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Handbook of
ANALYTICAL
THERAPEUTIC
DRUG
MONITORING
and
TOXICOLOGY

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SUPERCRITICAL FLUID EXTRACTION AS A SAMPLE PREPARATION TOOL IN ANALYTICAL TOXICOLOGY

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I. INTRODUCTION

One of the key challenges faced by analytical toxicologists is the development of highly sensitive, reliable techniques that can be performed in a rapid and economical fashion, preferably through automation. The accuracy of the analytical techniques used for trace analyte detection depends on the adequacy of the sample preparation method used to isolate the target species from the sample matrix. Biological samples pose unique challenges to the analytical chemist from the standpoint of sample preparation and extraction. Compounds of interest must be released and recovered from complex matrices, such as tissue, blood, urine, and hair before the application of high-sensitivity

posed by such matrices. Indeed, quantitative SFE recoveries of toxicologically relevant analytes, such as drugs-of-abuse, therapeutic drugs, veterinary drug residues, pesticides, herbicides, and PCBs have recently been reported from tissues, hair, urine, and blood (see "Applications of SFE in Analytical Toxicology"). These developments have coincided with advances in our fundamental understanding of analyte-SF-matrix interactions and the role of modifiers and additives in SFE.

The following sections provide the reader with a basic introduction to SFE, with particular emphasis on the practical and applied aspects, as well as the advantages and limitations of the technology as they relate to biological samples. It is the authors' goal to provide sufficient background and examples to establish a sound foundation of knowledge, so that potential and current users can make informed decisions regarding SFE method development and implementation in the analytical toxicology laboratory. For a more detailed treatment of the fundamental principles and theoretical aspects of SFE, the reader is referred to several recent reviews.⁵⁻¹¹

II. SOLUBILITY MEASUREMENTS OF ANALYTES IN PURE SUPERCRITICAL FLUIDS (SFs)

Knowledge of the solubility of analytes in SFs is helpful in assessing whether a particular compound has the potential for extraction by this technology. Such measurements have been of interest to investigators for over a century. The solubility of a compound can be described as the concentration of that compound in the supercritical phase at equilibrium with the pure fluid. The first report of critical point phenomena was by Hannay and Hogarth¹² in 1879. They measured the solubilities of certain inorganic salts in supercritical ethanol. Since that time, other investigators have measured the SF solubilities of many compounds, both organic and inorganic in nature. The most extensive study to date was reported by Francis¹³ in 1954. His pioneering investigation included the solubilities of 261 organic compounds in near-critical CO₂ (6.2 MPa, 25°C). His data is still useful to investigators, because it attempts to classify the solubilities of compounds according to functional group, such as esters, alcohols, carboxylic acids, amines, heterocyclics, amides, nitriles, and phenols.

Stahl and Glatz¹⁴ investigated the solubilities of 35 steroids in SF-CO₂ at 40°C over the pressure range of 8 to 20 MPa. Using the results of this study as a basis, they constructed a table describing the relationship between substituents on the steroid structures and their solubilities in SF-CO₂. The results demonstrated that increasing the number of free hydroxyl groups on the basic backbone structure increased the minimum pressure at which the steroid could be extracted. Similarly, when a carboxyl group is added to the structure, as in the case of the bile acid desoxycholic acid, an even higher minimum pressure was required to begin extraction. This concept was elucidated earlier by Giddings et al.¹⁵ as the "threshold density," which is defined as that density (or pressure) at which detectable solubilization of the analyte commences.

Stahl and Willing¹⁶ extended their earlier investigation of threshold densities to a series of alkaloids extracted at 18 to 23°C and 8 to 10 MPa, and, in a later investigation,¹⁷ they studied this alkaloid series at higher temperatures and pressures. The results of the latter study are listed in Table 5-1. Among the opium alkaloids tested, codeine had a threshold pressure of 9 MPa, whereas morphine displayed only slight solubility at 20 MPa, because of its increased polarity. In addition to SF-CO₂, these investigators examined the solubilities of the alkaloids in SF nitrous oxide (SF-N₂O) and SF trifluoromethane (SF-CHF₃ or fluoroform). This is one of the few investigations in the literature that reports the solubilities of organic compounds in fluids other than CO₂. With the exception of codeine, all of the drugs that they examined were more soluble in SF-CHF₃ than in CO₂ or N₂O. The solubility of morphine was also determined by these workers; however, they did not include the results for this compound in the table, because it exhibited little solubility in SF-CO₂ (5 µg/g at 20 MPa, 40°C). Although this value may be low compared with other opium alkaloids, its solubility may be sufficient for its extraction as a trace level residue in a biological matrix. From these investigations of the solubilities of natural products in SFs, Stahl¹⁸ conceived of what he termed the solubility "rules of thumb" that are summarized below:

FIGURE 5-1. Solubility vs. density isotherms of cholesterol (\square), stigmasterol (\circ), and ergosterol (\diamond) in pure CO_2 at 35°C . (From Wong, J. M. and Johnston, K. P., *Biotechnol. Progr.*, 2, 29, 1986. With permission.)

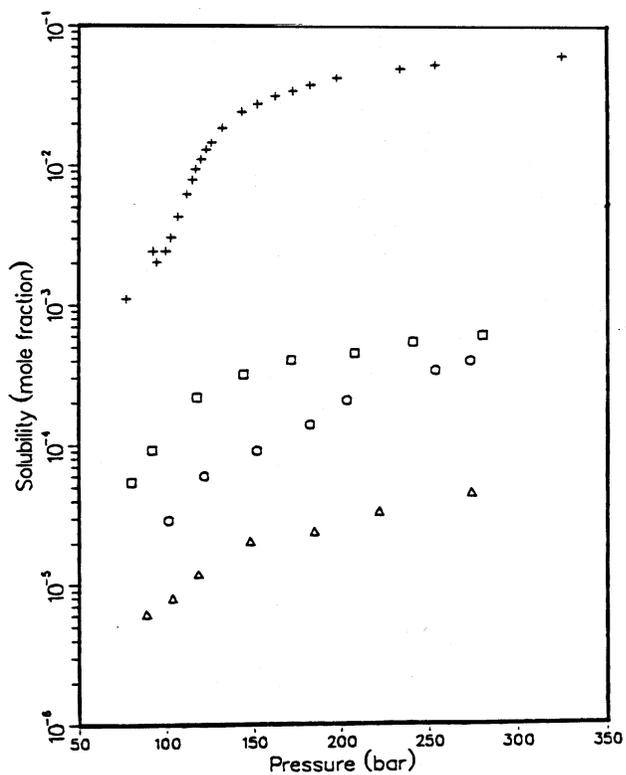
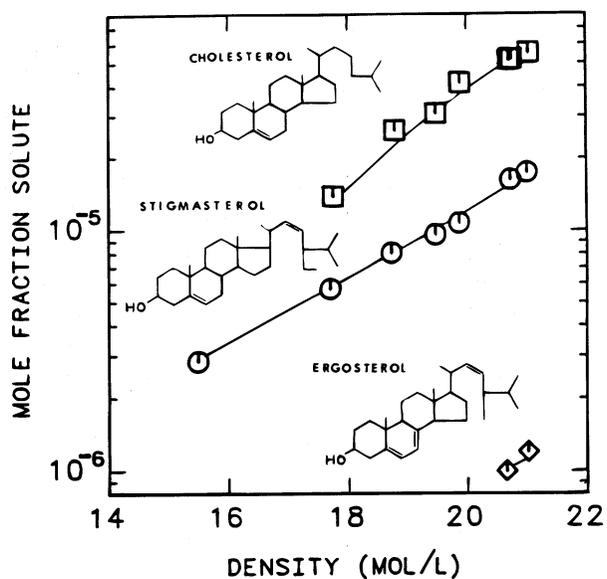


Figure 5-2. Comparison of solubility vs. pressure for penicillin V, cholesterol, monocrotaline, and naphthalene in pure CO_2 . \square , Penicillin V, 334.85 K (Ref. 21); \circ , cholesterol, 333.15 K (Ref. 19); \triangle , monocrotaline, 328.15 K (Ref. 22); and +, naphthalene, 328.15 K (Ref. 23). 1 bar = 0.1 MPa (From Ko, M., et al., *J. Supercrit. Fluids*, 4, 32, 1991. With permission.)

A more common and practical approach to increasing the solvent strength of the SF extractant has been to incorporate small amounts of polar organic solvents, called modifiers or co-solvents, in the primary fluid.^{32,33} A wide variety of solvents have been used as modifiers in SFE, including MeOH and other alcohols, water, acetonitrile, methylene chloride, toluene, organic acids (e.g., acetic acid), organic bases (e.g., aniline, diethylamine, and triethylamine), and others. The selection of modifiers has been largely empirical in the past; however, recent SFE studies^{26,33-36} have significantly advanced our fundamental understanding of the role of modifiers and the interactions that occur among analytes, SFs, modifiers, and matrix binding sites. This improved understanding has resulted in the potential for more rational method optimization choices and some predictive capability with respect to modifier selection. These studies have likewise demonstrated that, in many cases, the modifier has a more important role in facilitating analyte release from matrix binding sites (rather than improving bulk solubility of the analyte); this idea will be explored in more detail in the following section. Nonetheless, when the goal is to increase the solvent strength of the extraction fluid, a useful starting point is to employ a modifier that is capable of selective interactions with the target analyte and that is a good solvent in the liquid state for the analyte.⁷ Langenfeld et al.³³ recently reported a detailed evaluation of nine CO₂-modifier mixtures for a variety of analyte-matrix combinations and discussed their results in terms of selective interactions of the modifiers with the target analytes (e.g., hydrogen bonding, dispersion, and induced dipole interactions). In an earlier study, Page et al.³⁷ provided a detailed summary of modifiers that have been used in SFC and their potential interactions with solutes; this information can provide a useful starting point for method development in SFE.

A unique approach for enabling the extraction of very polar or even ionic compounds is *in situ* derivatization, complexation, or ion-pairing of the analyte to form a less polar and, therefore, CO₂-extractable species. This approach is potentially very useful for toxicological applications, especially for the extraction of polar drugs from biological samples. The derivatization, complexation, or ion-pairing reaction can take place directly in the SFE vessel during the extraction and is often referred to as “on-line,” “in-line,” or “simultaneous” derivatization (complexation and ion pair)/SFE. Quaternary ammonium salts, such as trimethylphenylammonium hydroxide and tetrabutylammonium hydroxide, have been used as ion-pairing reagents in the SFE of acid herbicides, microbial phospholipid fatty acids, wastewater phenolics,³⁸ and sulfonated aliphatic and aromatic surfactants in sewage sludge.³⁹ The analytes were extracted as their quaternary ammonium ion pairs, with subsequent methylation in the injection port of the GC to form esters or ethers (i.e., here the quaternary ammonium salt acts as both an ion-pairing and injection port derivatization reagent). Hills et al.⁴⁰ have added commercially available silylation reagents directly to the sample before SFE to facilitate the extraction of polar analytes from coffee beans, roasted tea, and marine sediment. In addition to forming less polar analyte derivatives, the silylation reagent is thought to aid the extraction process by displacing analytes from active matrix sites. Metals and organometallics have been extracted from solids and aqueous media using a variety of *in situ* chelation, derivatization, and ion-pairing methods.⁴¹⁻⁴⁴ An ion pair/SFE method has been reported for the recovery of the β -adrenergic agonist clenbuterol from food matrices⁴⁵ using a salt of camphorsulfonic acid as the ion-pairing reagent. Recently, Chatfield et al.⁴⁶ described the simultaneous SFE and methylation of acidic analytes, including drug compounds, from aqueous media after adsorption of the anionic forms of the analytes onto ion-exchange resins. Methyl iodide was used as the methylation reagent. Such an approach may have potential usefulness for the recovery of drugs and metabolites from urine samples.

In a remarkable recent development, Johnston and colleagues⁴⁷ have demonstrated the solubilization of a protein by SF-CO₂ containing a fluoroether surfactant which, together with water, forms micelles in the CO₂ phase. The surfactant was observed to dissolve in CO₂, forming aggregates containing water droplets with properties approaching those of bulk water. The micelles enabled the solubilization of bovine serum albumin, a protein whose solubility in neat CO₂ is essentially zero. This development has important and far-reaching implications for the isolation of highly polar compounds previously thought to be unextractable using CO₂.

sample, however, quantitative recoveries were achieved. This dramatic improvement in extractability is postulated to result from a displacement mechanism by which triethylamine (TEA) competes for binding sites in the hair matrix, effecting release of cocaine (see "Applications of SFE in Analytical Toxicology").

V. THE PRACTICE OF SFE

A. SFE INSTRUMENTATION

Conceptually, SFE instruments are simple in design. Modern commercial or laboratory-assembled instruments range from very basic systems operated manually to highly sophisticated automated devices. However, in principal, they are all designed using five basic components: a pumping system, a constant temperature oven or bath, an extraction vessel to contain the sample matrix, a restrictor to decompress the SF, and a trapping device for collecting extracted analytes.

Figure 5-3a depicts a highly simplified schematic of such an SFE instrument. The SF (e.g., CO₂, freon, or a modified SF) is directed to a pump where the fluid is compressed to the appropriate pressure, which is indicated on the gauge shown connected in-line with the pump. Then, the compressed fluid is directed to the constant temperature oven or bath, where it passes first through a heat exchanger before flowing into the extraction vessel containing the sample matrix. The compressed fluid, now in its supercritical state, diffuses through the sample matrix and exits the vessel laden with the extracted analyte. Finally, the SF passes through the restrictor, where it is decompressed at its tip. The SF, now in the gaseous state, bubbles through the solvent in the cold trap collector, deposits the extracted analytes in the collection solvent, and vents as a harmless gas.

With that overview of the SFE process, we will next examine in some detail the role of the individual system components shown in Figure 5-3 (a to c).

1. Pumps

A wide variety of pumps are used in both laboratory-assembled and commercially produced SF extractors. Two of the most commonly used types are syringe and reciprocating pumps. These pumps are familiar to any analyst who has operated a high-performance liquid chromatograph. SFE pumps should be capable of maintaining constant pressures up to levels of 68 MPa (10,000 psi). For many extraction applications, an upper pressure limit of 35 to 40 MPa is sufficient; however, many analytes of interest to toxicologists are polar in nature and may require an instrument having the 68

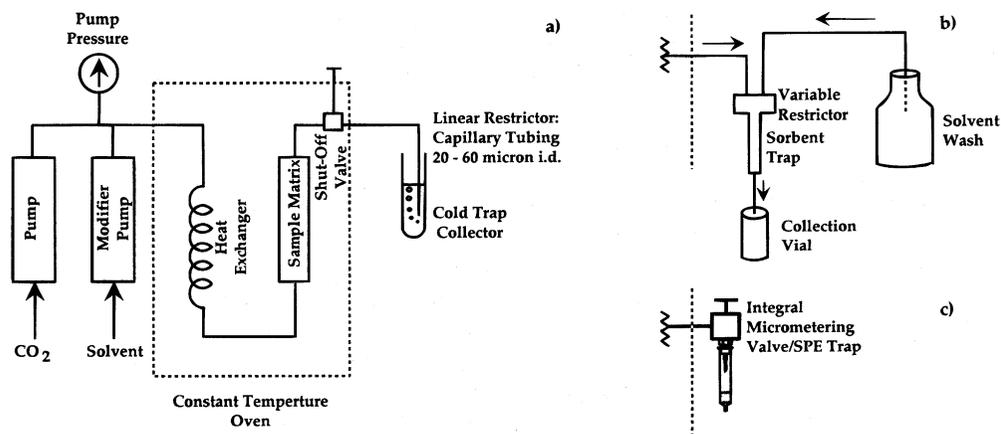


FIGURE 5-3. Schematic illustration of a SFE apparatus displaying three types of restrictor/analyte collector designs. a) Linear restrictor/cold trap collector (off-line solvent trap); b) variable restrictor/sorbent assembly; and c) integral micrometering valve/SPE assembly.

may have volumes of 20 to 30 ml, necessitating significantly larger extraction vessels (see Figure 5-4 and the discussion in “Pre-SFE Sample Preparation”). Therefore, if the SFE instrument is to be used for urine, blood, or tissue samples, an oven of sufficient size to accommodate larger extraction vessels must be considered.

3. Extraction Vessels

The first requirement of any SFE vessel is that it be able to withstand the stresses of high pressures. Today, a wide selection of vessels for working pressures (WP) up to 68 MPa (10,000 psi) are available from suppliers such as Keystone Scientific and Valco Instruments Co. These vessels, typically fabricated from 316 stainless steel tubing, have been designed with burst pressures of at least four times the allowable WP, giving the vessels a considerable safety margin. Some commercially available vessels have WPs lower than 68 MPa. However, even if the intended application for the vessel is less than that pressure, it is still advisable to use the higher rated vessels.

Vessels of the same internal volume (1 to 50 ml) are available in sizes ranging from long and narrow to short and wide. Some controversy exists in the literature regarding the optimum vessel geometry for analyte extraction.^{55,56} Reported examples describe the influence of vessel geometry on the SFE recoveries of analytes such as PAHs from environmental matrices; however, the findings may not be entirely relevant to biological matrices. Vessel geometry requirements may also be influenced by the method used for analyte trapping. For example, methods have been developed whereby the extracted solutes are trapped on sorbents placed in the same extraction vessel as the sample matrix (see “Applications of SFE in Analytical Toxicology”). For these applications, long, narrow vessels with 14-mm internal diameters provide the best results.

4. Variable and Linear Restrictors

Restrictor design is a critical element in the efficient operation of any SFE system. These devices control the system pressure and fluid flow. In their most elementary form, restrictors may

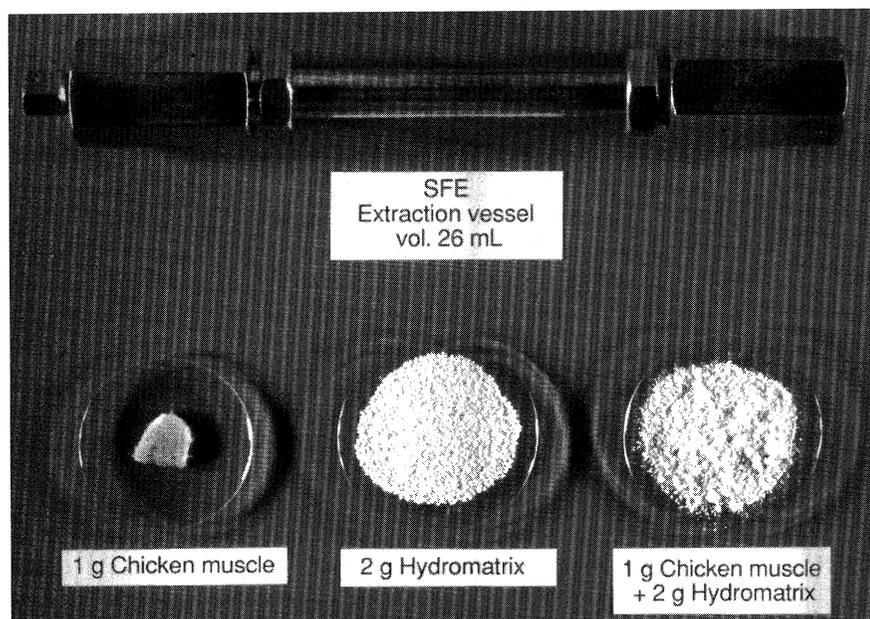


FIGURE 5-4. Pre-SFE processing of a tissue sample. (Left) Homogenized chicken muscle sample and (right) blended tissue/sorbent mixture.

Alternative sorbent-trapping techniques offer potentially attractive advantages over solvent-trapping methods. Commercial instruments are available that use this collection technique. A generic representation of such an SFE instrument is shown in Figure 5-3b. Up to the point where the SF enters the restrictor in this schematic, the device is similar to that depicted in Figure 5-3a, except that in Figure 5-3b the SF enters an electronically controlled variable restrictor that is connected to a column filled with a sorbent material. The sorbent in the column is chosen by the analyst for its affinity for the extracted analytes and may be any of the materials typically used in high-performance liquid chromatography (HPLC) columns. This restrictor/collector design does not provide for sorbent removal from the column for off-line processing. Instead, the restrictor/collector assembly is equipped with a wash station that is used to elute the extracted analytes from the sorbent bed into a collection vial. Variations on this design feature multiple solvent bottles that allow the sorbent bed to be eluted with solvents of increasing polarity, thereby effecting a partial fractionation of the trapped analytes. Solid sorbent trapping using a restrictor/collector assembly offers several advantages over solvent trapping, including reduced potential for loss of volatile analytes, minimal release of solvent vapors into the atmosphere, and greater selectivity in trapping desired analytes.

Despite these advantages, sorbent trapping by the technique shown in Figure 5-3b may pose problems for some applications involving biological matrices:

1. The sorbent column in these instruments may hold only a small amount of sorbent material (<1 g), whereas larger amounts of sorbent may be necessary to trap trace residue(s) in tissue samples containing large amounts of fat.
2. Because the sorbent bed cannot be removed from the instrument for off-line processing, the extracted residue eluted to a liquid sample vial may require transfer to an SPE column for further clean-up.

An SFE instrument designed in Agricultural Research Service/United States Department of Agriculture (ARS/USDA) laboratories⁵⁸ was fabricated specifically to address limitations encountered with solvent and sorbent-trapping techniques then available in commercial instrumentation. The focus of this design (Figure 5-3c) was the quantitative extraction of veterinary pharmaceuticals from biological matrices at parts per billion and parts per million levels. The SFE unit extracts two samples simultaneously; however, the representation shown in Figure 5-3c depicts only one extraction channel. This SFE has a manual rather than electronic variable restrictor that can be connected to a standard, off-the-shelf SPE column through an interface adapter. This integral restrictor-collector arrangement allows the decompressed gas, laden with extracted analytes, to be focused directly above the sorbent bed of the SPE column, thus reducing the potential for analyte losses as may be caused by additional transfer steps. The SPE column may be filled with varying amounts of any sorbent material selected for maximum analyte retention. SPE columns may be quickly removed from the interface adapter after SFE for further off-line analysis by subsequent manual or automated processing. The section on "Applications of SFE in Analytical Toxicology" describes some extractions from urine and tissues conducted using this instrument. This instrument design was transferred to a manufacturer, and a relatively low-cost commercial version is now available on the market.

B. INTRODUCTION OF MODIFIERS IN SFE

The introduction of modifiers in SFE, either for the purpose of increasing analyte solubility in the SF or for facilitating analyte release from matrix binding sites, is generally accomplished in three different ways.^{5,7,8,59} One approach involves the use of premixed cylinders containing known concentrations of organic modifier(s). These cylinders are commercially available and can be connected directly to the SFE supply pump. The modified fluid is thus delivered directly and continuously to the extraction vessel. This approach is not particularly convenient for method development, nor is it economical because a large number of tanks with a range of modifier types and concentra-

For most analytical-scale SF applications, the static extraction mode is seldom used alone. However, in unique cases where it is used without an additional dynamic step, the shut-off valve of Figure 5-3 would be opened and the system would quickly decompress to atmospheric pressure with no additional SF entering the system from the pump. During decompression, analytes soluble in the SF at higher pressures could potentially precipitate and redeposit in the extraction vessel and throughout the fluid lines before reaching the collection trap, resulting in overall poor product recoveries. For that reason, a static extraction period is normally followed by an extended dynamic flow period, during which the pump supplies a continuous stream of fresh SF to the system fluidics. The fresh SF diffuses through the sample matrix in the vessel and solubilizes additional analyte in the process. Samples may be extracted dynamically without resorting to a static hold period; however, many investigators have reported more favorable results using a coupled static/dynamic extraction rather than a simple dynamic step. During method trials, it is advisable to try various combinations of static and dynamic modes with and without the presence of modifiers and/or complexing and derivatizing reagents to achieve satisfactory analyte recoveries.

D. PRE-SFE SAMPLE PREPARATION

The degree and complexity of analyte/sample matrix pretreatment needed before SFE is sample dependent. Samples such as soil or river sediments containing environmental pollutants generally require only a grinding operation to achieve uniform particle size before packing the material in the extraction vessel. Similar techniques are also used with some polymers that may be ground with dry ice or liquid nitrogen to achieve a uniform sample bed.

On the other hand, with the exception of hair, biological samples (such as blood, tissue, and urine) require careful pre-SFE preparation and handling. Unlike soil samples, biological materials such as these cannot be simply packed into the extraction vessel without pretreatment. Although SF-CO₂ possesses high diffusivity and low viscosity, it does not uniformly penetrate dense sample matrices such as bulk tissue, and direct extraction of such samples with no pretreatment will result in poor analyte recoveries. Instead, tissue samples should first be quickly homogenized in a blender with precautions taken to prevent warming. (*Note:* all tissue processing operations should be conducted with chilled samples to prevent pre-SFE analyte losses.) Blood and urine, of course, do not require homogenization and can be mixed directly with an adsorbent material.

The blended tissue sample must then be desiccated and dispersed before it is ready to be packed into the extraction vessel. This step is accomplished by mixing the sample with an adsorbent material that serves two purposes. First, the adsorbent acts as a drying agent, forming a free-flowing mixture that can be uniformly packed in the vessel, and, second, the adsorbent disperses the biological sample over a wide surface area. This gives the SF greater access to the target analyte than would occur in a nonadsorbent-treated sample.

Several types of adsorbents have been used in SFE applications, including sodium sulfate, magnesium sulfate, normal- and reversed-phase SPE sorbents, alumina, and Celite 566 (Hydromatrix from Varian Sample Preparation Products). The choice of adsorbent used for individual sample types is dictated by the nature of the sample, its water and fat content, and other variables. (A few examples of the use of adsorbents with biological matrices will be given in this section. Other illustrations of their uses with blood, urine, and tissues are described in the section on "Applications of SFE in Analytical Toxicology.")

Biological matrices, such as blood, urine, and tissues, generally must be desiccated and dispersed before SFE. Typically, these samples are mixed with an adsorbent such as sodium sulfate, magnesium sulfate, or Hydromatrix. There are advantages and limitations associated with the use of these adsorbents. For instance, sodium sulfate is a good desiccant; however, it may dissolve in the presence of large amounts of water. Magnesium sulfate has also been used as an SFE desiccant; however, it may bind too tightly to the sample matrix and thus restrict fluid flow. Hydromatrix, first reported as an SFE dispersing agent by Hopper and King⁶² in 1991, does not have these limitations.

and is the more common approach reported in the SFE literature. Any appropriate measurement technology may be used, including GC, GC-MS, HPLC, supercritical fluid chromatography (SFC), and spectroscopic methods. Off-line SFE/immunoassay methods have recently been reported for the rapid screening of analytes in a variety of matrices, including cocaine in hair,⁶⁶ phenobarbital in liver tissue,⁶⁷ and pesticide residues in meat products.⁶⁸ Capillary electrophoresis, with laser-induced fluorescence detection, has been used for the analysis of PAHs extracted from contaminated soils using SFE.⁶⁹ In each case, the type and volume of the SFE collection solvent (for SFE methods involving liquid solvent trapping of extracted analytes) or elution solvent (for SFE methods involving solid-phase trapping of extracted analytes) can be tailored to meet the sensitivity and selectivity demands of the specific analytical technique used. Regardless of the analytical method used, potential interferences derived from modifiers, collection or elution solvents, and co-extractables must be carefully monitored.

On-line analysis of SF extracts involves the direct coupling of the extraction step with the measurement step (i.e., analytes are directly transferred from the SFE system to a chromatographic or other analytical system). The direct interfacing of SFE to chromatographic systems is made possible because CO₂ is a gas at ambient conditions and is therefore readily removed. The obvious advantages of on-line analysis are the elimination of sample handling steps between extraction and analysis, and the sensitivity enhancement that arises because the entire extract is transferred to the analytical system. On-line SFE approaches are potentially very useful for trace analysis when only limited amounts of sample are available. SFE has been directly coupled with capillary GC, GC-MS, capillary and packed SFC, and, less commonly, HPLC.^{4,8,11,28,70} On-line SFE can be more difficult to implement because of hardware considerations (i.e., interface design). In addition, the use of modifiers in on-line SFE/GC and SFE/GC-MS methods is not straightforward because of the production of large solvent peaks in the chromatographic system that can degrade chromatography and cause overpressure conditions in the MS. Finally, the need for additional post-SFE clean-up steps may preclude direct interfacing approaches for some biological sample applications. Nonetheless, for simple substrates, where only SF-CO₂ is used, the potential exists for automated on-line analysis.

VI. APPLICATIONS OF SFE IN ANALYTICAL TOXICOLOGY

A. SFE OF TISSUE SAMPLES

1. SFE for the Recovery of Pesticides from Tissues

The recovery of chemical residues from tissue samples by SFE presents unique challenges to the analyst, compared with the SFE of analytes from other matrices. The challenges arise because of the varied nature of the constituents found in tissues. Depending on the tissue site (i.e., liver, heart, muscle, adipose, etc.), the sample will contain varying amounts of fat (lipid) and water. SF-CO₂ readily solubilizes the constituents of a lipid mixture,⁷¹ including fatty acids, diglycerides, triglycerides, and minor lipid components (such as pigments and sterols), whereas water is soluble to a lesser extent in this fluid. The chemical residue of interest will, therefore, constitute only a very small portion of the total SFE extract. The target analyte(s) then must be separated from the co-extracted lipids before chromatographic analysis. (For the purposes of this discussion, the collection of analytes after fluid decompression will be referred to as off-line collection or trapping.) Depending on the properties of the target analyte(s), this separation process may require multiple steps to isolate the analyte(s) from the undesired lipid components. To circumvent this problem, researchers have devised various techniques to simplify the post-SFE clean-up process through alternative analyte collection schemes. If a multiple-step clean-up process is required after SFE, the benefits of this technology may be negated, and non-SFE isolation techniques should be considered. In the applications that follow, we will discuss some of the approaches investigators have taken to facilitate the separation of target analytes from unwanted co-extracted artifacts.

tissue. Their SFE method was developed for use in epidemiological studies to assess the potential role of these pollutants in breast cancer. In the reported method, the fat in adipose tissues was trapped on a neutral alumina sorbent bed layered above the sample in the extraction vessel. The extraction process occurred in two steps and resulted in the quantitative recovery of eight pesticides and PCBs. These authors reported that a post-SFE clean-up of the extract by adsorption column chromatography was necessary to avoid interferences in the GC-ECD analysis. Their results indicated that levels of these pesticides and PCBs in breast cancer cases were higher than those in the control subjects, suggesting the need for further studies.

Ashraf-Khorassani et al.⁸² are pursuing a novel approach for the isolation of pesticides and PCB extracts free of artifactual fat. Instead of using SF-CO₂, they have conducted experiments with MeOH-modified CHF₃. SF-CHF₃ exhibits much less affinity for fat than does CO₂ under similar conditions. (King and Taylor⁸³ reported that 54.4% of the fat from avian adipose tissue [0.2 g on glass beads] is extracted by CO₂, whereas only 0.45% [w/w] of the fat from the same tissue is extracted using CHF₃). Ashraf-Khorassani and co-workers used 10% MeOH in CHF₃ to extract rendered fat containing OCPs. Unlike the SF-CO₂ methods previously described, no adsorbent was packed in the extraction vessel for fat retention. Using the modified CHF₃, the extracted OCPs, free of fat contaminants, could be analyzed directly by GC-ECD without post-SFE extract clean-up. The authors have only extracted a few samples using MeOH/CHF₃; however, their preliminary findings indicate that CHF₃ may simplify post-SFE analysis of pesticide and PCB extracts.

2. SFE for the Recovery of Drugs and Other Chemical Residues from Tissues

Only a few research groups have reported data on the SFE recovery of pharmaceutical residues from tissues. The majority of these studies have been conducted using animal tissues, with only one reported use of human tissues. Several of these investigations have used conventional off-line collection strategies for extracted analytes. As in the case of pesticide recoveries from tissues using off-line collection, post-SFE purification steps are typically used to prepare the pharmaceutical analytes for chromatographic analysis. However, unlike most SFE applications for pesticides in tissues, additional post-SFE purification of pharmaceutical extracts may not be necessary in all cases, because pharmaceutical compounds are generally polar and lend themselves to alternative collection techniques that may obviate the need for post-SFE clean-up. Examples of the use of alternative collection techniques will be discussed later in this section.

The earliest report describing the isolation of pharmaceutical compounds from animal tissue samples was in 1989 by Ramsey et al.⁸⁴ These workers attempted to isolate four veterinary drugs (dienestrol, diethylstilbesterol, hexestrol, and trimethoprim) from freeze-dried pig kidney with pure CO₂ using an on-line SFE/SFC/MS-MS system. The drugs were trapped after SFE on an amino-SPE sorbent column, whereas co-extracted fat was swept to waste. The drugs were eluted from the SPE column directly into the SFC-MS by switching the SF from CO₂ to MeOH-modified CO₂. The drugs were then detected with high specificity by tandem MS. Recoveries were not stated; however, the authors concluded that the detection limits of the method did not meet the stringent controls for drug residues in meat set by regulatory agencies.

Other investigators have also experienced difficulties in extracting pharmaceuticals from freeze-dried or desiccated tissue samples. For example, Jimenez-Carmona et al.⁴⁵ attempted to extract clenbuterol with unmodified SF-CO₂ from lyophilized liver (20 µg/g) premixed with an ion-pairing reagent, the ammonium salt of camphorsulfonic acid. Clenbuterol is a β-adrenergic agonist that is used illegally in Europe and the U.S. as a growth promoter in meat-producing animals, and is monitored by both U.S. and European regulatory agencies. Using an SFE instrument containing a variable restrictor similar in design to that shown in Figure 5-3b, Jimenez-Carmona et al. obtained poor clenbuterol recoveries (12%) from the lyophilized liver samples. The authors provided no explanation for the low recoveries. Subsequent work by Parks et al.⁸⁵ suggests that the difficulty may have been caused in part by the lack of water in the liver samples used by the previous investigators. Parks et al. used SF-CO₂ for the isolation of the veterinary drug zoalene (3,5-dinitro-*o*-toluamide)

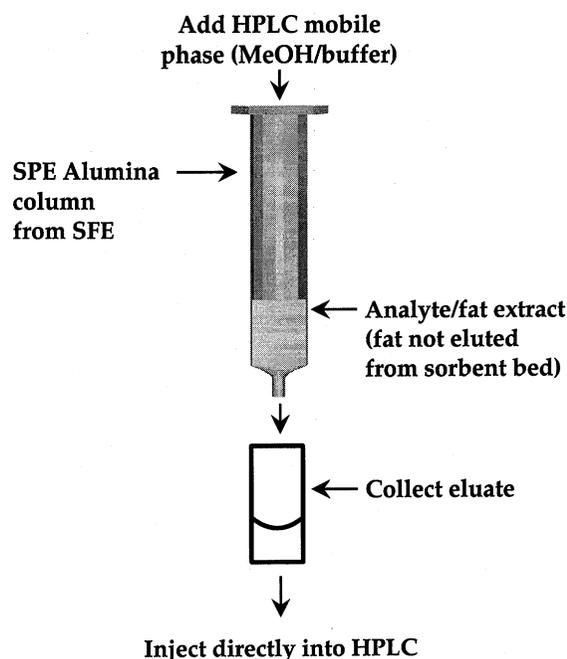


FIGURE 5-5. Post-SFE processing of extracted nitrobenzamide analytes on an SPE column removed from the SFE instrument shown in Figure 5-3c.

purification and interfere with chromatographic detection of the target analytes. It may be possible to minimize this problem by changes in the composition of the eluting solvents; however, if interferences persist, alternative techniques may be required.

Parks and Maxwell⁶³ recognized this problem during a study of the SFE of three sulfonamide drugs from chicken tissues. In this investigation, recoveries from three tissues (liver, breast, and thigh) were compared. Tissues were fortified with the sulfonamides (sulfamethazine, sulfadimethoxine, and sulfaquinoxaline), mixed with Hydromatrix (Celite 566), and extracted with pure SF-CO₂ at 40°C and 68 MPa. The drugs and co-extracted endogenous material were first collected on off-line alumina SPE columns. Recoveries of the three drugs from the various tissue sites are listed in Table 5-3. The recoveries of all three drugs were low and inconsistent with this off-line collection technique. In addition, sulfonamide peak areas were difficult to integrate because of high background ultraviolet (UV) interferences that may be observed in the HPLC chromatogram shown in Figure 5-6a. Cross et al.⁹⁰ also isolated sulfonamides from chicken livers using an off-line solvent-trapping system of the type shown in Figure 5-3a. Recoveries were higher than those reported by Parks and Maxwell;⁶³ however, to achieve these recoveries, the researchers increased the polarity of the SF by adding 25% MeOH to the CO₂. Incorporation of the MeOH modifier resulted in the need for additional post-SFE clean-up operations to prepare the extract for HPLC analysis. High concentrations of polar modifiers in the SF also limit the choice of off-line collection to solvent trapping, because extracted solutes collected on sorbent beds would be eluted during the extraction process due to the presence of the modifier (Figure 5-6b).

To minimize this problem of co-extracted interferences, Parks and Maxwell⁶³ devised an alternative trapping technique illustrated in Figure 5-7, which depicts a detailed section of the complete SFE apparatus shown in Figure 5-3c. The extraction vessel in Figure 5-7 is connected through fluid transfer lines to a shut-off valve that is in turn connected to the variable restrictor interfaced to an SPE column. Two sorbent traps are used in sequence to collect extracted material: one consists of a packed sorbent bed contained in the extraction vessel (in-line), and the second is a conventional SPE column attached to the micrometering valve (off-line). In Figure 5-7, SF-CO₂ enters the bottom of the extraction vessel, diffuses through the sample matrix, and solubilizes target analyte(s) and endogenous co-extractables, then contacts the in-line sorbent bed, where the polar target analytes

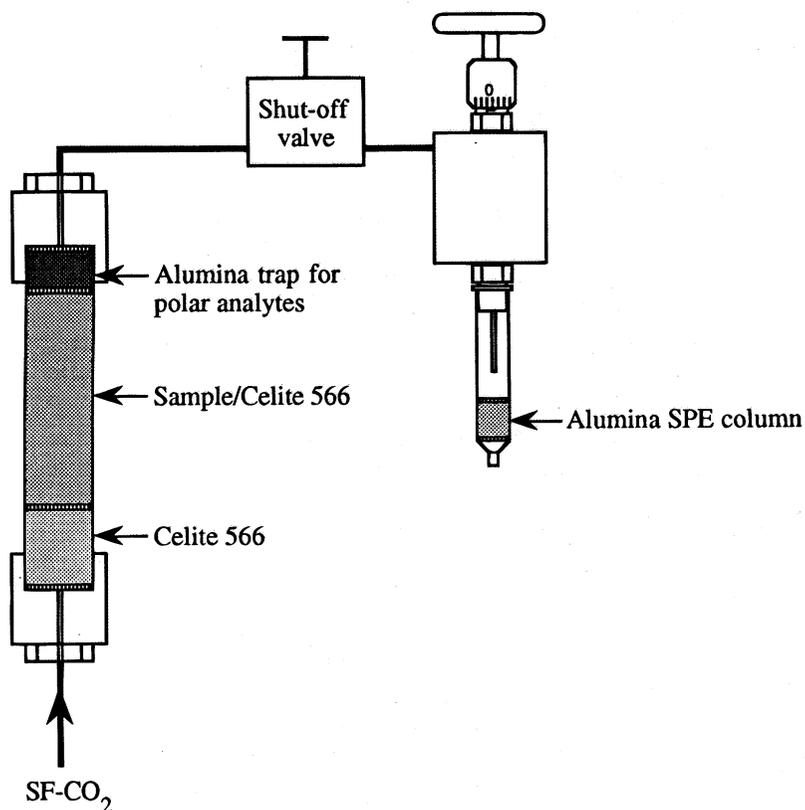


FIGURE 5-7. Schematic illustration of the extraction vessel-micrometering valve/collection assembly section of the SFE shown in Figure 5-3c. The extraction vessel is prepared for polar solute retention on an in-line sorbent bed.

are adsorbed from the SF and retained. The SF, now containing only fat and other endogenous tissue material, is vented through the off-line sorbent bed. Consequently, the analytes of interest are retained on the in-line sorbent bed, whereas interfering endogenous components are collected in the off-line SPE column. The degree to which polar analytes in this process are bound to the in-line adsorbent is dependent on the characteristics of analytes, their affinity for the sorbent, and the density of the SF. This process is one of continuous analyte adsorption and desorption on the in-line bed, necessitating an understanding of analyte-sorbent interactions to ensure successful in-line trapping.

The ability of the in-line technique to retain polar analytes while other endogenous materials remain solubilized is demonstrated by the results in Table 5-3 for the in-line and off-line recovery⁶³ of sulfonamides from chicken tissues. Recoveries of the three sulfonamides using in-line trapping are uniformly high and consistent from tissue to tissue, whereas the results from the off-line SPE columns are poor and vary from tissue to tissue. Similarly, a comparison of the HPLC chromatograms in Figure 5-6a (off-line recovery) and Figure 5-6b (in-line recovery) illustrates the improved HPLC separation possible with in-line trapping.

Off-line analyte recovery has been used by Magard et al.⁹¹ in the extraction of steroids from animal tissues. Androsterone, a steroid responsible for the boar taint odor in pork, was extracted from fortified boar fat using SF-CO₂. The recovered androsterone was detected by GC-MS. However, whereas the SFE procedure extracted 77% of the steroid, it also co-extracted 10% of the fat, thereby increasing the potential for regular fouling of the GC column and the MS ion source. Although steroids are constituents of fats and are easily extracted by SFE at moderate temperatures and pressures, they too, like sulfonamides, may be retained on in-line sorbent beds. Parks and co-workers⁹²

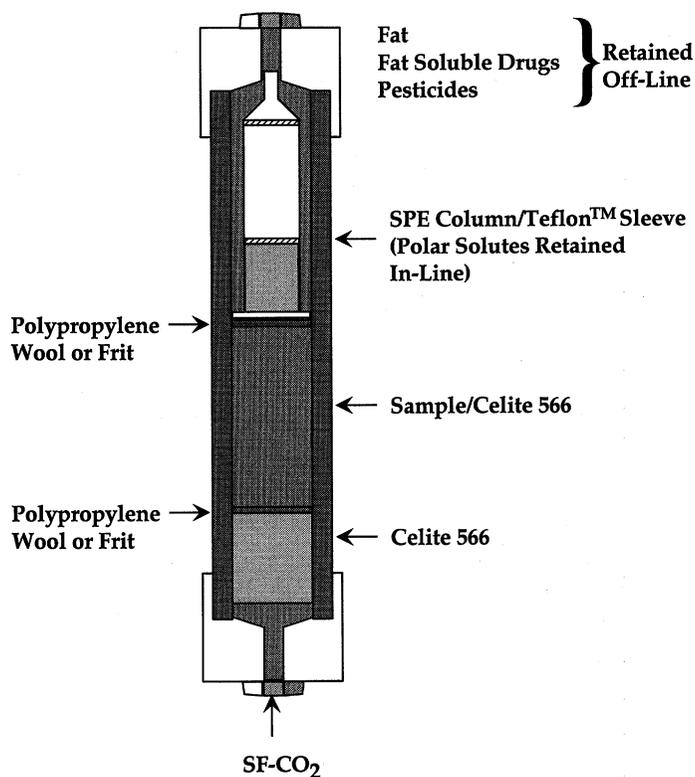


FIGURE 5-8. Cutaway illustration of an extraction vessel prepared for in-line trapping on a standard, commercial SPE column.

B. SFE OF HAIR SAMPLES

Sachs and co-workers^{94,95} first reported the application of SFs to the isolation of drugs from hair in 1992. They demonstrated recoveries of opiates and cocaine from hair using CO₂ modified with ethyl acetate, but found that extraction rates and reproducibility of the SFE technique under these conditions were not comparable with wet chemical methods. The approach taken by Sachs was to increase the solvent strength of the SFE fluid by incorporating a modifier that was a good solvent for the target analytes; however, this approach failed to consider the influence of strong analyte–matrix binding interactions on analyte extractability.

Subsequently, Edder et al.⁹⁶ reported excellent recoveries of opiates (codeine, morphine, 6-monoacetylmorphine [6-MAM], and ethylmorphine) from drug user and standard reference (fortified) hair using CO₂ modified with a mixture of methanol, TEA, and water (85:6:6:3%, v/v). SFE was performed for 30 min (dynamic) at 40°C and 25 MPa on 50-mg portions of prewashed, pulverized hair. A laboratory-built, two-pump system with a mixing chamber was used to combine and deliver the modified fluid to the extraction chamber. Under the conditions used, the multicomponent extraction fluid is, strictly speaking, in the subcritical rather than supercritical state, and the authors refer to their method as subcritical fluid extraction. Analytes were collected by immersing the restrictor in a few milliliters of methanol (i.e., off-line solvent trapping), and extracts were analyzed using GC-MS after a propionylation derivatization step. Water was again found to be essential for efficient recoveries of the target analytes from the hair matrix.

Based on results obtained on the standard reference hair (prepared by soaking drugs into the hair), the method was found to be linear in the concentration range of 0.5 to 2 ng/mg for the four opiates tested, with coefficients of variation ranging between 3 and 10%. Replicate analyses of blank

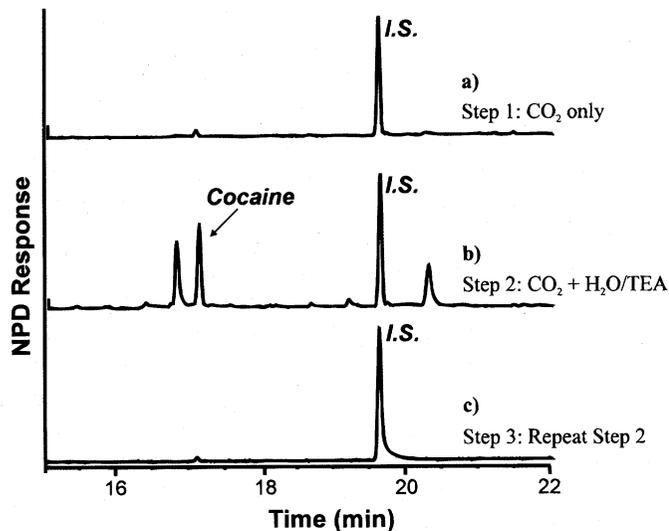


FIGURE 5-10. GC-nitrogen-phosphorus detection chromatograms of sequential SF extracts obtained on a 50-mg sample of a chronic drug user's hair. In all cases, SFE was performed at 40.5 MPa and 110°C with a 10-min static step and 30-min dynamic step. I.S., internal standard (*n*-propylbenzoyllecgonine).

sample, and a final extraction (chromatogram c) under the same conditions illustrated that complete extraction of cocaine was obtained during step 2 of this sequence. After SFE, the hair was subjected to a 24-hour acid incubation/SPE procedure; analysis of the resulting extract showed no remaining detectable cocaine.

Further experiments demonstrated that cocaine could be efficiently recovered from an inert matrix such as Teflon™ wool using pure, unmodified SF-CO₂ (Figure 5-11), suggesting that desorption of the drug from matrix binding sites was a rate-limiting step in the extraction of cocaine from hair. It was hypothesized that the role of TEA was one of competitive displacement of cocaine

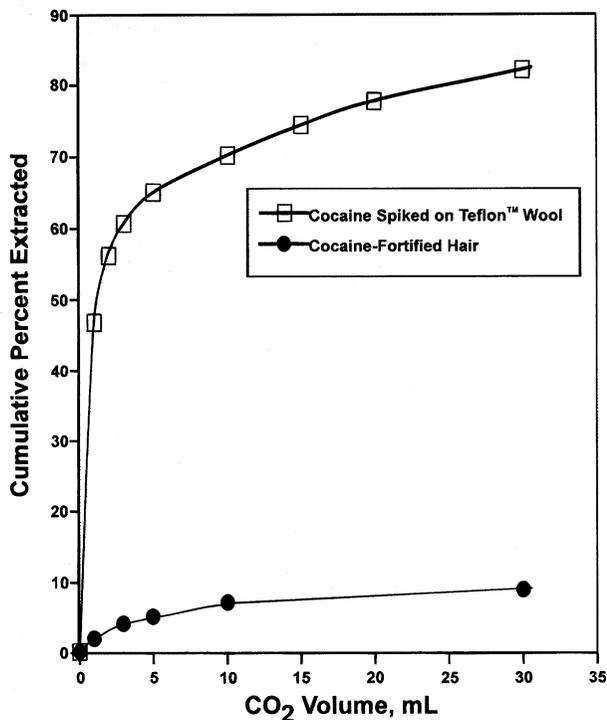


FIGURE 5-11. Extraction profile comparison illustrating the influence of matrix on cocaine extractability using pure SF-CO₂. Hair was fortified with cocaine by soaking it in a dilute dimethylsulfoxide (DMSO) solution of the drug for 1 month, with subsequent removal of the solvent. SFE conditions: 100% CO₂, 40.5 MPa, 110°C.

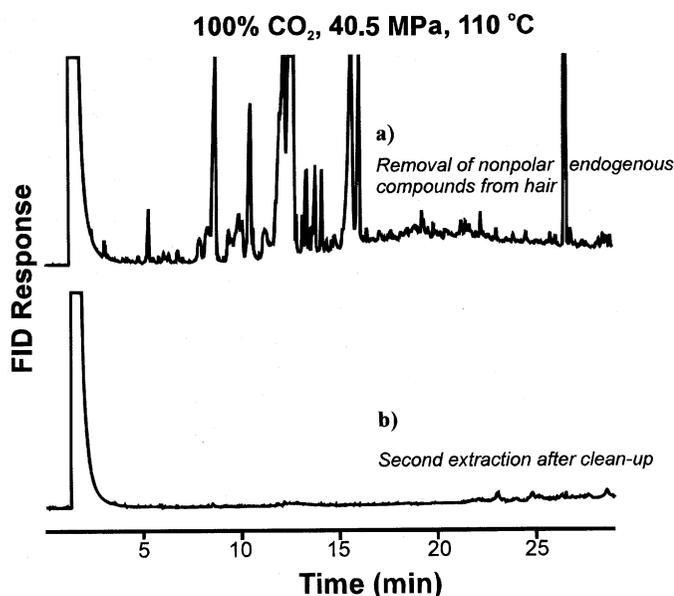


FIGURE 5-13. GC-flame ionization detector (FID) chromatograms showing sequential SF extracts obtained on a 150-mg sample of powdered hair from a non-drug user (blank control). A short preclean-up step with pure CO₂ efficiently removed nonpolar surface oils and waxes from the hair that could potentially interfere with chromatographic analysis.

using the SFE conditions described previously, and extracts were analyzed for the presence of cocaine using a commercially available solid-phase RIA kit. Issues unique to the immunochemical analysis of SFE-generated extracts were studied. MeOH (the SFE collection solvent) had only a minor impact on immunoassay performance; however, the presence of TEA/H₂O (the SFE modifier mixture) profoundly degraded assay performance, producing a 60% suppression in assay response. To preserve RIA sensitivity, SF extracts were evaporated under nitrogen to remove the modifier and reconstituted in MeOH for RIA analysis. SFE-RIA analysis of a series of drug-free hair samples established an RIA cut-off value for distinguishing between a negative and presumptive positive cocaine sample at an SF extract concentration of 1.2 ng/ml, or a hair concentration of 0.07 ng/mg. The robustness of the SFE-RIA method was demonstrated by the analysis of a variety of hair samples from both drug users and nonusers, and the quantitative SFE-RIA findings correlated well with values obtained by an acid incubation/GC-MS method.

C. SFE OF URINE SAMPLES

Direct extraction of analytes from aqueous samples using SFE is a particularly challenging analytical problem for several reasons.^{5,70,99,100} First, the extraction vessel must be able to retain the liquid sample and prevent its mobility during the extraction period. Second, although water is only sparingly soluble in SF-CO₂,¹⁰¹ this limited solubility can be problematic when one considers that, for trace analysis, it would be necessary to extract large aqueous sample volumes to recover sufficient analyte for quantification. Large volumes of SF-CO₂ would consequently be required for extraction, with the potential for carryover of considerable amounts of water to the collection vial or solid-phase trap. The solvated water can additionally cause plugging of linear restrictors because of ice formation during decompression. Finally, the direct isolation of polar analytes from a polar liquid matrix (water) using a relatively nonpolar extractant (CO₂) is a particularly difficult task from an analytical method development standpoint. Using a specially designed CO₂ recycling system and an extraction vessel geometry that minimizes “splashover” of water, Hedrick and Taylor^{99,100} have demonstrated the direct extraction of phosphonates, phenols, triprolidine, caffeine, and pseudoephedrine from aqueous solutions. However, the analytes were not at trace levels, and, in addition to the requirement of specialized hardware, this direct approach precludes the use of polar modifiers.

D. SFE OF BLOOD SAMPLES

The extraction of blood samples by SFE poses some of the same analytical challenges discussed herein for urine samples. The most common approach has been to preconcentrate the analytes or load the sample onto selective solid-phase sorbents before SFE. Johansen et al.¹⁰⁴ report recoveries of PCBs from blood serum using an on-line SFE-GC method after loading of the serum sample (up to 5 ml) on a C₁₈ sorbent. Residual water was retained in the extraction vessel by placing a small amount of basic alumina directly in the vessel. A separate column containing basic alumina was also placed downstream from the sample vessel to retain co-extracted lipid material. Removal of water and lipid material was found to be crucial for maintaining chromatographic performance in the on-line SFE-GC procedure. Extracts obtained by the SFE procedure displayed fewer chromatographic interferences, compared with extracts generated by traditional LLE procedures, resulting in more reliable quantification. Absolute recoveries, however, were slightly lower with the SFE procedure. The method was used to examine total PCB concentrations down to 25 ng/l.

Liu and Wehmeyer¹⁰⁵ have used SPE with SF elution for the trace analysis of the naturally occurring flavonoid compound flavone from dog plasma. The plasma sample (1.0 ml fortified at 50 ng/ml) was preconcentrated on a pre-conditioned commercially available C₁₈ column. The SPE column was placed directly in the SFE vessel and eluted with 5% MeOH-modified CO₂ at an extraction temperature and pressure of 50°C and 15 MPa, respectively, and an extraction time of 10 min. Flavone recoveries for the SPE-SFE method ranged from 89 to 96% for concentrations ranging from 10 to 250 ng/ml. The method performance compared favorably with traditional aqueous-organic solvent SPE elution. The advantages of the SPE-SFE elution method included easier removal of the eluent after extraction and the potential for increased selectivity by varying extraction pressure and temperature.

The SFE of temazepam from whole blood has been reported by Scott and Oliver¹⁰⁶ after loading of the blood sample onto a selective sorbent material. The sorbent was subsequently eluted with 5% ethyl acetate-modified CO₂ for 10 min at an extraction temperature and pressure of 65°C and 20.7 MPa, respectively. Analyte recoveries were greater than 80%. SFE results on authentic forensic blood specimens correlated well with results obtained by traditional SPE over the concentration range of 1 to 8 mg/l.

Combined SPE-SFE has also been demonstrated for the recovery of mebeverine alcohol from blood plasma.¹⁰⁷ The plasma was applied directly to preconditioned C₁₈ SPE columns; the columns were subsequently washed to remove protein, and the sorbent was removed and placed in the SFE vessel. The analyte was recovered using 5% MeOH-modified CO₂ at 35.5 MPa and 40°C for a 10-min collection interval. Addition of an organic amine (0.1 ml TEA) to the plasma sample before extraction was necessary to effect release of the analyte from sorbent binding sites.

Finally, Karlsson and co-workers¹⁰⁸ have reported the SFE of corticosteroids from fortified blood plasma (93 nmol/l) after deposition of 500- μ l portions of the plasma on filter paper. SFE recoveries exceeding 80% could be obtained from the plasma samples, compared with only 28% SFE recoveries when the pure compound was spiked on filter paper. It was postulated that the improved extractability from plasma was caused by the presence of water in the sample, which can act as a polar modifier in CO₂.

VII. CONCLUSIONS

This extensive review demonstrates that significant progress has been made in the development of analytical SFE technology for the isolation of trace analytes from biological matrices. Resourceful and creative approaches have been used by researchers to improve extraction selectivity and sensitivity for biological applications, illustrating the potential of this technology as a sample preparation tool in analytical toxicology. The recent report by Johnston and co-workers⁴⁷ describing the solubilization of a protein by aqueous microemulsion droplets in SF-CO₂ suggests exciting possibilities for the SFE of highly polar molecules. Although the application of SFE in analytical toxicology is relatively new, much can be learned from the voluminous SFE literature

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