

On-line SFE involves the coupling of the extractor directly to an analytical device while off-line SFE uses an autonomous trapping system. Off-line supercritical fluid

extractions have several advantages. With an off-line extraction larger sample sizes can be tolerated. Another advantage is that the analytes extracted can then be analyzed by multiple techniques. Injection size can also be better controlled in off-line analysis so that column overload is avoided. The analytical instrumentation employed is free to assay other samples as well. There are numerous parameters that must be investigated in off-line SFE in order to achieve quantitative recovery of the analyte(s) of interest. These variables include SF solvent type, density, time of extraction, and mass of SF. Other variables that could be considered are extraction temperature and the use of modified supercritical fluids.

One of the most important parameters to optimize in off line extractions is analyte trapping. An adequate trap must first catch the analyte and then release the analyte for assay when it is rinsed. There are two trap types, solvent or solid phase. Solvent traps are simply 1-4 mL of solvent in a vial. Solvents commonly used included methlylene choride, methanol and acetonitrile. Glass beads, stainless steel beads and HPLC column packings have all been routinely used as adsorbents in solid phase traps. Equally important in solid phase trapping is the type and volume of rinse that is used to remove the analyte from the trap.

The effects of density on solvating power have been previously discussed. The ability to change the solvating

power of the supercritical fluid can be an aid in obtaining a selective extraction. For example, using different densities may allow different classes of analytes to be selectively extracted from the same matrix. The chamber or extraction temperature can influence this situation. First, the temperature will affect the density of the supercritical fluid. At constant pressure the density of a supercritical fluid will decrease as temperature increases. This would imply a decrease in solvating power. Temperature also influences the analyte. For volatile analytes, an increase in temperature will increase the vapor pressure of that analyte. An increase in vapor pressure can increase the solubility of the analyte in the supercritical fluid (3) .

The amount of supercritical fluid and extraction time needed to achieve a quantitative recovery of the analyte can vary widely. Interaction between the matrix and the analyte(s) usually governs these variables. Despite this a rule of thumb for extraction volumes has been established (16). It has been determined that a minimum of three to ten void volumes of SF are necessary to achieve 100% extraction. This rule implies that the smallest possible extraction thimble should be used to maximize efficient removal of the bulk analyte.

In order to remove an analyte from a matrix, the analyte must be soluble in the solvent. Solubility alone is not enough however. In addition, the solvent must be able

to overcome the forces holding the analyte to the matrix, i.e. the analyte must have a preference for the SF solvent. In many cases, pure CO_2 is unable to overcome these matrix forces. Modifiers have been used to increase the efficiency of supercritical fluid extraction. Compounds commonly used as modifiers (9,17) for CO_2 SFE are methanol, methylene chloride, hexane, benzene, and toluene. By increasing the polarity of the SF solvent, extractions of more polar analytes such as drugs and drug metabolites are feasible. The effect of modifiers on matrices is not well understood (17). It is believed that modifiers increase the solvating power of the solvent and compete for active sites on the matrix (15). This allows for the release and transport of the analyte from the matrix into the extraction fluid. This demonstrates why analytes may be extractable from one matrix but unextractable from another.

F. Off-line SFE: Trapping

Without high efficiency in the trapping and recovery of analyte(s) from the trap, analytical SFE is not feasible. Although many types of traps have been investigated, the most common types of traps (15) are solvents or solid phase adsorbents.

Solvent traps, in principle, are simple to use. The analyte rich supercritical fluid passes through the

restrictor and decompresses into a vial containing from 1 to 4 milliliters of solvent. Commonly used solvents are methylene chloride (15) or hexane (17).

Solvent traps are also mechanically easy to assemble. A fused silica restrictor, either linear or tapered, is placed in a vial containing the solvent of choice. After the analyte has been deposited into the trap, there is no need for rinsing as is necessary with a solid phase trap. The major disadvantage of solvent traps is loss of analyte due to the aerosol effect (17,18,3). The aerosol effect is caused by the rapid expansion of supercritical fluid upon decompression due to the fact that 1 mL of supercritical CO_2 results in a decompressed gas volume of approximately 500 mL (9). Volatile analytes are the most susceptible to losses resulting from this effect. It has been shown that quantitative results can be obtained using a solvent trap. In these experiments, the flow rate was slow (19), (i.e. < 1 mL/min of SF), and/or the extraction time was short (19,20) i.e. (< 30 min). An additional experimental problem can be restrictor plugging.

Work performed by Wright et al. (21) showed the extent to which the aerosol effect can influence results by the extraction of six model compounds off of a XAD-2 resin. The model polycyclic aromatic compounds were chosen to cover a wide range of volatility. In this experiment two types of traps were used. The open vessel trap relied on cryo-

trapping at 0°C whereby a restrictor was placed into the neck of a round bottom flask. The alternate method investigated used a sealed vessel cooled by liquid N_2. In this method, all the supercritical CO_2 was immediately solidified upon entering the flask, thus preventing the escape of volatile analyte(s). The extraction used a liquid CO_2 flow rate of 4-6 mL/min, a temperature of 150°C, and a pressure of 410 bar. Extractions used approximately 250 mL of liquid CO_2 each and were performed in triplicate.

For the six model compounds, the average recovery ranged from a low of 0% to a high of 8.2% with a relative standard deviation of 10% for the first method described. When identical extractions were performed using the sealed trap, the range of recovery was from 25% to 95% with a relative standard deviation of 10%. This study emphasized the need for adequate trapping in supercritical fluid extractions in order to combat the aerosol effect.

The alternative to solvent trapping is to use a solid phase absorbent. The types of solid phase traps commercially available include glass or stainless beads and HPLC column packings such as, silica, C-18, or C-8 bonded silica. An advantage of solid phase trapping is that the type of trap adsorbent can be adjusted to best fit the type of analyte being trapped. However, solid phase traps are mechanically more difficult to interface to the extractor. With solid phase traps the supercritical fluid decompresses

into the trap and the expanded gas must pass through it. As a result, a gas tight interface between the end of the restrictor and the trap is required to prevent analyte losses. An additional rinsing step is needed with solid phase traps because the analyte must be removed from the trap. The inefficient rinsing of traps has been shown to lead to low recoveries by Mulcahey (22). It has also shown that it may be necessary to match the polarity of the analyte to that of the trap. This is particularly true in the case of volatile compounds. Mulcahey's data have shown less than quantitative recoveries for C10 to C14 aliphatic hydrocarbons when diol and amino traps were used. In the aforementioned case, cryogenic trapping due to expanding CO_2 would be the primary mechanism of trapping. However,when non-polar traps such as C-8 and C-18 were employed quantitative recoveries for these compounds were obtained.

More recently, inert materials such as glass or stainless steel beads, in conjunction with cryogenic trapping, have also been used. Even though data on these types of trapping materials are still sparse, it appears that these traps will be appropriate for intermediate volatile compounds.

G. SFE in the Food Industry

Because CO_2 is non-toxic, supercritical CO_2 extraction is very appealing in the food industry. Supercritical fluid extractions have been used for a wide variety of analytes and matrices in this context. Examples include the extraction of trichothecene mycotoxins from wheat (23) and fat from meat products (24). Studies have also been conducted on the extraction of analytes from hops, coffee, tea, tobacco, and spices (25). Flavors and fragrances from natural compounds have been successfully extracted using SF (26). Although many examples of the use of SFE in the food industry can be sited, a review of the literature revealed only one such study on animal feed. Locke (27) investigated the use of SF CO_2 for the extraction of menadione (vitamin K_3) from a rat feed. The rat food sample was spiked with menadione by first dissolving it in methylene chloride and then adding appropriate aliquots of this solution to the rat feed. This study employed a 20 minute static extraction utilizing SF CO_2 at a pressure of 8000 psi and a temperature of 60°C. The analyte was trapped in a 6 X 3/4 inch o.d. stainless steel tube filled with silica gel. Locke quotes a recovery of 90.5% with a RSD of 2.2% when extracting the vitamin at the 1 mg/g level. It was also reported that this method of extraction did not extract any lipophilic material from the rat feed. Therefore, no further sample cleanup was

required. The menadione was quantitated using high performance liquid chromatography and an electrochemical detector.

III. EXPERIMENTAL

A. Extractions: Liquid Trap

A modified Suprex (Pittsburgh, PA) Model 200A (Figure 2) supercritical fluid chromatograph was used for the preliminary work on this project. The Suprex 200A featured a 250 mL syringe pump capable of pressures in excess of 5000 psi. The instrument was computer-controlled and could operate in either a density or pressure programmed mode. Supercritical conditions were maintained by placing the extraction vessel in a GC type oven and using a fused silica outlet restrictor. Modification involved the placement of an extraction vessel in-line after the pump. A 1 mL HPLC precolumn from Upchurch Scientific (Oak Harbor, WA) served as the extraction vessel. A 60 cm length of 50 micron fused silica at the outlet of the extraction vessel served as a linear restrictor. A ten milliliter volumetric flask with vented cap containing acetonitrile solvent was used as a trap. SFC grade CO_2 was obtained from Scott Specialty Gases (Plumbsteadville, PA).

B. Extraction of rat feed: Liquid trapping

Primary extraction studies were done on the modified Suprex 200A previously described. For this series of

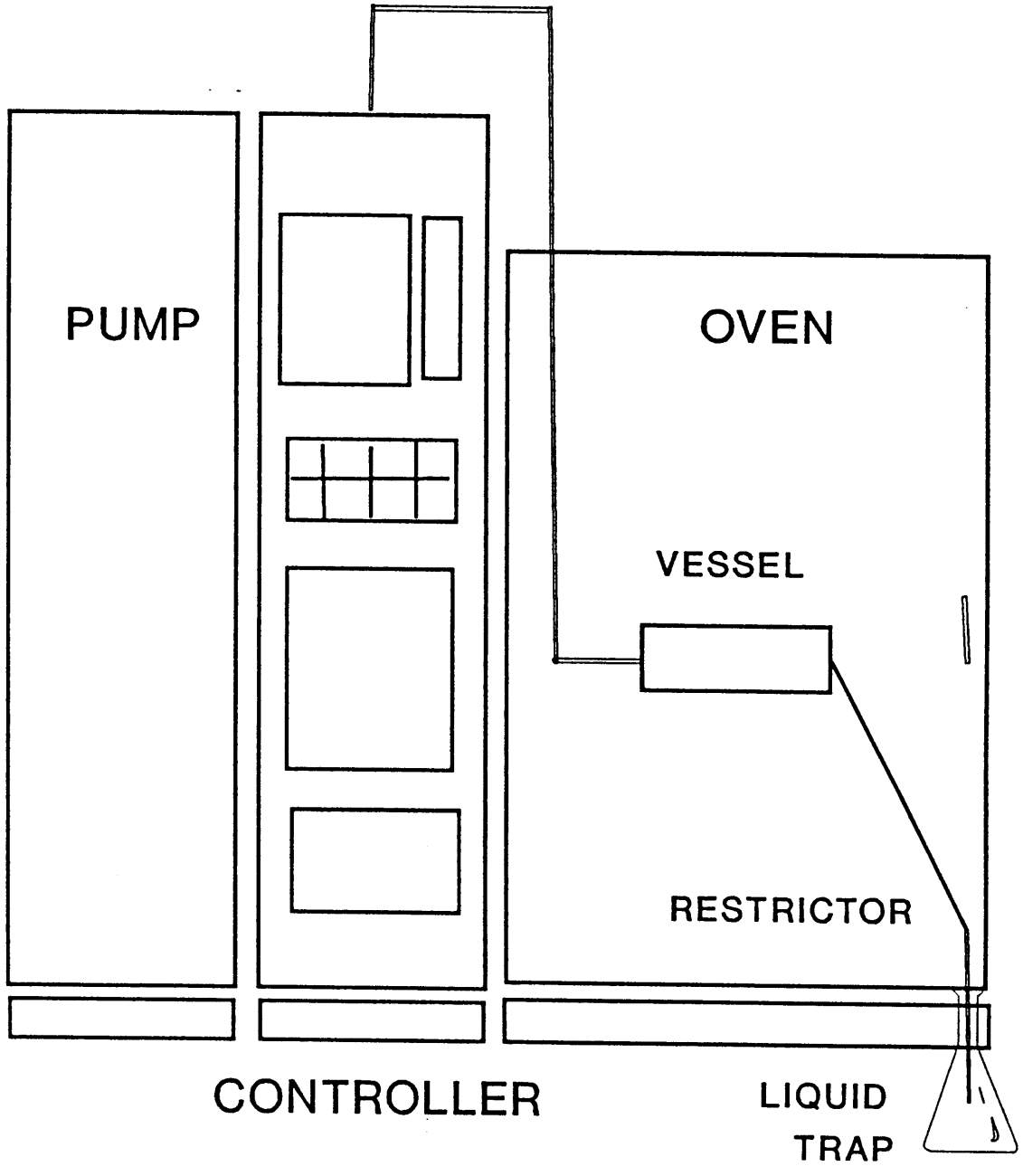

Figure 2. Modified Suprex 200A.

extractions oven temperature was maintained at 50°C and a liquid CO_2 flow rate of 1 mL/minute was used. The CO_2 was held at a pressure of 350 atm. The density of CO_2 at this temperature and pressure is 0.90 g/mL. The sample size extracted was 400 mg. Four milliliters of acetonitrile was used as the trapping solvent.

C. Extractions: Solid Phase Traps

The bulk of the extractions were performed on the Hewlett-Packard (Avondale, PA) Model 7680A Supercritical Extractor (Figure 3). Supercritical fluid enters the HP 7680A from any of three solvent tank reservoirs. The system is designed to use one pure CO_2 source as well as two modified CO_2 sources. From the tank the solvent passes to the pump. The pump is a cryogenically cooled dual-head reciprocating type capable of pressures up to 5000 psi, flow rates up to 4 mL/min and densities up to 0.9 g/mL. The heads of the pump are maintain at 5°C using CO_2. From the pump, the supercritical fluid can either go though the by-pass loop or though the chamber containing the extraction vessel. The by-pass is used to allow the instrument to reach the desired supercritical parameters before the extraction begins. During the extraction, the solvent

(HEWLETT-PACKARD Co.)

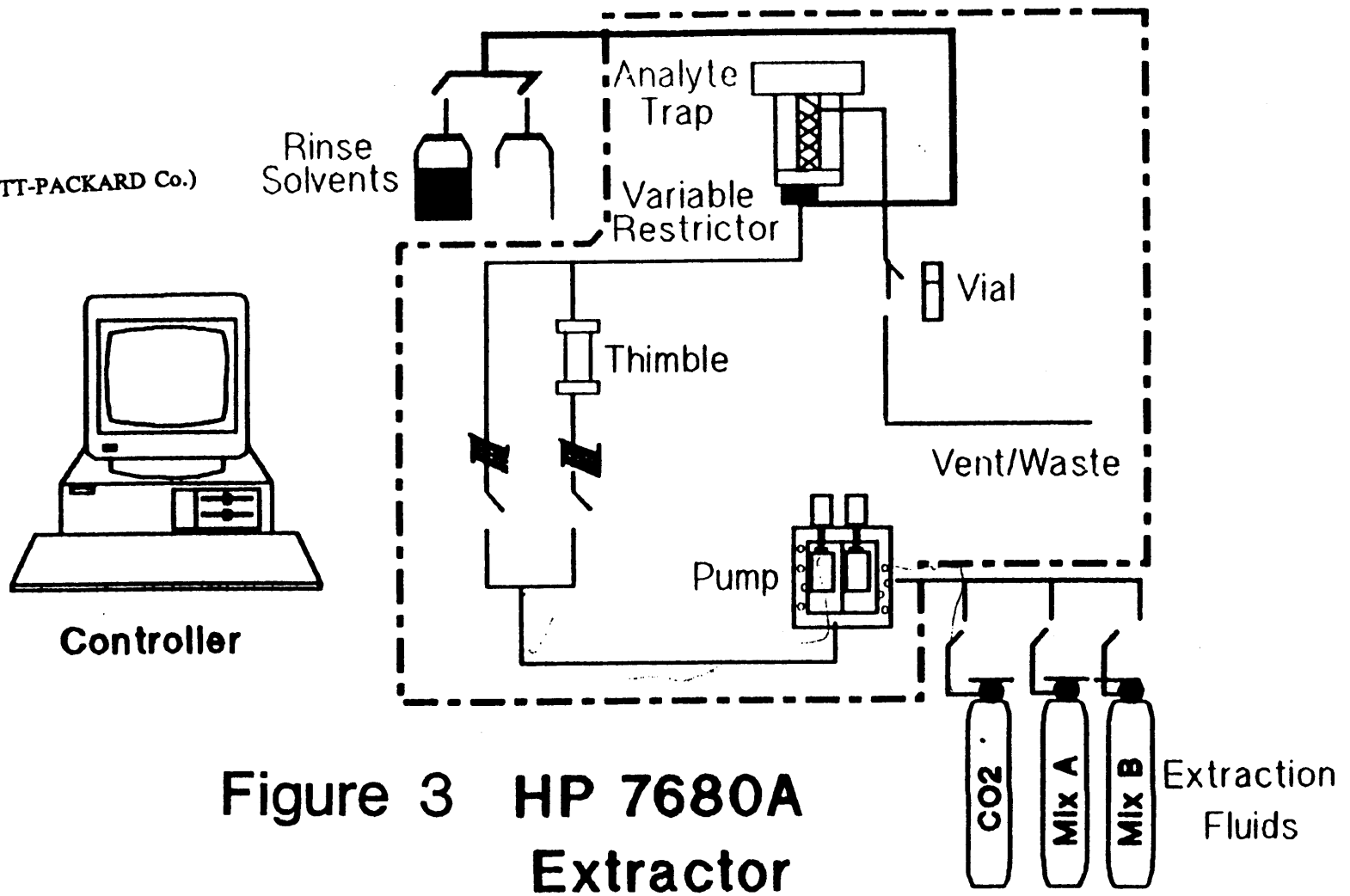

Figure 3 HP 7680A Extractor

passes through the chamber region where the extraction vessel is located. This area of the instrument is thermally controlled, with a temperature range of 40°C to 80°C. After the solvent passes through the extraction vessel it then enters the restrictor. The HP 7680A employs a computer-controlled needle valve type, variable restrictor. After it leaves the restrictor, the supercritical fluid solvent is decompressed into a solid phase trap. Traps were packed with 40 μm C-18 particles or 100 μm stainless steel beads. Both were commercially available. The restrictor and trap areas were independently thermally controlled with temperature ranges of 5°C to 80°C. Rinsing of the trap was accomplished by flushing it with a liquid solvent. The HP 7680A allowed for a choice of two rinse solvents. The analyte is washed from the trap and into a fraction collector equipped with 2 mL glass vials. SFC grade CO_2 from Scott Specialty Gases was also used with this instrument.

D. Assay of xenalipin

The drug extracts were assayed by high performance liquid chromatography (HPLC) using a Tracor (Austin, TX) Model 995 isocratic HPLC pump connected to a Valco (Austin, TX) model EQ-60 LC injector, a Spectro Monitor III (Houston, TX) ultraviolet (UV) detector and a Spectra-Physics

(Houston, TX) Model SP-4200 integrator. A 4.6 mm X 250 mm octadecyl silica (ODS) column from Keystone Scientific (Bellefonte, PA) was utilized. This assay required a HPLC mobile phase composed of 50% acetonitrile and 50% water with the pH adjusted to approximately 4, using a 50 millimolar sodium acetate buffer solution. A flow rate of 1.5 mL/minute and UV detection at 254 nm were also employed. The injection volume utilized for the liquid trap study was 20 μL while a reduction to 10 μl was necessary for the solid phase trap studies. The LC chromatogram of xenalipin shows good peak shape and a k' approximately equal to 2 (Figure 4).

Standard solutions of the drug were prepared from the pure crystalline drug which was obtained from Burroughs-Wellcome Company (Research Triangle Park, NC) and were analyzed using the previously described HPLC method. The purpose of analyzing standard solutions of the drug was to construct a calibration curve in order to quantitate the drug extracts. The resulting calibration curve had good linearity with a correlation coefficient (r) of 0.999 (Figure 5).

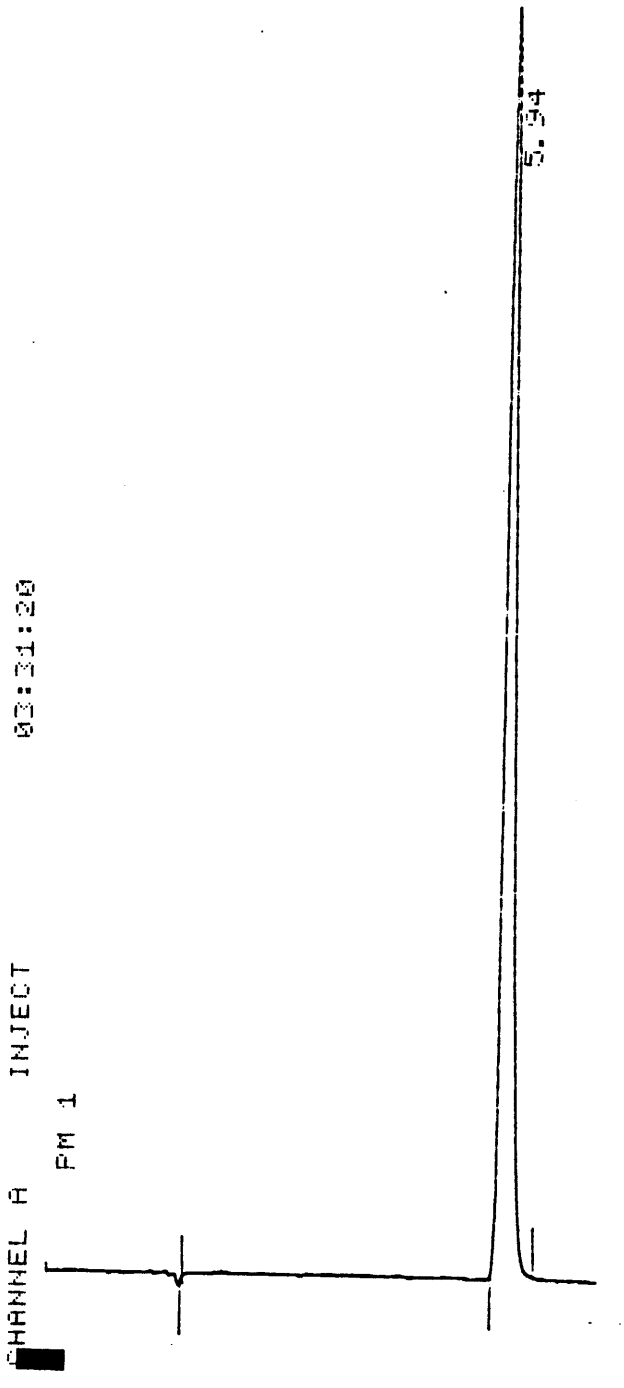

Figure 4. LC of Xenalipin

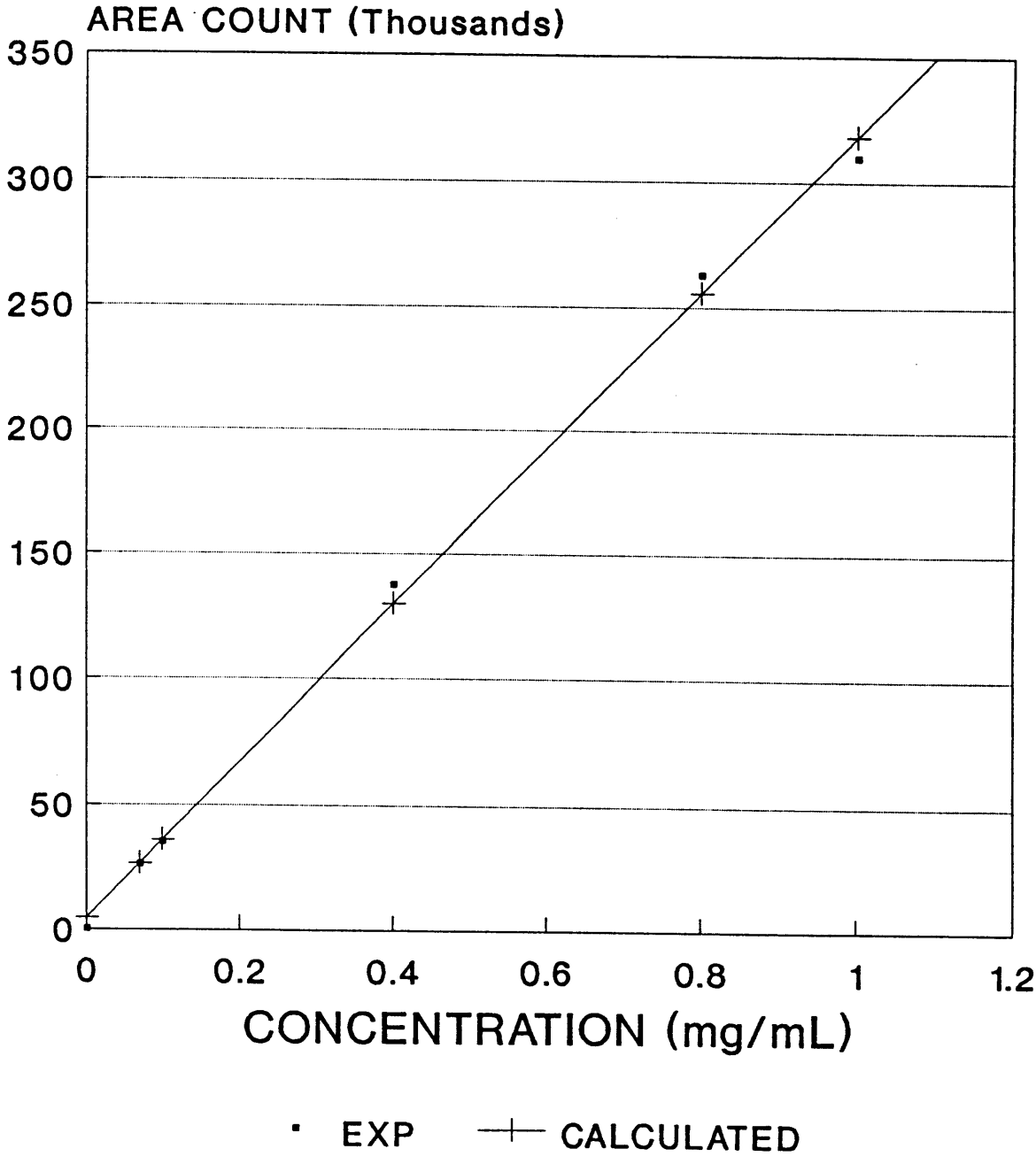

Figure 5. Calibration curve for Xenalipin.

E. Extraction of rat food: Solid Phase Trapping

Extraction studies with solid phase trapping were performed on the HP 7680A SFE. The study began by examining the trap packing materials (stainless steel beads or ODS), as well as different rinse solvents. Subsequently, the effect of flow rate, density, and number of thimble volumes used of CO_2 were also investigated. During these studies the following parameters were held constant: chamber temperature at 50°C, equilibration time was 0.30 minutes, the thimble volume was 1.5 mL and the nozzle temperature was 55°C. The trap temperature was held at 5°C. Unless otherwise stated the compressed CO_2 flow rate was 2 mL/min, density was 0.90 g/mL, and 41.1 thimble volumes of supercritical fluid solvent were used. During the washing of the trap, nozzle temperature was at 45°C and the trap temperature was increased to 40°C.

IV. RESULTS AND DISCUSSION

A. Extraction of rat feed: Liquid Solvent Trapping

Initially, the Suprex system described previously was used. Prior to attempting the extraction of the drug from the matrix, an extraction of the blank rat food matrix was performed. This was necessary in order to ensure that no compound extracted from the matrix itself would interfere with the analysis of the drug during the HPLC assay. These extractions produced a whitish-brown precipitate in the acetonitrile solvent trap, thus showing that part of the matrix was mobile and therefore extractable. Removal of this precipitate was achieved by filtering the solvent through a 2 μm filter (Fisher Scientific, Raleigh, NC) and into a 10 mL volumetric flask. The filtered acetonitrile extract was then diluted to 10 mL with the solvent strength being adjusted to 50% water/50% acetonitrile. The analysis of the extracts indicated that despite the presence of the precipitate in the trapping solvent, nothing was detected with the HPLC-UV assay. Because of this, it was determined that the rat food would not interfere with further HPLC analysis of the drug extracts.

After discovering that there were no contaminants in the extract of the blank matrix, a time study was performed utilizing the Suprex system. Rat feed containing one

percent drug by weight was used for this study. This sample was provided by Burroughs-Wellcome and was used as received. The following extraction times were investigated: 20, 30, 40, and 60 minutes. All extractions were done in triplicate. The 20 minute extraction yielded 68% recovery of the drug with a relative standard deviation (RSD) of 9.8%. The percent recoveries for the 30, 40, and 60 minute extractions were 75%, 28%, and 37%, respectively (Figure 6). The RSD's of these extraction were 9.7%, 8.2%, and 13.7%, respectively.

These results were less than adequate as the rate of recovery was too low (< 95%) and the RSD's were too high (> 5%). As expected the initial 20 minute extraction gave reasonable results with respect to extraction theory. Increasing the extraction time to 30 minutes also increased the recovery as expected. As the extraction time was increased from 30 to 40 minutes recovery rates showed a substantial decrease contrary to what would be expected. It has been shown in the literature that the recovery should increase to a point and then plateau. (28) The 60 minute extraction recovery rate increased slightly from the 40 minute extraction, but still remained dramatically lower than the 30 minute extraction recovery.

Because of the high RSD's obtained with these data, statistical verification of their significance was necessary. In order to calculate a 99% confidence limit,

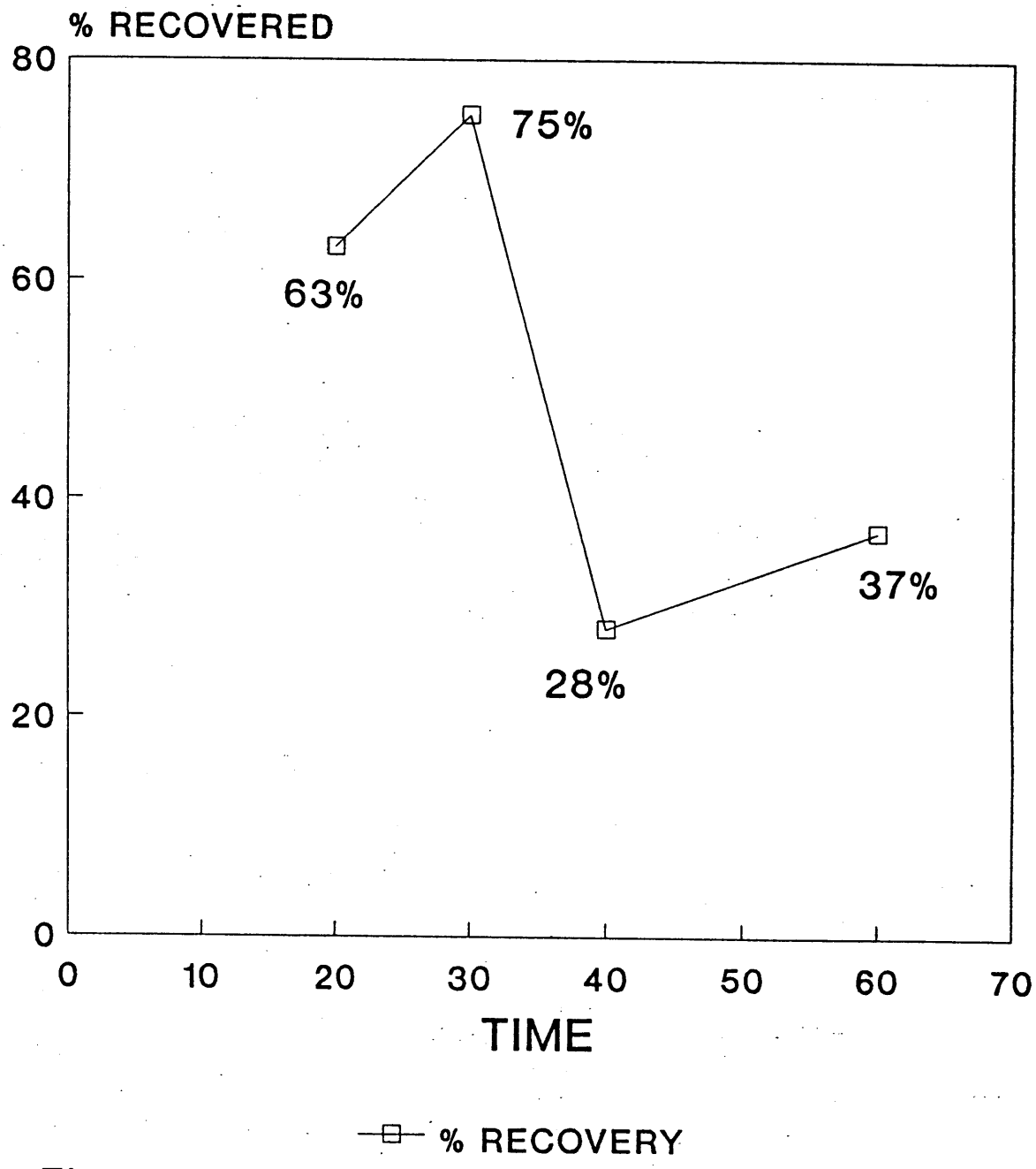

Figure 6. Xenalipin recovery by SFE
Time Vs. average percent recovery

pooled standard deviation was used which is shown below (29):

$$s^2 = \{(n_1 - 1)z^2 + (n_2 - 1)y^2)\}/(n_1 + n_2 - 2).$$

In the above equation, s is the pooled standard deviations, n_1 is the number of repetitions of the first experiments, n_2 is the number of repetitions of the second experiment, and y and z are the standard deviations of the first and second experiments. Then, using the pooled deviation, the confidence interval (t) was calculated using the following equation:

$$t = (x_1 - x_2) \; / \; s \; (1/n_1 + 1/n_2)^{1/2}$$

where x_1 and x_2 are the average percent recoveries of experiments one and two, and n_1 and n_2 are the number of repetitions of each experiments. The critical value for the confidence interval (t) when $n_1 = n_2 = 3$ is equal to 4.60. If the calculated value of (t) is greater than the critical value, the difference in the value of x_1 and x_2 is considered to be real, i.e. the two numbers are statistically different. If the calculated value of (t) is less than the critical value, the numbers x_1 and x_2 are proven to be statistically the same.

Evaluation of the above data was performed using this statistical test. Comparison of the data from the 20 and 30 minute extractions resulted in a pooled standard deviation value of 6.90 and a (t) value of 1.24 indicating that there was no difference in these values. Evaluation of the extraction data from the 30 to 40 minute runs produced a pooled standard deviation of 5.29 and a (t) value of 10.9. This (t) value indicated a significant change in the recovery of the drug. The final extraction interval, from 40 to 60 minutes, had a pooled standard deviation of 3.94 and a (t) value of 2.80 again indicating no statistical difference in the recoveries.

Based on the results of the statistical testing, further explanation of the profound drop in recovery between the 30 to 40 minute extraction interval was required. As previously stated, the recovery from the 30 minute extraction was 75% as contrasted with the 28% recovery during the 40 minute. Previous studies have documented that an aerosol effect can result in the loss of an analyte when a liquid is utilized as a trap due to the great increase in volume of CO_2 as it expands to a gas.(3) As the supercritical CO_2 decompresses, the trapping solvent which contains the analyte is turned into an extremely fine mist, i.e. an aerosol, which can be lost to the atmosphere. As an analyte becomes concentrated in the trapping solvent, the loss of even minute amounts of solvent can significantly

reduce recovery. Additionally, one could hypothesize that if all of the extractable analyte had been removed from the matrix within 30 minutes, the loss of analyte due to this aerosol effect may be greater than the amount extracted during the 10 minute period from 30 to 40 minutes. In addition, this same aerosol effect may be partially responsible for recoveries being much less than 100%. Because of these problems, solvent traps were shown to be inadequate for the extraction of xenalipin from the rat food matrix.

However,low extraction recoveries could also be due to matrix effects. One type of matrix effect lies in how the analyte interacts with the matrix. The analyte may be bound or unbound to the matrix. The unbound analyte can be described as laying freely on top of the matrix or simply in the mixture without direct interaction with it. Bound analytes have direct interaction with the matrix which can be a physical and/or chemical phenomenon. Two examples of chemical interaction would be van der Waals (9) interaction between the analyte and matrix molecules and/or other type of chemisorption such as hydrogen bonding. Physical binding could also be due to the analyte molecules being physically trapped within the matrix. This would be probable if there is a crystallization step in the preparation of the sample.

Another possible matrix effect is inhomogeneity of the analyte-containing matrix which can also cause low,

irreproducible extraction recoveries. Furthermore, if the matrix is inhomogeneous, assumptions made about the amount of analyte actually present in a given amount of matrix become invalid. This implies that an extraction recovery of less than one hundred percent could be attributable to having less analyte in a given sample than was expected.

B. Extraction of pure drug: Solid Phase Trapping

Due to the inadequacy of the liquid trap, a solid phase trapping system was employed by using the HP 7680A supercritical fluid extractor. The solid phase trap was used in order to eliminate extract losses due to the aerosol effect. In order to address the other concerns of inhomogeneity and actual amount of analyte in the sample available for extraction, a method for the extraction of pure drug (i.e. no rat feed matrix) was devised.

To accomplish the extraction of the pure drug, it was necessary to deliver a known amount of drug (approximately one milligram) into the extraction vessel in a reproducible manner. Several possible ways of achieving this were considered.

The method that proved to be most successful was pipeting a known amount of drug solution dissolved in methylene chloride into the extraction vessel. As the extraction vessel has frits at both ends where liquid could

escape it was necessary to hold the solution in place until the methylene chloride evaporated. Slices of filter paper were cut and folded in accordion fashion and inserted into the vessel to serve this purpose. A filter paper blank was extracted in order to check for interferences. None were found when the extract was analyzed by the HPLC assay. Because of the lack of response, filter paper was deemed to be an appropriate support to hold the drug during extraction. Eppendorf pipets were used to deliver the solution onto the filter paper.

Unfortunately, Eppendorf pipets are designed to deliver accurate and precise volumes of aqueous solutions only. As methylene chloride has a much lower surface tension than water, the volume of solution actually delivered was significantly less than what was expected. Because of this, calibration of the pipet was necessary. This was accomplished by pipeting what was thought to be 0.200 mL of a methylene chloride solution of xenalipin into a ten milliliter volumetric flask. The methylene chloride was then allowed to evaporate off in an oven heated to 40°C. After evaporation, the drug was then dissolved in a solution of 50% acetonitrile/50% water and assayed. Results of this analysis indicated (Figure 7) that the average (n=8) peak area obtained for the xenalipin was 29954 with an RSD of 1.22%. This average area count was used as the 100 percent recovery value on all pure drug extractions.

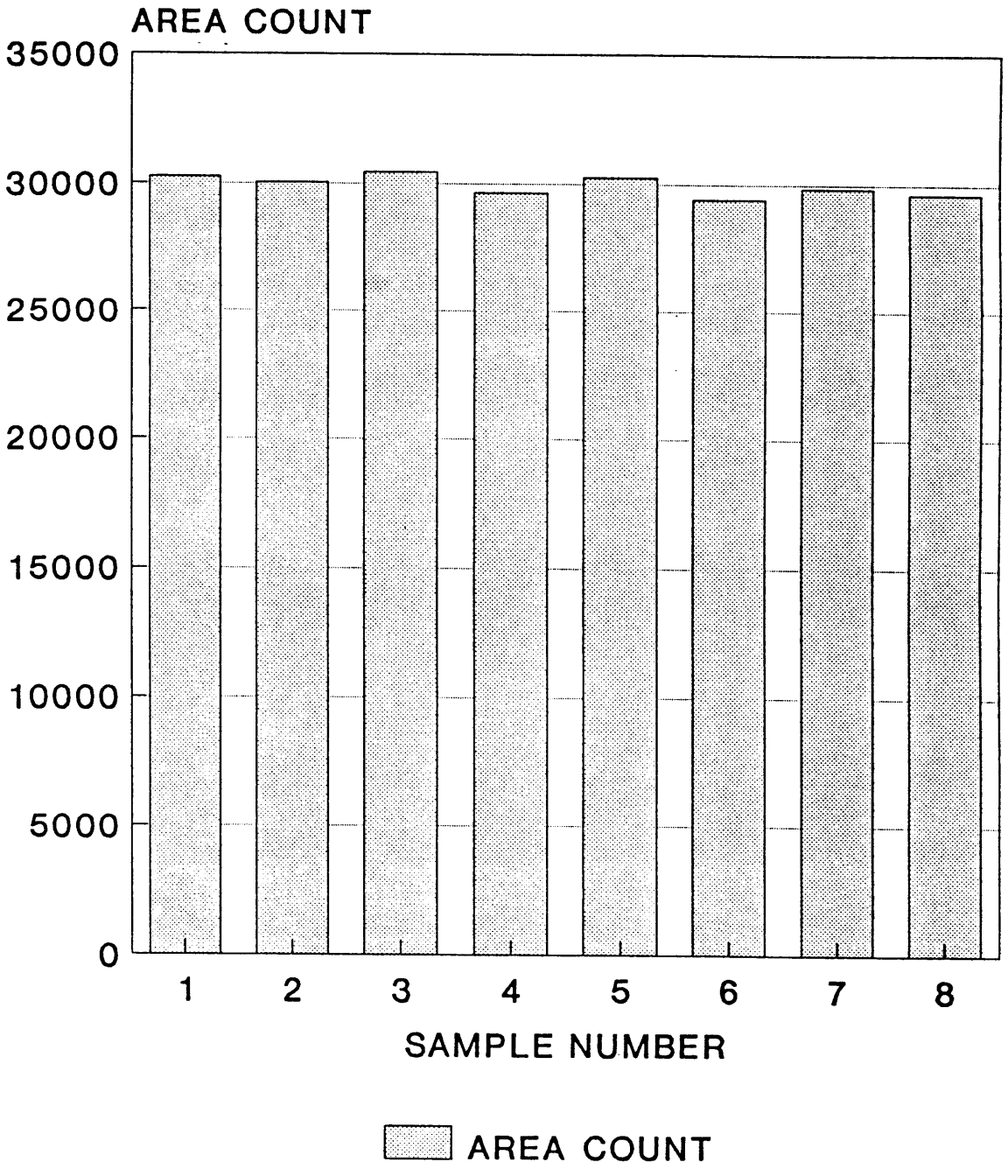

Figure 7. Calibration of pipet delivery

A decision was made to optimize the recovery of the drug from the trap first rather that the extraction parameters themselves. In order to optimize the recovery, three parameters needed to be considered: trap type, rinse type and amount of rinse used. The traps evaluated were those commercially available with the HP 7680A which were either 40 μm C-18 particles or 100 μm stainless steel beads. Two different rinses were evaluated, 50%/50% acetonitrile/water and 100% acetonitrile. These rinses were chosen not only because of their ability to remove the analyte from the trap but also because they facilitated the HPLC assay. By choosing a rinse solvent that matched the HPLC method's mobile phase the need for possible solvent exchange steps and chromatographic interferences by other solvents were eliminated. The 50%/50% acetonitrile/water rinse was the most compatible with the assay method. The 100% acetonitrile rinse was also compatible although dilution with water was required so that the solvent strength ultimately matched the mobile phase. The first parameter to be optimized was the amount of rinse solvent needed to clean the trap. To accomplish this, a 30 minute extraction of pure xenalipin using a flow rate of 2 mL/min and a CO_2 density of 0.90 g/mL was performed. The nozzle, chamber, and trap temperatures were at the settings previously stated. These parameters resulted in the extraction vessel being swept 41 times with CO_2 during the

course of the extraction. After the extraction, the trap was rinsed with five 1 mL aliquots of each rinse solvent being evaluated. Each 1 mL aliquot was then assayed using the HPLC method. Each series of extractions was done in triplicate.

Using this procedure, each trap and each rinse solvent was evaluated. It was determined that in both cases studied, nearly all the analyte was removed with the first milliliter of rinse solvent. The second milliliter of rinse solvent proved to remove the remainder of the analyte, in that no analyte was detected in the third or any additional aliquots of rinse solvent. To ensure proper removal of the analyte from the trap all subsequent extractions used 3 mL of solvent to rinse the trap.

In the evaluation of the 50% acetonitrile/50% water rinse, the stainless steel trap yielded an average drug recovery of 88.7% with a RSD of 5.01%. Using the same rinse the ODS trap provided a drug recovery of 90.0% but the relative standard deviation was slightly higher at 6.90% (Figure 8). Using identical conditions, triplicate extractions of pure xenalipin using 100% acetonitrile as the rinse were performed employing both traps. This study showed the recovery of drug using the stainless steel trap to be 95.3% recovery with a RSD of 4.60%. The ODS trap had an average drug recovery of 92.4% and a RSD of 7.13% (Figure 9) when this solvent was utilized.

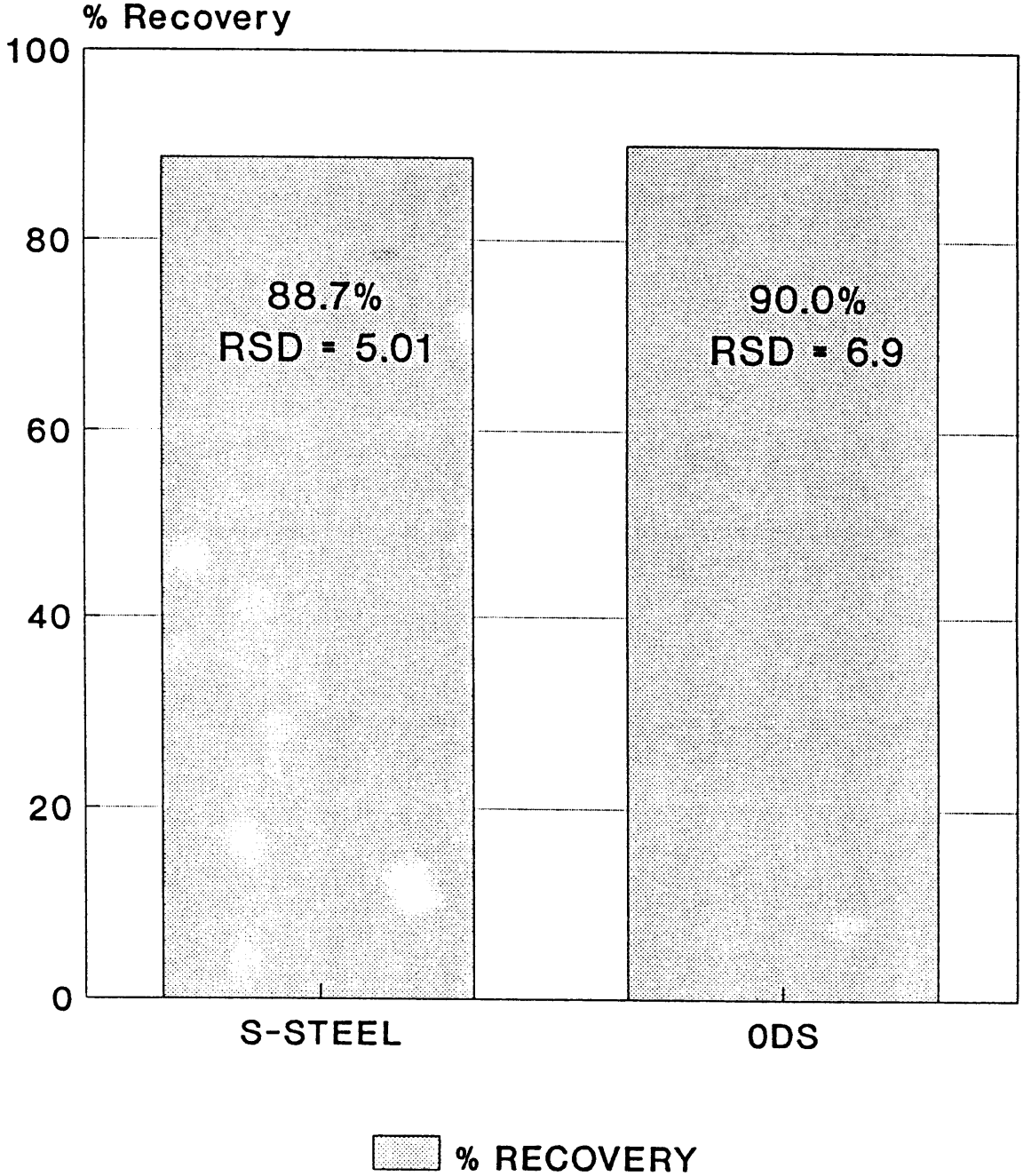

Figure 8. Recovery of pure drug using ACN/H20 wash

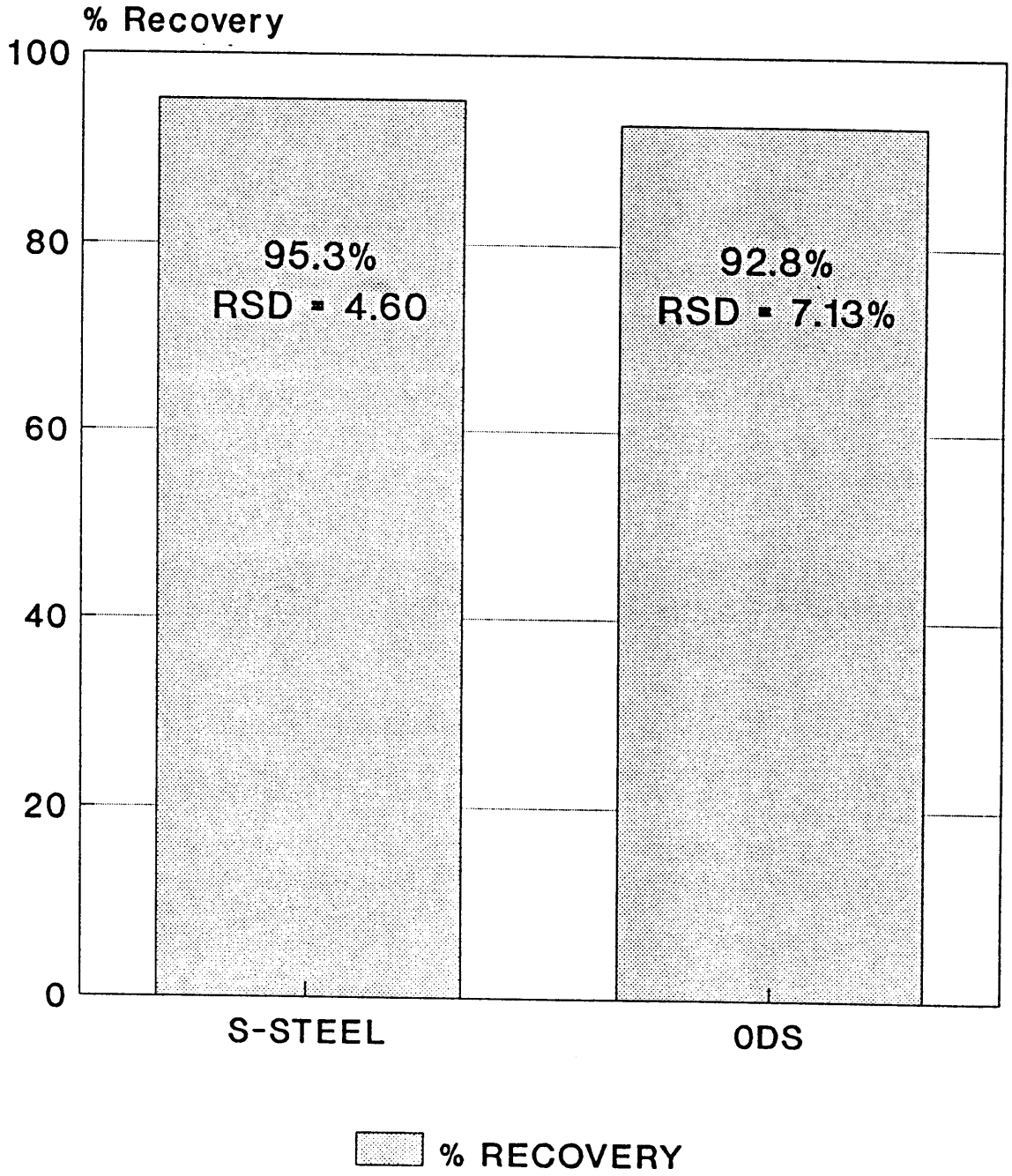

Figure 9. Recovery or pure drug using ACN wash

With triplicate analysis and with the given RSD's, there were not enough experiments performed to allow for a meaningful statisical analysis in this case. The stainless steel trap using 100% acetonitrile yielded both the highest recovery and the lowest RSD. In addition, the recoveries obtained had lower RSD's for both solvents when stainless steel was used as the trapping material. Both of these results indicated that the stainless steel trap is consistently more reproducible. On the basis of these results, stainless steel was chosen as the trap and 100% acetonitrile as the rinse for all further studies.

Extraction recovery as a function of density was also studied. For this study, CO_2 densities of 0.45, 0.65 and 0.90 g/mL were investigated. The flow rate of liquid CO_2 was held at 2 mL/min and time was varied in order to sweep the vessel 41 times with CO_2. At a density of 0.90 g/mL the average (n=3) percent recovery was 95.3% with a RSD of 4.60%. At a density of 0.65 g/mL, percent recovery dropped to 87.4% with a RSD of 4.70%. When the density was reduced to 0.40 g/mL the percent recovery decreased dramatically to 32.6%. The results of this study are shown graphically in Figure 10. This plot takes the shape of the classic curve when comparing CO_2 density versus percent recovery or solvating power (28). Because the solvating power of supercritical CO_2 increases with increasing density this is exactly the type of curve theory predicts.

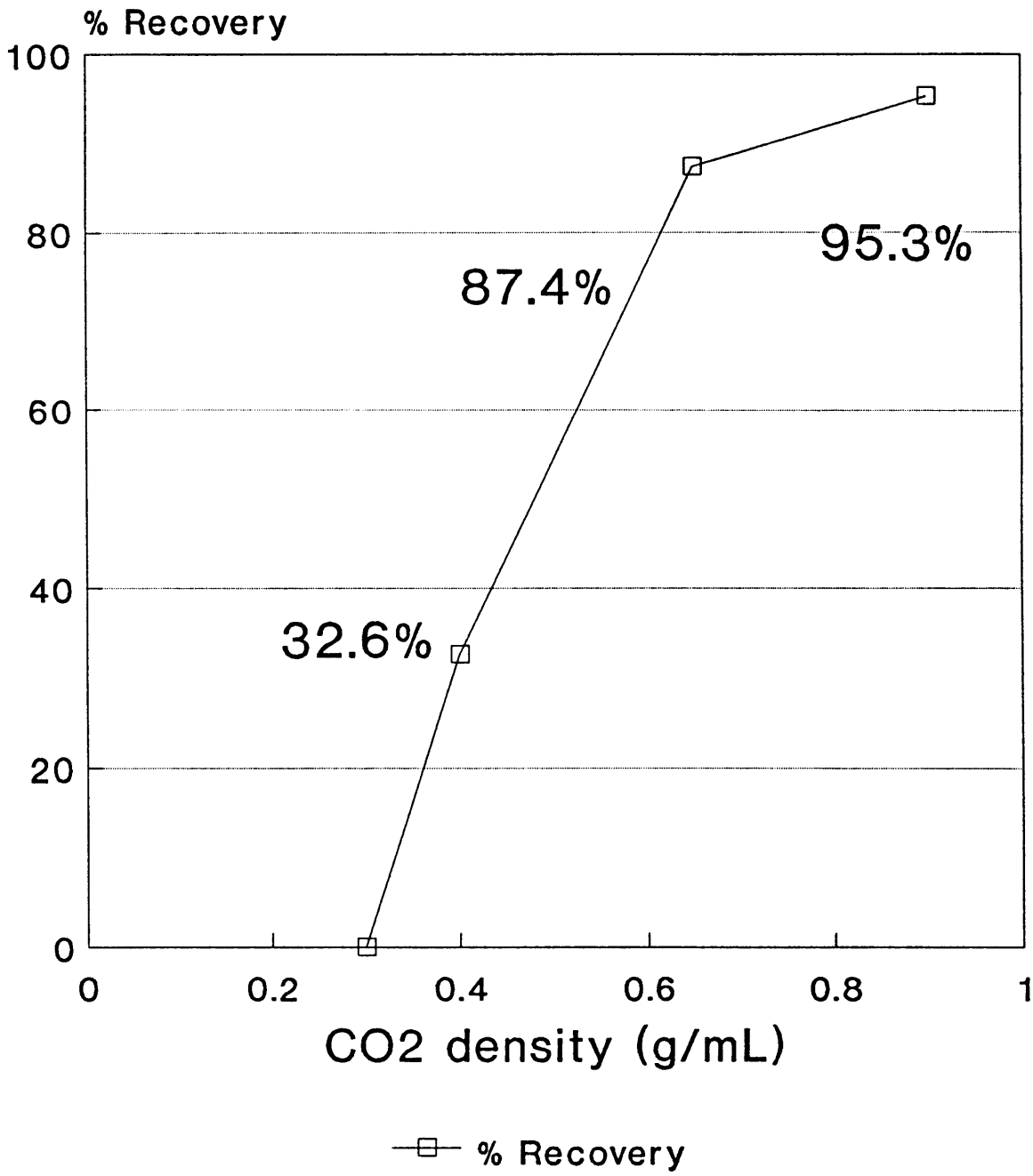

Figure 10. The effect of density on percent recovery

In the study of drug recovery versus the number of vessel volumes, two vessel volumes were selected: 20.5 and 41 vessel volumes. The flow rate of CO_2 was maintained at 2 mL/min and the other extraction parameters were as previous stated. At 20.5 vessel volumes the recovery was 62.7% and at 41.0 vessel volumes the recovery was 95.3%. The results of these extractions were as expected (Figure 11). At a fixed density, as the amount of SF solvent is decreased the amount of analyte extracted should decrease.

From the extraction of pure xenalipin, optimum extraction parameters were established. For extraction of drug from the rat food matrix, the conditions established were: a CO_2 flow rate of 2 mL/min, a density of 0.90 g/mL and 41 vessel volumes resulting in an extraction time of 30 minutes. The recovery of the analyte from the trap was achieved by using a stainless steel trap and a 100% acetonitrile solvent rinse. Other extraction parameters included chamber temperature of 50°C, thimble volume of 1.5 mL, nozzle temperature of 55°C and a trap temperature of 5°C. During the rinsing of the trap, the nozzle and trap temperatures were held at 40°C.

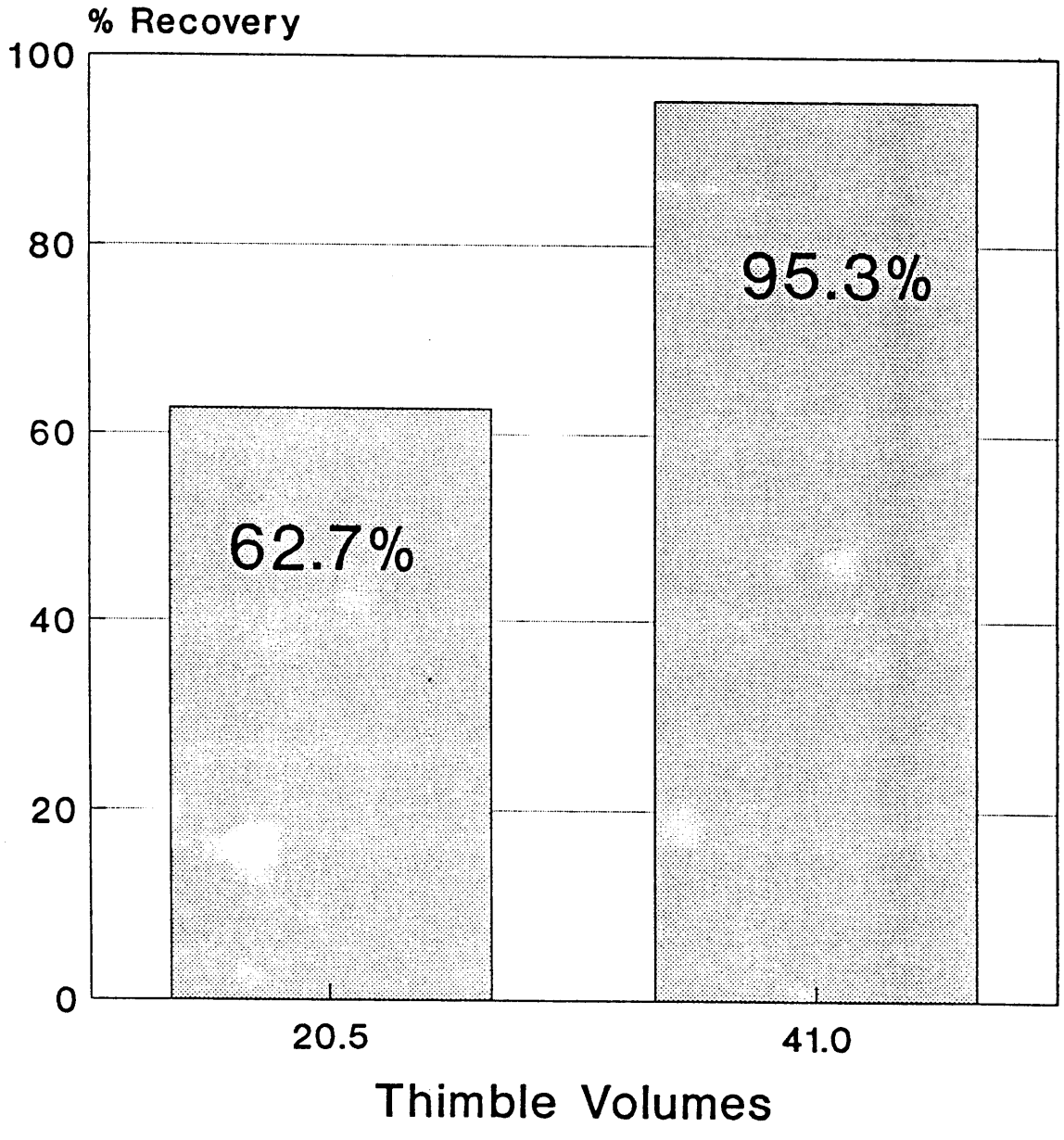

Figure 11. The effect of thimble volumes on percent reovery

C. Extraction of drug from rat feed: Solid Phase Trapping

Using these extraction parameters, the drug/rat feed matrix as received from the pharmaceutical company, which will be designated as the "crystalline matrix", was extracted. Using a 100 mg sample, the extraction was done in triplicate (Figure 12). The results of these extractions yielded recoveries of 62.4%, 59.0, and 85.1% with a RSD of 20.6% These results suggested serious problems with inhomogeneity of the analyte/matrix signaled by the lower than anticipated drug recoveries and high RSD's. Close visual inspection of the sample matrix revealed additional evidence of inhomogeneity. On an irregular basis, individual crystals of xenalipin could be sorted from the matrix. This suggests that size distribution of the crystals was not uniform, making homogeneous sampling difficult.

To address the problem of inhomogeneity, a new method of spiking the rat food was implemented. This will be designated as the "solvent matrix". A known weight of rat feed was placed in a beaker and a solution of the drug in methylene chloride was added to the rat feed to create a slurry. The slurry was mixed vigorously and the solvent was then allowed to evaporate. The mixture was again mixed vigorously. The final concentration of xenalipin in the rat feed was 1% by weight.

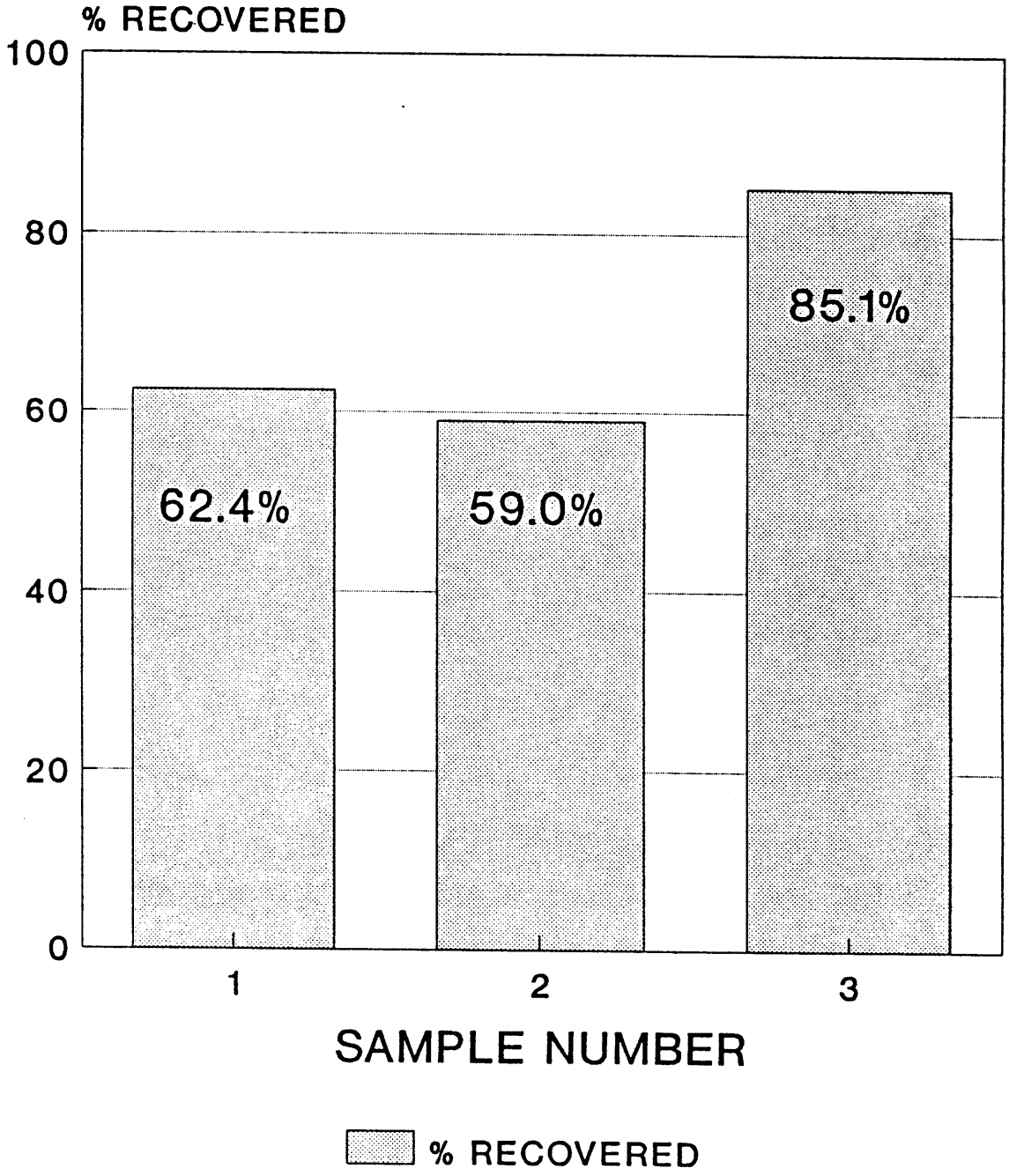

Figure 12. Extraction of drug from the crystalline matrix.

The extraction of this matrix was performed using a 100 mg sample size. The recoveries of the triplicate extractions were 85.7%, 82.2% and 84.2% with a RSD of 2.09% (Figure 13). The reduction in the RSD of a full order of magnitude indicated that the original matrix was inhomogeneous.

An additional study was performed in an attempt to create a homogenous matrix by reducing the particle size of the xenalipin. The crystalline drug was ground into a fine powder using a mortar and pestle. This will be referred to as the "ground" matrix. Although the grinding reduced the particle size of the drug, the powder formed was not free flowing and tended to cluster together. Upon adding this powder to the rat feed each cluster of xenalipin needed to be manually broken apart and mixed into the rat feed. This process continued until all visual signs of clustering were removed from the matrix. This matrix was extracted using the optimum parameters discussed previously. The recoveries of xenalipin from the triplicate extraction were 81.6%, 85.6% and 100.6%, respectively (Figure 14). The average recovery was 89.2% and a RSD of 11.2%. The recovery from the "ground matrix" was 5% higher than from the solvent matrix. Statistical evaluation showed, however, that with the given RSD's and repetition of extractions, there is no difference in these recoveries. These results are no surprise given the number of experiments performed or the

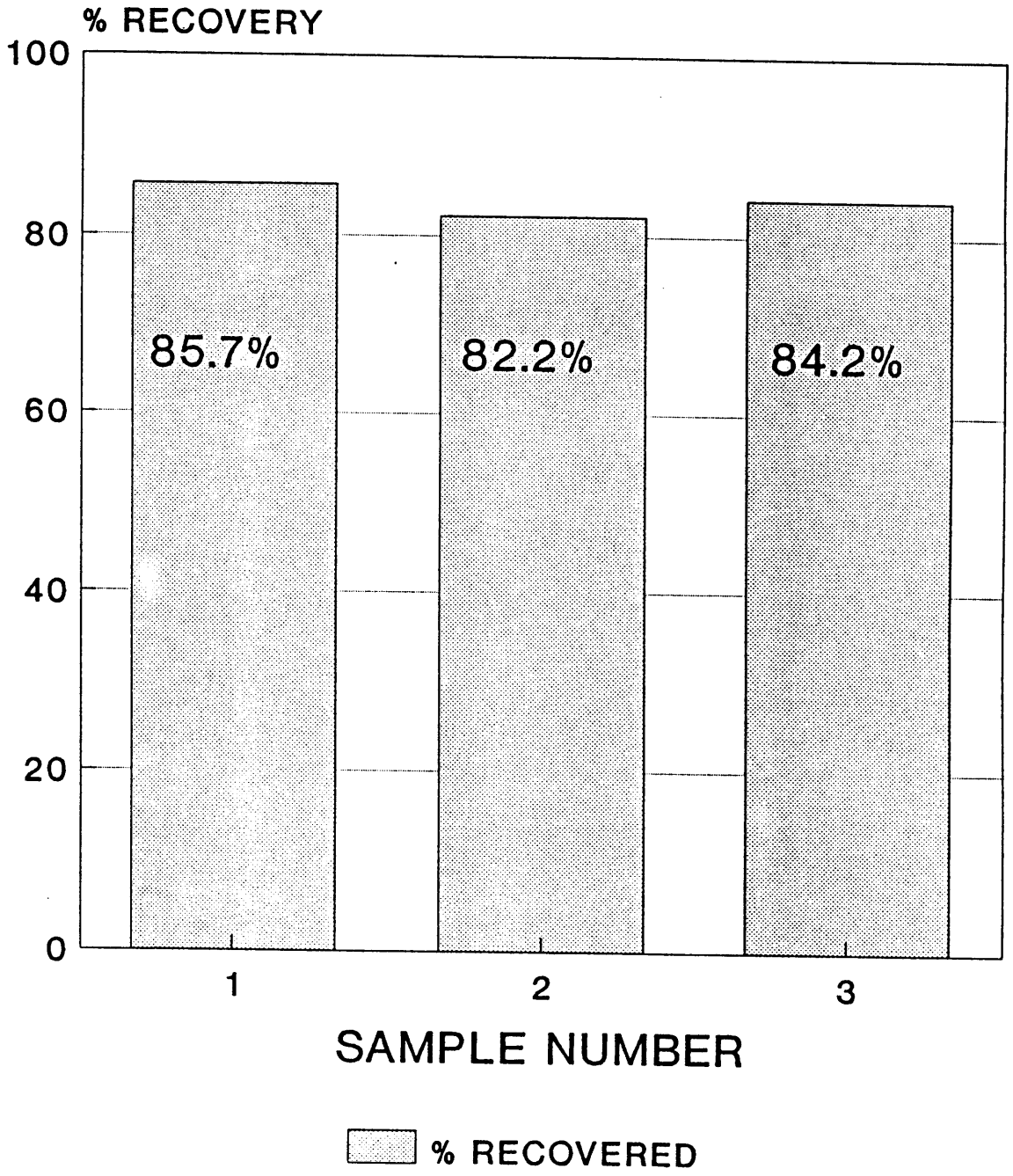

Figure 13. Recovery of drug from the solvent matrix.

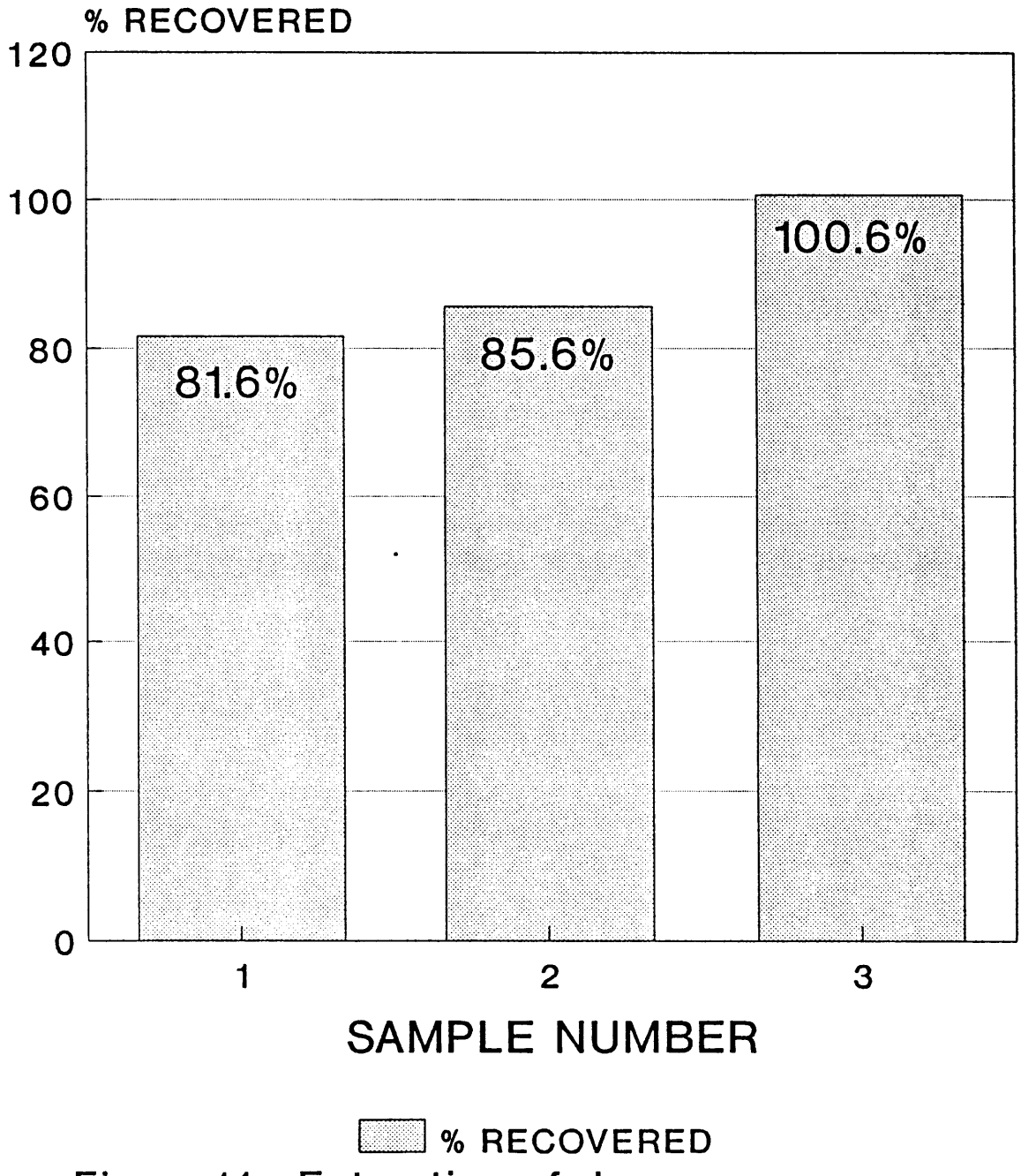

Figure 14. Extraction of drug from the ground matrix

"n" value. If the n value for this experiment was increased while the average drug recoveries remained the same, more valuable information could have been gained from this experiment. The reason for this is that as the n value increases the critical value for t is greatly reduced. In the preparation of the solvent matrix, not only the drug is dissolved in the methylene chloride, but also components of the matrix could also be dissolved. As the methylene chloride evaporated, both the drug and the soluble components of matrix will crystallize simultaneously. As this process continues, some of the crystallized drug may be bound in and on the crystals of the matrix. This might make these crystals of drug more difficult to extract which may result in lower drug recoveries. The ground matrix could also be subject to binding with the matrix, but only if surface interactions should occur.

The solvent matrix had a RSD of 2.09% while the ground matrix had a RSD of 11.2%. Although the size of the xenalipin particles were substantially reduced, the powder created had a tendency to cluster together. This clustering phenomena prevented adequate mixing of the matrix and the drug. The recrystalization of the drug into the matrix furnishes a more uniform distribution of the drug throughout the matrix.

V. Conclusion

The goal of this study was to use supercritical CO_2 for the extraction of xenalipin from a rat feed matrix with a RSD of less than 5% and an average recovery of 95% or greater. Preliminarily, this goal was achieved for the extraction of 1 mg of pure drug from filter paper. The parameters necessary for this extraction were a CO_2 density of 0.90 g/mL with a flow rate of 2 mL/min. A stainless steel trap held at 5°C was used and it was washed with 3 mL of acetonitrile. These parameters were then extended to the extraction of the drug from the rat feed matrix.

In an attempt to reach the above goals three types of matrices were extracted. The first was the crystalline matrix, which was the drug in the crystalline form mixed with the rat feed that was prepared by the pharmaceutical company. The second matrix was prepared in-house by dissolving xenalipin in methylene chloride, mixing the solution with rat feed and allowing the solvent to evaporate. This was called the solvent matrix. The final matrix, the ground matrix, was also prepared in house by manually grinding the drug, and then adding it to the rat feed.

The RSD of the crystalline matrix was 20.6% with a recovery of 68.8%. The RSD for the solvent matrix was 2.09, with an average recovery of 84.2%. The ground matrix had a

RSD of 11.2% with a recovery of 89.2%. These RSD's reflected the homogeneity of the sample. In the case of the crystalline drug, the particles were the largest of the three matrices studied, as well as, a large size distribution. These factors made the uniform distribution of xenalipin in the matrix difficult. This is exhibited in the very high RSD values.

Grinding of the crystals reduced the size of the crystals and increased their uniformity, which was generally believed to cause the high RSD's seen within the crystalline matrix. In the grinding process, the xenalipin lost its ability to flow freely and a clustering phenomena was observed. Despite these phenomena, the RSD of the ground matrix was approximately half of that of the crystalline matrix. This indicated that the ground drug is able to be more uniformly distributed throughout the matrix. The lowest RSD was produced by the solvent matrix.

Recovery of xenalipin from the matrix was based on two criteria, the first being crystal size and the second being drug matrix interaction. Although the size of the particle does not affect the ultimate solubility of the compound, it will affect the rate at which the compound dissolves. The smaller the particle the faster the dissolution process.

The recovery of the drug from the crystalline matrix was 68.8% and a RSD of 20.6%. With this high RSD any conclusions about recoveries would be little more than

speculation, but this matrix had substantially larger particle sizes for the drug than did the pure drug sample used in developing the extraction method. The percentage of recovery from the solvent matrix was 84.2% while the ground matrix had a recovery of 89.2%. Assuming that these numbers are different and that the size of particles was relatively close, the matrix effect had a larger impact in the solvent matrix than it did in the ground matrix. This result is most likely due to the co-crystallization of both compounds in the matrix and the drug itself in the solvent matrix.

This study illustrates that CO_2 has ample solvating power to meet the goals for quantitative extraction of xenalipin from the filter paper. Additionally, pure CO_2 has shown the ability to reproducibly extract the drug from the solvent matrix.

The extraction of xenalipin from the rat food matrix proved to be more difficult. Although the goal for reproducibly extracting the drug from the matrix was achieved using the solvent matrix, quantatative extraction (>95%) has yet to be attainted. Additional research using modified CO_2 should be attempted in this area.

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VITA

Dale C. Messer was born on September 21, 1954 in St. Cloud, MN. He received his B.S. in Chemistry and Education from St. Cloud State University in May, 1977. He is married to the former Ruth Steman and has two children.

Prior to entering the graduate program at Virginia Polytechnic Institute and State University in June, 1989, Dale worked with emotionally disturbed adolescents and as a high school math and science instructor in North Carolina and Virginia. He completed his M.S. degree in Analytical Chemistry under the direction of Dr. Larry T. Taylor in June of 1991, and is currently continuing his graduate studies at VPI, working toward his PhD.

Abstract

Supercritical fluid extraction (SFE) continues to be explored as a feasible alternative to traditional Soxhlet and other types of extraction. In many cases SFE is cleaner, faster, and less expensive than the traditional types of extractions. This investigation has focused on the evaluation of Supercritical CO_2 extraction as a quantitative method for recovery of a hypolipidemic drug (tradename xenalipin) from an animal feed matrix.

Initial emphasis of this study focused on the recovery of xenalipin from a the animal feed matrix employing a liquid solvent trap. By studying the effect of time of extraction versus recovery of drug, the liquid trap was shown to be inadequate.

Further studies implemented the extraction of xenalipin from a filter paper matrix and the use of solid phase traps. This method of inquiry was use to evaluate the degree of recovery as related to the parameters of CO_2 density, extraction time, and flow rates of CO_2.

After achieving quantitative extraction of xenalipin from filter these parameters were transferred to the animal feed matrix. The animal feed matrix was prepared using three different methods. Each matrix was evaluated for homogeneity of sample and ability to achieve quantitative extraction.