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Development of Supercritical Fluid Extraction (SFE) for the Determination of Polychlorinated Biphenyls in Tissues of Aquatic Biota

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Development of Supercritical Fluid Extraction (SFE) for the Determination of Polychlorinated Biphenyls in Tissues of Aquatic Biota

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Michael O. Gaylor

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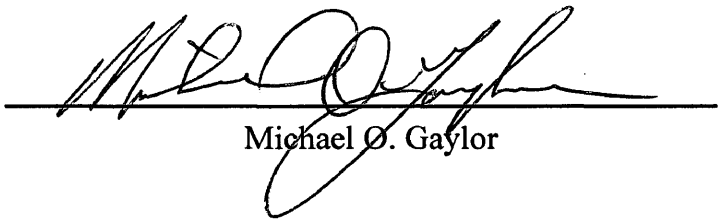

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Approved December, 1997

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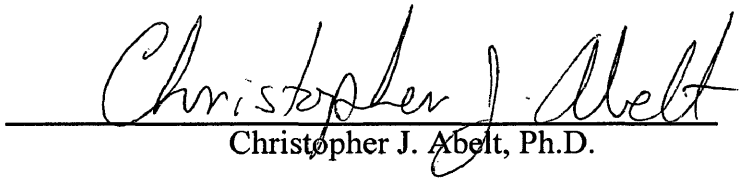

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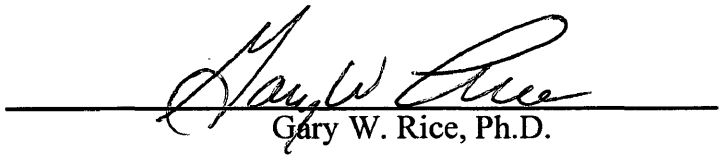

Gary W. Rice, Ph.D.

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ABSTRACT

Extraction methods commonly used for PCB determinations in environmental samples generally rely on classical liquid organic solvent approaches, such as Soxhlet extraction and column elution. These techniques are time consuming, laborious, tedious, non-selective and require copious volumes of solvents. Ultimately, these solvents must be disposed of as hazardous waste. Extracts obtained require numerous post-extraction purification steps, such as gel permeation chromatography (GPC), florisil and silica column cleanup. Alternatively, supercritical fluid extraction (SFE) using CO_2 and cryogenic, solid-phase analyte trapping presents a rapid, safe and more analyte-selective alternative for PCB extraction from biological matrices. An automated SFE-based method was developed using unmodified supercritical CO_2. PCB extraction was quantitative following a 10 minute static and 30 minute dynamic extraction at a liquid flow rate of 3 ml/min. Extraction pressure and temperature were 350 atm and 150°C, respectively. No organic modifiers were required. Lyophilization was the preferred sample drying approach. The method included use of activated neutral alumina oxide (Al_2O_3) in the extraction cell and permitted on-line elimination of > 99% of co-extracted lipids. Extracted PCBs were collected on a cryogenic trap containing a 1:1 (w/w) mixture of C_{18}:Unibeads. PCBs were eluted with 2 ml isooctane at 90°C, reducing organic solvent consumption by two orders of magnitude. In contrast to conventional organic solvent-based approaches, SFE extracts required no post-extraction purification and minimal solvent reduction prior to GC-ELCD analysis. Excellent results were obtained from laboratory-amended and field-incurred matrices. Established SFE conditions were verified as "optimum" by systematic manipulation of individual extraction and trapping parameters. Validation of the method was achieved by application of SFE to the extraction of PCBs from a Canadian Research Council candidate certified reference material (CRM; carp homogenate). A SFE method for the extraction of total lipids from tissue samples was also developed. Total lipid values comparable to conventional extraction methods were obtained using unmodified CO_2 at a pressure of 450 atm and temperature of 150°C. Lipid extraction was complete following a combined 10 minute static and 30 minute dynamic extraction at a liquid flow rate of 6 ml/min. Use of organic solvents and glassware was eliminated completely. Lipids could be determined gravimetrically by the difference in vessel weight before and after extraction. PCB levels in edible musculature did not correlate well with % total lipids. The robustness of the SFE-based method was confirmed by successful PCB and total lipid extraction and analysis of 644 (1288 total) lyophilized edible fish tissue samples collected from Virginia fresh water river systems.

Development of Supercritical Fluid Extraction (SFE) for the Determination of Polychlorinated Biphenyls in Tissues of Aquatic Biota

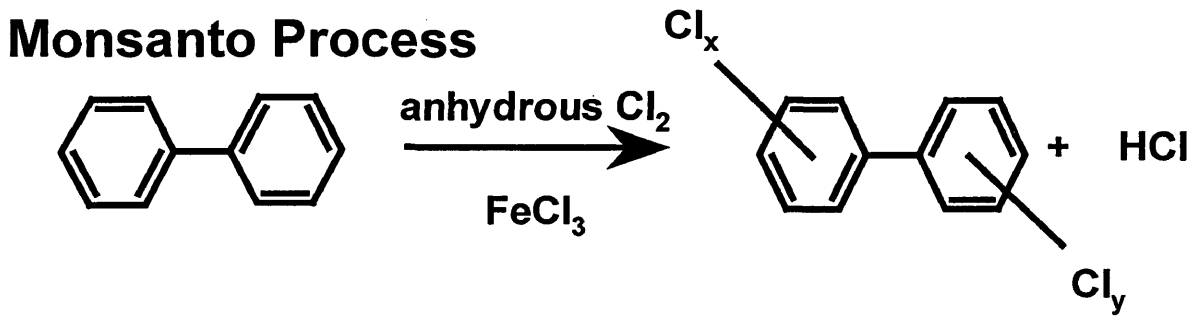
INTRODUCTION

Polychlorinated biphenyls (PCBs), a class of synthetic organic chemicals, are persistent, lipophilic environmental pollutants that tend to bioaccumulate in the tissues of aquatic organisms. Their widespread environmental presence poses considerable risks to ecosystems and human health (Lang, 1992). Proliferation of their use in industry and manufacturing since 1930 has led to their deposition in at least trace levels in virtually every compartment of the environment. Because of their potential toxicity, PCBs are of great concern to the scientific and regulatory communities, as well as the general human population.

PCBs were first synthesized in 1881 (Schmidt and Schulz, 1881). Global commercial production of PCBs began in 1929 and continued through 1977 (Erickson, 1992). The prominent manufacturers include Monsanto (Saint Louis, MO, USA), Kanegafuchi Chemical (Tokyo, Japan) and Bayer (Leverkusen, Nordrhein-Westfalen, Germany). PCBs were produced as complex mixtures and first marketed under the trade name Aroclor™ by Monsanto (Lang, 1992). Other trade names include Kanechlor, Clophen and Delor. Aroclors were synthesized in large batches by the percolation of chlorine gas through molten biphenyl in the presence of an appropriate catalyst. The primary synthetic mechanism for PCB production in the Monsanto approach was electrophilic substitution of chlorine atoms on a central biphenyl molecule in the presence of an $FeCl_3$ catalyst (Figure 1). The result was a mixture of 209 PCB isomers (or

congeners) ranging in degree of chlorination from mono- through decachlorobiphenyl.

Figure 1. *A schematic of the Monsanto synthetic approach to PCBs.*



Degree of chlorination in this reaction was controlled by manipulation of reaction thermodynamics to yield the desired physical and chemical properties. These same thermodynamics also favored certain substitution patterns, resulting in differences in congener abundance and composition among the various Aroclor formulations (Albro and Parker, 1979). Aroclors were assigned four digit numerical designations based on their composition. The first two digits identify the starting reagent biphenyl (C-12), while the last two digits indicate the weight percent of chlorine in the mixture (Lang, 1992). For example, Aroclors 1242, 1248, 1254 and 1260, the most common formulations, contain 42, 48, 54 and 60 weight percent chlorine, respectively.

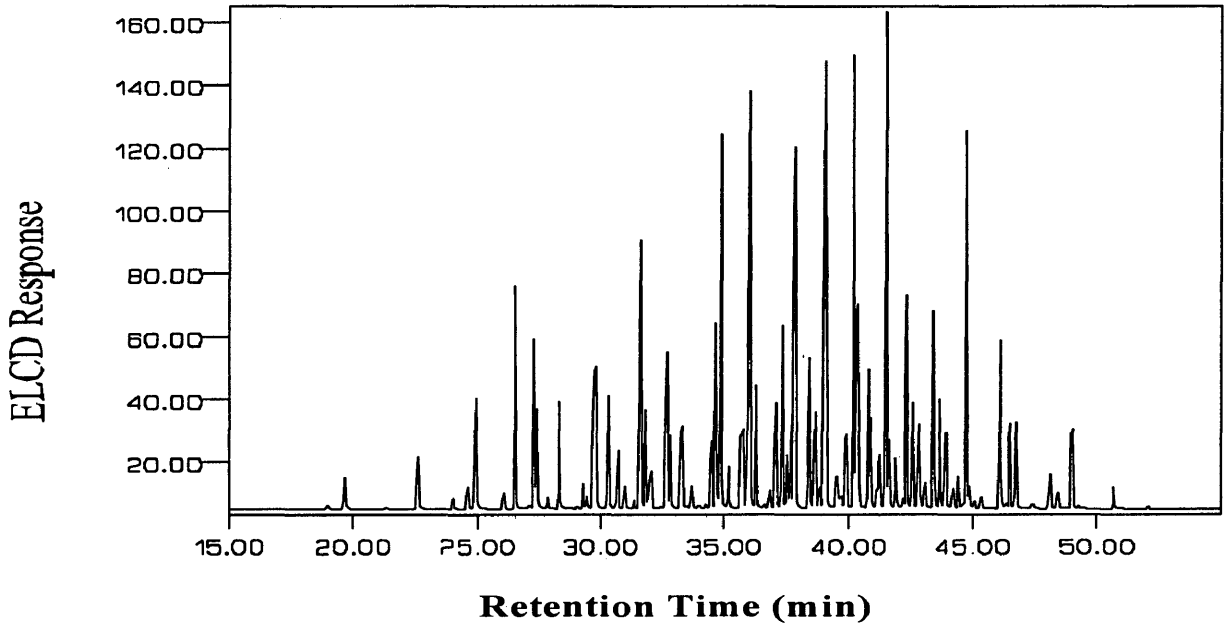
Commercial PCB mixtures have been used in a wide variety of industrial and electrical applications. These have been classified (WHO, 1976) into three distinct use categories: controllable closed systems, uncontrollable closed systems and dissipative uses (Erickson, 1992). Controllable closed system applications were considered

relatively resistant to environmental release during the life of the product and include dielectric fluids in capacitors and transformers. Uncontrollable closed systems permitted leakage over time and included heat transfer and hydraulic fluid systems. Dissipative uses allowed direct introduction of PCBs into the environment and included lubricating and cutting oils, additives in pesticides, copy paper, adhesives, sealants, plastics and plasticizers in paints. Dissipative applications of PCBs in the U.S. were banned in the late 1970's. However, closed system applications (e.g. electrical transformers) now pose the greatest threats as their working lifetimes expire and they are disposed in the environment. Accidental discharge of PCBs is also a major route of introduction.

A hallmark of PCBs is their extreme chemical and thermal stability. Ironically, these same robust physico-chemical properties that made them desirable in electrical and industrial applications are those responsible for their widespread dispersal and persistence in environmental compartments. Owing to their great complexity, PCB mixtures pose difficult challenges to the analytical chemist interested in extracting, purifying and quantitating them in environmental matrices, particularly biota (see Figure 2). PCBs are resistant to thermal degradation, oxidation and reduction, hydrolysis and attack by acids and bases. PCBs are non-polar, exhibit low or no flammability and have extremely low aqueous solubilities and vapor pressures, and high octanol-water partition coefficients (Brinkman and de Kok, 1980; Moore and Ramamorthy, 1984).

Because of their chemical properties, PCBs readily sorb to suspended particulate matter, accumulate in sediment strata and can concentrate in organisms upon release into the aquatic environment. While there has been considerable debate regarding actual

Figure 2. *Electrolytic conductivity detector (ELCD) chromatogram of a standard mixture of Aroclors 1242, 1254 and 1260. See text for GC conditions.*



mechanisms of distribution and uptake of PCBs by aquatic organisms, it is generally agreed that PCB accumulation in biota occurs through two different pathways: 1) passive partitioning directly from the water column into tissue lipid deposits (bioconcentration), and 2) trophic-level accumulation through successive predator-prey interactions (Moriarty and Walker, 1987). Recent studies have shown that increases in tissue PCB burdens are attributable to increases in lipid content from one trophic level to the next (Leblanc, 1995). These findings indicate that trophic-level transfer (or bioaccumulation) is likely the dominant mechanism in aquatic food webs. Once taken up, PCBs can induce a variety of toxic effects, including body weight loss; skin (chloracne), hepatic, endocrine

and reproductive dysfunction. PCBs have also been shown to be carcinogenic and teratogenic (Lang, 1992). Most of these effects are preceded by metabolization via enzyme systems (e.g. oxygenase; McConnel, 1980)

As apex members of the global food web and consumers of copious quantities of seafood, humans are highly predisposed to the toxic effects of PCBs. Effects reported in humans include hepatic dysfunction, dermal lesions, respiratory disorders, severe ocular dysfunction, various neurological symptoms, endocrine disruption, immunodeficiency and reproductive disorders (Kuratsune, 1980; Jones, 1989). To effectively understand, address and remediate the myriad hazards of PCBs in complex biological matrices, improved analytical methods are needed that can provide scientific and regulatory agencies with accurate and reliable data on PCB congener concentrations within environmental compartments.

Most environmental studies of PCBs rely on lengthy, complex chemical analyses. Current methods are costly in terms of project budgets, data turnover time and exposure of workers to harmful chemicals (Huhnerfuss and Kallenborn, 1992). The current study focuses on isolating and quantifying PCBs in biological samples, specifically, aquatic biota tissue. The analysis process may be broken down into extraction, multi-step purification and ultimately compound identification and quantification. While refinements in extract purification and analyte quantification/identification techniques have been made, few improvements have been made in the initial and most time consuming step, sample extraction. In addition, concerns have arisen regarding the actual

efficacy of conventional extraction approaches (de Boer, 1988). If the analytes are not quantitatively separated from the matrix initially, all subsequent analysis steps are effectively useless, regardless of their sophistication.

Commonly used extraction procedures for PCB determinations in environmental samples are labor intensive, tedious, not amenable to automation and exhibit minimal selectivity. Historically, this crucial analysis step required significant quantities of solvent to achieve quantitative removal of target analytes from the matrix. Many of these solvents are highly toxic or flammable. All are expensive in the high purity grades needed for trace analyses. Despite significant environmental reform in other segments of society, spent solvent continues to be stored and disposed of as hazardous waste. This outcome contradicts the desired goal of environmental research, that of improving environmental quality. Disposal costs are also an increasing strain on research budgets, as state and federal regulations have become increasingly more restrictive.

As an example, a benchmark extraction approach is Soxhlet extraction (Snyder et al., 1992; Meyer et al., 1992). This technique continuously refluxes the sample with recirculated solvent for up to 48 hours. Extracts produced must be reduced in volume, with attendant concentration of extract impurities and potential evaporative losses of analytes. Delicate glassware must also be used and then laboriously cleaned. The remaining extract typically requires multi-step purification to remove co-extracted material, prior to liquid (LC) or gas chromatographic (GC) analysis (Hale and Greaves, 1992). The time required to complete each group of samples is typically about five days.

In addition to tissue contaminant burdens, knowledge of tissue lipid content is also important. Since partitioning of non-polar organic pollutants such as PCBs is directly related to this parameter, total lipid determinations are essential for elucidating PCB disposition in biological samples. Total lipid concentrations are widely used to normalize contaminant body burdens. As with PCBs, lipid extraction methods are laborious, time consuming, not amenable to automation and require copious quantities of glassware and organic solvents. The most widely used lipid quantitation method, developed by Bligh and Dyer (1959), requires significant sample manipulation and use of chloroform, a highly toxic solvent.

To modernize studies of organic pollutant transport, fate and effects, extraction methodologies are needed that improve analyte recovery, minimize hazardous solvent usage and accelerate and streamline the overall analysis process. An ideal extraction technique would be rapid, simple, inexpensive over the long-term, minimize off-line evaporative concentration steps, ensure quantitative recovery of analytes without degradation or loss of volatile species and produce little or no solvent wastes. The method should produce an extract that requires no further post-extraction purification (e.g. gel permeation, silica gel and florisil chromatography). Finally, the extract should also be amenable to direct introduction to gas chromatographic (GC) systems.

Off-line supercritical fluid extraction (SFE) with solid phase cryogenic trapping has the potential to satisfy all of these criteria. SFE has emerged in recent years as an environmentally-benign analytical technique that has significantly improved the

extraction of trace organic pollutants, such as PCBs, from environmental samples (Meyer et al., 1992; Camel et al., 1993; Hale and Gaylor, 1995). During SFE a gas, usually CO_2, is elevated beyond its critical point and brought into contact with the sample matrix. In this region fluid viscosities and densities approach those of gases and liquids, respectively. The result is an extraction solvent with solubilizing power approximating that of hexane, that is "tunable" as a function of temperature and pressure and will dissolve non-polar organic pollutant compounds effectively. In the supercritical region, fluid surface tension and viscosity are practically zero, thus allowing greatly improved mass transfer of solutes from the matrix (Camel et al., 1993; Bayona, 1993; McHugh and Krukonis, 1994). The result is a substantial increase in the kinetics of the extraction process. The practical advantages of SFE for PCB determinations in environmental matrices are rapid extractions (30-60 minutes), improved analyte selectivity, no post-extraction cleanup, virtual elimination of hazardous liquid solvent usage and substantial waste minimization. A comparison of the physical properties of a supercritical fluid (SF) to those of gases and liquids is given in Table 1.

Table 1. *Physical properties of a typical supercritical fluid, liquid and gas.*

Parameter	Supercritical Fluid	Liquid	Gas
Density (g/cm^3)	0.2-0.9	0.8-1.0	$(0.6\text{-}2.0) \times 10^{-3}$
Diffusivity (cm^2/s)	$(0.5\text{-}3.3) \times 10^{-4}$	$(0.5\text{-}2.0) \times 10^{-5}$	0.01-1.0
Viscosity (g/cm s)	$(2.0\text{-}9.9) \times 10^{-4}$	$(0.3\text{-}2.4) \times 10^{-2}$	$(0.5\text{-}3.5) \times 10^{-4}$

The critical point of a substance was first observed in 1822 by Baron Cagniard de la Tour. However, the efficacy of SFs in solubilizing chemical compounds was not realized until 1879 when Hannay and Hogarth reported that metal halides were soluble in supercritical ethanol and carbon tetrachloride (Riekkola and Manninen, 1993). The solubility of solid organic substances was reviewed by Rolwinson and Richardardson (1959). Their work demonstrated that a SF or "dense gas" could dissolve solids to the same extent as liquid solvents. Since that time SFE has been applied to an increasing number of industrial, engineering and chemical process applications. The most obvious in recent years has been the decaffeination of coffees and teas using supercritical CO_2 as an alternative to toxic organochlorine solvents (e.g. methylene chloride and trichloroethane). Decaffination was the first industrial-scale use of SFE (McHugh and Krukonis, 1994). Other recent process applications of SFE have included extraction of bittering acids from hops, extraction of oligomers from polymer formulations, extraction and purification of pharmaceuticals, extraction of oils and fats from soybeans and determination of extractable fats in food products (Friedrich et al., 1982; Lembke and Engelhardt, 1993; McHugh and Krukonis, 1994).

A fluid is said to exist in a supercritical state when the pressure and temperature of the substance are simultaneously elevated above its critical point. As the critical point is approached, its isothermal compressibility approaches infinity, leading to drastic changes in molar volume and density. In this region the equilibrium boundary layer between liquid and vapor is no longer distinguishable. Regardless of pressure increase at

constant temperature, it is not possible to shift the equilibrium towards formation of a liquid. Alternatively, increasing temperature at constant pressure will not produce a vapor (McHugh and Krukonis, 1994). The uniqueness and applicability of this phase to compound separations lies in the ability to tune the density of the fluid. By manipulating the temperature and pressure, it is possible to adjust fluid solubilizing power. This property permits class-selective extractions of compounds of differing polarity and molecular mass with a single SF, as well as the extraction of thermally-labile compounds (Hawthorne, 1992).

All substances have a critical point. However, few exist within the useful range of modern laboratory instrumentation and hardware. A list of common SFE solvents and their critical parameters is given in Table 2. It can be seen from this list that, other than ethylene, CO_2 has the most easily-attainable critical constants. Supercritical CO_2 is non-polar, relatively inert, non-toxic, non-flammable and relatively inexpensive, thus making it ideal for use in the environmental. Supercritical CO_2 is also an excellent solvent for the removal of numerous lipophilic solutes from a wide variety of sample matrices. Subcritical CO_2 has no permanent dipole and can solubilize mainly non-polar chemical species. However, in the supercritical region, a modest dipole can be induced with incremental adjustments in pressure. Induced dipole interactions, coupled with the Lewis base characteristics of supercritical CO_2, make it possible to dissolve non-polar to moderately polar chemical species (Hawthorne, 1990; Camel et al., 1993). Relevant environmental pollutants in this polarity range include PCBs, polycyclic aromatic

hydrocarbons (PAHs), aldehydes, esters and organochlorine pesticides.

Table 2. *Common SFE solvents and their critical parameters (adapted from King and France, 1992)*

Compound	Critical Temperature (K)	Critical Pressure (MPa)	Critical Density (g/ml)
Ethylene	283.0	5.12	0.23
Carbon Dioxide	304.1	7.39	0.47
Nitrous Oxide	309.6	7.26	0.46
Propane	369.8	4.26	0.22
Sulfur Hexafluoride	318.8	3.76	0.75
Methanol	513.4	7.99	0.27
Water	637.0	22.1	0.32
Ammonia	405.4	11.3	0.24
n-Pentane	469.8	3.37	0.23

A fundamental consideration when developing SFE is the solubility of the target compounds. In the supercritical region, solubility is strongly dependent on fluid pressure, temperature and density (King, 1989; Pipkin, 1992). Therefore, it is reasonable that analyte solubility theory might provide a convenient theoretical approach to optimizing SFE. This approach was placed on firm quantitative ground by Giddings et al. (1968), when the concept of the Hildebrand solubility parameter was applied to the solubility of

selected organic compounds in dense gases. Theoretical development was based upon extrapolation of the van der Waals equation of state for gases to organic liquids (Hildebrand and Scott, 1950). It was found that the equation is quite reasonable for many liquids.

In addition to solubility parameter considerations, it has been proposed that four fundamental variables are paramount to a full understanding of solute behavior in a SF (King, 1989). The first of these is miscibility pressure, defined as the minimum pressure at which the analyte begins to dissolve in the SF. This pressure has been described as the "threshold pressure" (Giddings et al., 1969). The threshold pressure permits a modicum of *a priori* knowledge regarding target analyte solubility by which to begin optimization of an extraction/fractionation protocol. The second parameter is the pressure at which the solute attains maximum solubility in the SF. This concept allows approximation of the maximum analyte solubility by relation of the solubility parameter of the gas to its critical and reduced state properties (Giddings et al., 1968). According to this approach, maximum solubility is achieved when the solubility parameter of the extracting fluid equals that of the solute. The third parameter is the fractionation pressure range, in which analyte solubility ranges from the threshold level to its maximum in the SF. In this range, it is theoretically possible to perform selective fractionation of multiple solutes by manipulating the SF pressure/density. Finally, knowledge of an analyte's physical properties is very important in SFE. Of these, the melting point can be most pronounced in determining extraction efficiency. Compounds that exist as a liquid are solubilized by

a SF to a greater extent (King, 1989).

Knowledge of compound solubility behavior can provide a useful starting point to developing and optimizing a SFE-based extraction method. However, it is insufficient to guarantee high extraction efficiencies from complex "real-world" matrices such as those typically collected in the environment (Hawthorne et al., 1993). The supercritical solvent must also be able to overcome matrix-analyte interactions by competing with target analytes for matrix binding sites. Typically, several types of molecular adsorption forces are at work at the matrix-analyte interface, including dipole interactions, hydrogen bonding, complexation and π-π interactions (Friedrich et al., 1995). Disruption of matrix-analyte interactions generally requires overcoming the desorption energy associated with these forces before achieving complete dissolution in the extraction solvent (Alexandrou et al., 1992). Thus, SFE can be conceptualized as a two stage process: 1) disruption of matrix binding sites with subsequent desorption of the analytes and 2) solvation of the desorbed species into the extraction solvent. It is generally helpful, however, to subdivide the process further into smaller physical steps for greater understanding of the SFE process.

The currently accepted model of compound extraction from an environmental matrix using a SF assumes initial adsorption of the analyte onto an impermeable particle core that is surrounded by an organic layer (Pawliszyn, 1993). This is often reasonable, given the heterogeneous nature of most environmental matrices. Starting with this simplified assumption, the extraction process can be modeled using basic engineering and

chromatography models of chemical species transport through porous media. To remove the analyte from the particle, it must be desorbed from the surface and diffuse through the organic layer to the matrix-fluid interface. The analyte must be solvated by the SF and diffuse through the static fluid in the matrix pores, finally reaching the bulk flowing fluid for transport through the interstitial spaces of the matrix. Once removed from the matrix, the analyte is thoroughly solubilized by the SF and is swept out of the extraction chamber by the flowing solvent. The general acceptance of this model among SFE researchers suggests that matrix-analyte interactions are the primary limiting step. These strong molecular forces can often be surmounted, however, through the use of elevated extraction temperatures and matrix modification with liquid organic solvents, or a combination of both (Taylor, 1995).

Numerous remedies have been proposed for overcoming matrix analyte interactions and improving SFE efficiency. Among these are the use of elevated extraction temperatures and pressures, SF flow rate and increased extraction duration. Matrix modification with liquid organic solvents has been examined rigorously (e.g. methanol, acetone etc.; Hawthorne, 1990; McNally, 1995). Increasing extraction temperature at constant pressure increases analyte vapor pressure and decreases cohesive energy density, thus improving analyte solubility and SFE kinetics (Prausnitz, 1969; King and France, 1992; Hawthorne and Miller, 1994). Increasing pressure at constant temperature increases SF density and can enhance solvating power (King and France, 1992). Elevated flow rates can improve extraction efficiency in two ways: 1) by

increasing the number of times the matrix is swept by the SF during the extraction; and 2) by rapidly extracting high concentration analytes that are loosely-bound to the matrix (e.g. 100 mg/g) and exposing more tightly sorbed species to the SF (Hawthorne, 1993).

These myriad extraction approaches have proven successful in varying degrees for a range of organic contaminants (e.g. PAHs, PCBs) in abiotic matrices (e.g. sediments, soils and fly ash). However, there have been very few studies examining the effects of individual parameters on SFE of persistent organic contaminants in biological matrices. Further, existing data have resulted in considerable uncertainty regarding the importance of these parameters on the SFE process. In an effort to address the lack of data in this crucial area of environmental research, a SFE-based method for the determination of PCBs in tissues of aquatic biota was developed and applied. Effects of individual SFE variables were examined systematically. Highlights of the method are speed of extraction, automation, high sample throughput and selectivity. SFE saves considerable time (hours vs. days) and solvent (mls vs. liters), compared with conventional liquid solvent-based extraction techniques.

STUDY OBJECTIVES

I. Development of SFE for the determination of PCBs in edible fish tissues.

1. Preparation and certification of VIMS reference materials (VRMs) using Soxhlet extraction (lyophilization vs. chemical desiccation).

2. Optimization of SFE parameters: temperature, pressure, extraction duration (kinetics), SF flow rate, solvent modifiers and solid-phase trapping.

3. Comparison of SFE and Soxhlet extraction for the determination of PCBs in a fish tissue VRM.

4. Comparison of PCB yields from a candidate certified reference material (CRM; fish homogenate) using the SFE method with those obtained during a National Institute of Standards and Technology (NIST) multi-laboratory intercomparison study.

3. Application of the SFE method to the determination of field-incurred and amended (surrogate) PCBs in edible fish tissue samples (n = 644) collected from fresh water river systems in Virginia: assessment of method "robustness" and precision.

II. Development of SFE for the determination of total extractable lipids in edible fish tissues.

1. Assessment of CO_2 flow rate effects on tissue total lipid extraction efficiency.

2. Comparison of SFE, Soxhlet extraction and column elution for extraction of total lipids from fish tissue.

3. Application of the SFE method to the determination of total extractable lipids in edible fish tissue samples (n = 644) collected for PCB determinations: assessment of robustness.

4. Correlation of PCB concentrations with % total extractable lipids.

MATERIALS and METHODS

Sample Preparation: Lyophilization vs. Chemical Desiccation

Pre-extraction sample preparation is a critical step for successful SFE. Two drying approaches have been commonly used: chemical desiccation and lyophilization. These were briefly assessed here. During initial method development, wet tissues were chemically desiccated by mixing with either diatomaceous earth (1:1 (w/w) Hydromatrix) or a 10:1 (desiccant:sample (w/w)) mixture of anhydrous sodium sulfate (Na_2SO_4). It was observed during preliminary SFE trials, however, that chemical desiccation was not adequate to completely dry tissue samples. Further, the copious sodium sulfate required drastically reduced space available within the extraction vessel for sample. Therefore, VRMs and field-collected tissue samples were freeze-dried (FTS Systems Inc., Stone Ridge, NY) in subsequent studies. Freeze drying involved initial freezing of the wet sample in a stainless steel pan. Pans were then placed in the freeze-drying chamber and water slowly removed under vacuum. During the process, the chamber temperature was raised and a stream of pre-purified nitrogen introduced to eliminate pump oil back-streaming, preventing sample contamination. Alumina blanks were freeze-dried along with tissue samples to monitor potential sample contamination. Analyte loss during freeze drying was assessed initially by spiking alumina blanks with PCB surrogate congeners 30, 65 and 204. Later, analyte losses were assessed during freeze drying of a

CRM.

Certification of VIMS Reference Materials (VRMs)

Optimization and validation work required the use of large quantities of homogeneous, previously-characterized sample material for comparison of SFE results with those obtained with the well-established Soxhlet extraction method. VRMs were used primarily for spiking studies onto which PCB congener standards could be amended. Native analytes were determined and quantitated in the VRMs where needed to "certify" PCB burdens. They could then be used to provide consistent "benchmark" matrices by which to compare results obtained using the SFE method, VRMs were prepared by combining and homogenizing several aliquots of field-collected fish and crab tissues. VRMs were extracted with Soxhlet extraction (described below) and analyzed in triplicate using accepted analytical protocols (Hale and Greaves, 1992).

Glassware Preparation

Glassware and utensils were thoroughly cleaned with detergent (Alconox) and soaked overnight in a 10% (v/v) solution of Contrad 70 and deionized water. After soaking, they were rinsed thoroughly with deionized water (Milli-Q) and acetone. Non-volumetric glassware was then baked at 400°C. Volumetric glassware was baked at 100°C to avoid deformation of the glass. Prior to use, hardware and glassware contacting the samples was rinsed with toluene, acetone, methanol and methylene chloride. All organic solvents used were residue grade or equivalent (Burdick and Jackson, Muskegon,

MI).

Soxhlet Extraction

Tissue VRMs (ca. 5 gm aliquots) were placed in fritted glass thimbles and refluxed using heating mantles with 400-500 ml of methylene chloride for 48 hours. An aliquot of the solvent extract was then removed for gravimetric determination of total extractable lipids. Soxhlet extracts required purification on an Autoprep Model 1002A gel permeation chromatograph (GPC; ABC Laboratories, Columbia, MO) to remove the copious quantities of co-extracted lipids. The GPC column was equipped with a glass column packed with Bio-Beads S-X3 resin (Bio-Rad Labs, Richmond, CA; Hale et. al., 1991). The column was eluted with 240 ml of a 1:1 (v/v) mixture of cyclohexane:methylene chloride solvents. GPC fractions containing the PCBs (100 ml) were reduced in volume under nitrogen. The remaining co-extractives were removed by passage over 1gm solid-phase florisil extraction columns (Burdick and Jackson) with 5 ml of methylene chloride. The solvent was then changed to hexane under nitrogen gas to permit detection with a halogen-selective detector. Large organic solvent volumes generated during soxhlet extraction and GPC purifications were reduced with a TurboVap LV or Turbo Vap II solvent evaporator (Zymark Corp., Hopkinton, MA).

Supercritical Fluid Extraction (SFE)

Initial SFE method development was conducted on a Prepmaster™ unit (Isco-Suprex Inc., Lincoln, NE). Final phases of development were conducted on an Autoprep

44™ (AP44) unit. Transition to this system was straightforward, as major functional components of the two systems are identical. The instruments are microprocessor-controlled and have variable-flow, heated restrictors. Both are equipped with dual-head reciprocating LC pumps, capable of delivering a liquid flow rate of up to 7 ml/min. Both use solid phase, temperature-controlled traps for analyte collection. The distinguishing feature of the AP44 is an autosampler carousel, capable of sequential extraction of up to 44 samples with collection of corresponding extracts directly into GC autosampler vials. Automation and the potential for diverse method development were the major impetuses for switching to the AP44. A generalized flow diagram showing the basic features and processes of a typical SFE system is shown in Figure 3. A more detailed schematic of the Isco-Suprex AP44 SFE unit is provided in Figure 4.

Figure 3. *Flow diagram of the general components and processes of a typical SFE system*

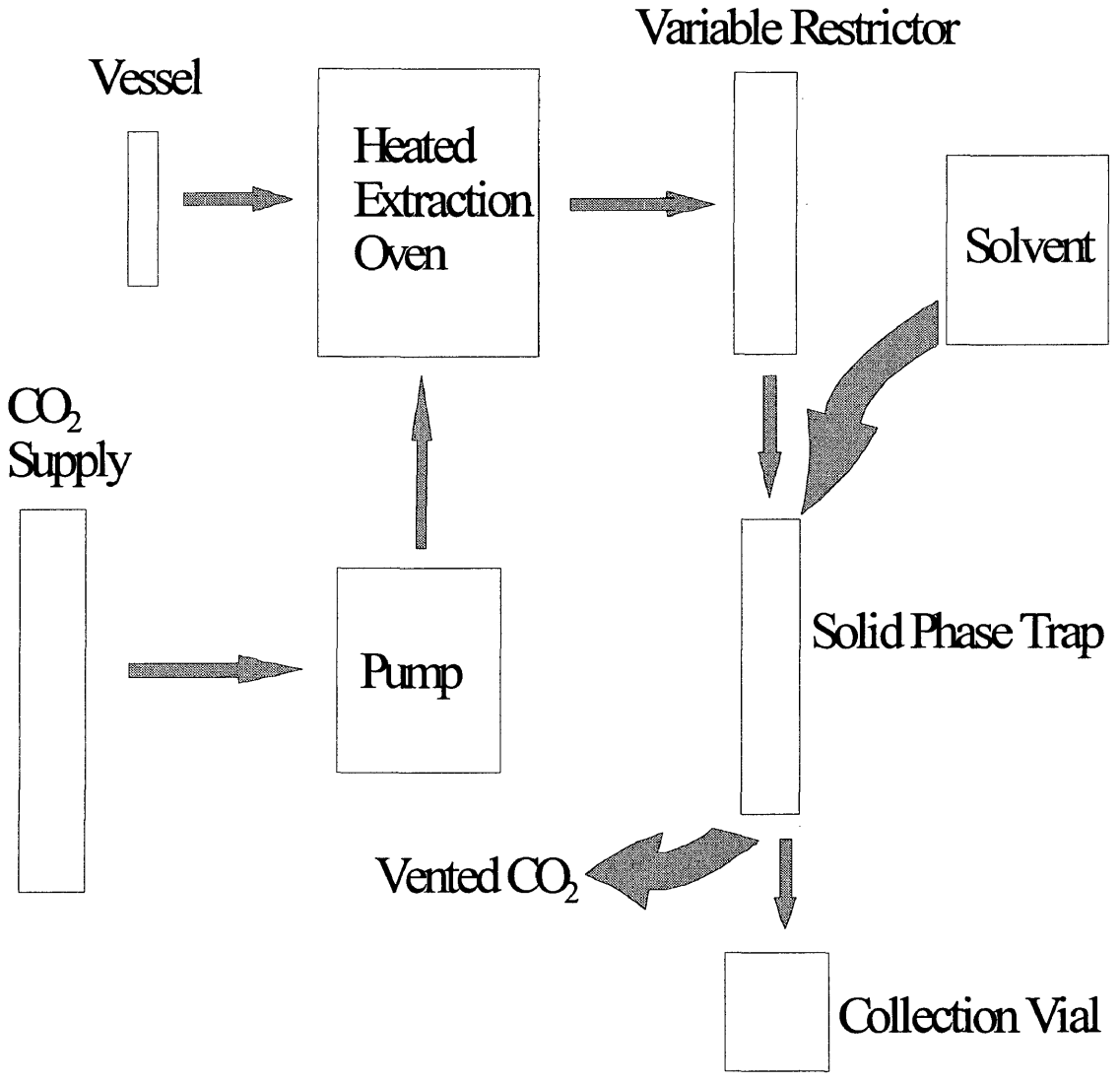
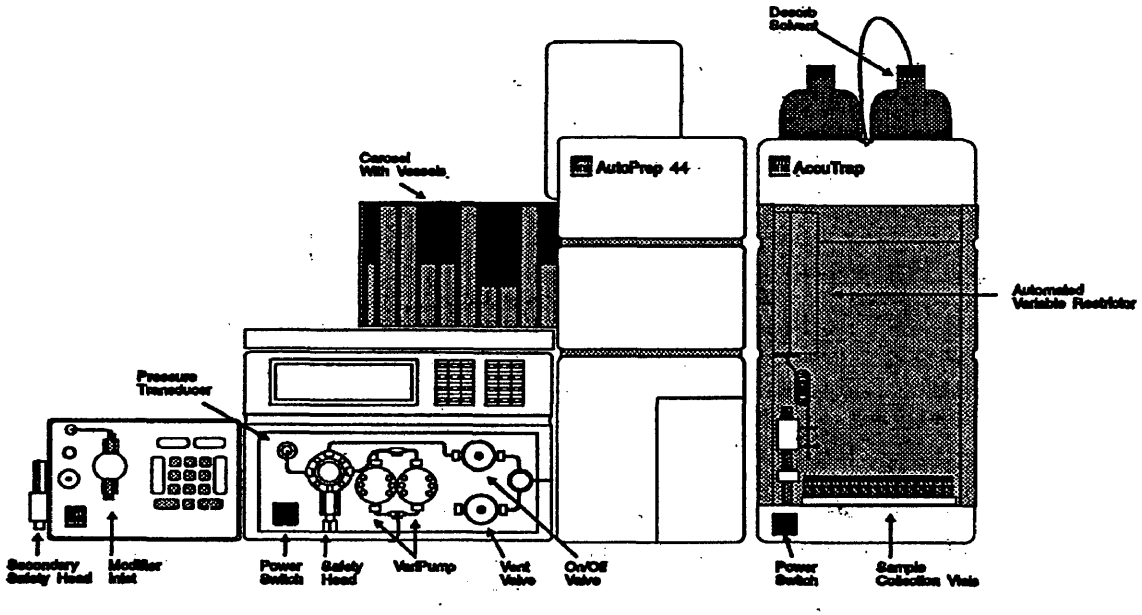


Figure 4. *Schematic of the Isco-Suprex AutoPrep44 SFE unit.*



Effect of Temperature/Pressure on PCB Yield

To examine the effects of temperature and pressure on SFE of PCBs, tissue VRMs were employed. Aliquots of freeze-dried fish tissue (ca. 1gm dry weight) were loaded into 10 ml stainless steel extraction vessels. The exit end of the vessels were then filled with approximately 6 gm of 150 mesh, neutral alumina oxide (Al_2O_3; Aldrich Chemical, Milwaukee, WI) for on-line retention of co-extracted lipids (Hale and Gaylor, 1995). The alumina was activated overnight by heating at 130°C. Prior to use, the alumina was allowed to cool in a desiccator to room temperature. The alumina required

no deactivation before use. Extraction vessels containing alumina blanks were interspaced with tissue VRMs to assess reagent purity and possible cross-contamination between samples. No interfering compounds were detected greater than 1% of the concentration of the target analytes. A PCB by-product standard containing IUPAC congeners 1, 3, 7, 30, 50, 97, 143, 183, 202, 207 and 209 (Ultra Scientific, Kingstown, RI) was added to the dried tissue and alumina blanks during optimization studies to assess matrix effects and the effects of increasing chlorine content and molecular mass on extraction efficiency. In addition to serving as procedural blanks, extraction efficiency of PCBs from alumina blanks was monitored. The by-product congeners are absent from common Aroclor formulations (Schultz et al., 1989) and proved ideal in this study for spiking directly onto tissue VRMs containing field-incurred PCB congeners.

Preliminary observations suggested PCB congeners could be extracted from freeze-dried fish tissue samples using a combined 10 minute static and 30 minute dynamic extraction step at 150°C and 350 atm (ρ = 0.56 g/ml). During dynamic extraction, a liquid flow rate of 3 ml/min (measured at the pump) of unmodified supercritical CO_2 (SFE-SFC grade, MG Industries, Richmond, CA) provided good recoveries of surrogate (IUPAC PCB congeners 30, 65 and 204) and native PCBs from these samples (Hale and Gaylor, 1995). It was also observed that a 10 minute static extraction step at 150°C was needed to allow thermal equilibration between the sample and the outer surface of the stainless steel extraction vessels. The variable restrictor was maintained at 100°C throughout the study to prevent plugging due to lipid/water co-

extraction and freezing attributable to Joule-Thompson cooling. These conditions were used as starting points around which extraction temperature and pressure could be varied to determine optimum settings for SFE of amended PCBs from tissue VRMs. Three temperatures (50, 100 and 150°C) at four pressure settings (100, 200, 300 and 400 atm) were examined in triplicate. Recoveries were summed at each set of SFE conditions to obtain an extraction profile for total PCBs.

Effect of Extraction Duration (Kinetics) on PCB Yield

Once SFE temperature and pressure parameters were established, the kinetics of the SFE process were examined to determine the impact of extraction time on extraction of amended PCBs from tissue VRMs. Aliquots of freeze-dried tissue VRM were prepared and extracted using SFE conditions established above. Extractions were performed in triplicate for 5, 10, 15, 20 and 30 minutes. PCBs were collected on a solid-phase trap containing a silica adsorbent mixture (see trap optimization section for details).

Effect of Supercritical CO_2 Flow Rate on PCB Yield

An important parameter when developing SFE methodologies is SF flow rate through the extraction cell. Here, the effect of CO_2 flow rate was evaluated by systematically varying this parameter during the extraction. Aliquots of freeze-dried tissue VRM were prepared and extracted using established SFE conditions. Extractions

were performed in triplicate at 0.5, 1.0, 2.0 and 3.0 ml/min. PCBs were trapped on a solid-phase trap as before (see trap optimization section). To assess the true importance of fluid flow rate on the SFE process, a study was conducted to determine whether CO_2 flow rate or number of vessel volumes swept by the SF controlled the extraction efficiency of PCBs from biota samples. Two sets of extractions were performed in triplicate: 1) a 30 minute extraction at 3 ml/min flow rate (90 ml total CO_2); and 2) a 90 minute extraction at 1 ml/min flow rate (90 ml total CO_2). To assess whether observed trends were consistent for SFE of real-world analytes, the same conditions were used to extract native PCBs from tissue VRMs. PCBs 52, 95, 110, 118, 153/132, 138/158 and 180 were selected and quantitated as representative analytes due to their prevalence in many environmental samples (Lang, 1992). The automated design of the SFE restricted the number of possible extraction vessel sizes usable. All vessels had the same diameters, but differed in length and thus volume. The largest vessels (10 ml) provided the greatest sample capacity. In order to maximize the amount of alumina needed for on-line lipid retention, these vessels were used in all subsequent PCB work.

Effect of Solvent Modifier on PCB Yield

Solvent modifiers are typically required to improve SFE efficiency of more polar organic contaminants (e.g. PAH) from environmental samples. As PCBs are non-polar, it was believed, based upon preliminary PCB recovery data, that organic solvent modifiers would not be needed to achieve quantitative recoveries of PCBs. To confirm this,

addition of methanol was investigated. Tissue VRMs were prepared and extracted using established SFE conditions. 10% (v/v) methanol was added directly to the SF CO_2 stream during the dynamic extraction phase using the AP44's external modifier pump. PCBs were trapped as before (see trap optimization).

Effect of Solid-Phase Trapping on PCB Yield

Several parameters were investigated: trapping material, trapping temperature, elution solvent and volume and elution temperature. Two types of trapping materials were examined: 1) 100-120 mesh silanized glass beads (Alltech, Deerfield, IL); and 2) a 1:1 (w/w) mixture of 20-30μm C_{18}-modified silica (Aldrich Chemical) and 80-100 mesh Unibeads 2S (Alltech). A 3:1 (w/w) mixture of C_{18}-modified silica and silanized glass beads also showed promise for quantitative PCB trapping, as previously demonstrated by Ashraf-Khorassani et al. (1996), but was not examined rigorously here. However, substitution of this mixture in the trap during several routine PCB extractions provided quantitative retention of surrogate PCB standards. Different trapping temperatures and elution regimes were also examined. Subsequent to these findings, a study of collection and elution temperature efficiency was conducted to determine optimum settings. Here, the PCB by-product standard was spiked onto a tissue VRM and extracted using established SFE conditions. The collection temperatures examined were 0, 30, 60 and 90°C. The importance of desorption and solvent temperature were assessed simultaneously. Surrogate congeners 30, 65 and 204 were spiked directly onto the

trapping material and the carrier solvent (hexane) allowed to dry. PCBs were then desorbed manually with 2 ml benzene (during preliminary trials) or isooctane, over a range of temperatures, to arrive at an optimum desorption temperature. After establishing a working desorption temperature, surrogate congeners were spiked onto tissue VRMs, extracted, trapped and desorbed at this temperature to confirm this value.

Comparison of SFE and Soxhlet Extraction

To assess the efficacy of the optimized method, PCB yields by the SFE of native PCBs from a tissue VRM (goldfish homogenate) were compared to those obtained using Soxhlet extraction. The tissue VRM was prepared as previously described and spiked with PCB surrogate congeners 30, 65 and 204. Aliquots (ca. 5 gm dry weight for Soxhlet extraction and 1 gm dry weight for SFE) were extracted using previously established conditions for both techniques.

SFE Method Validation: Application to a Certified Reference Material

To validate the SFE method more rigorously, carp homogenates (*Cyprinus carpia*, Certified Reference Material (CRM) Carp-1, National Research Council of Canada) were obtained and analyzed for PCBs using the SFE methodology. The results were compared to those obtained during a recent multi-laboratory intercomparison exercise overseen by the NIST (NIST/NOAA, NS&T/EPA EMAP, 1993). The carp homogenate was prepared and extracted using previously established SFE conditions.

SFE Method Robustness: Application to PCB Determinations in a Large Field Sample Set and Results of Analyses

Upon completion of the method development and validation phase of the study, it was desirable to establish the robustness of the method on a large data set. To accomplish this, the newly-developed SFE method was applied to the determination of field-incurred PCB congeners in 644 edible fish tissue samples collected from fresh water river systems in Virginia. Fish samples were collected by the Virginia Department of Environmental Quality (DEQ). Samples were prepared and extracted using established SFE conditions. In order to continuously monitor method accuracy and precision, samples were spiked with PCB surrogates 30, 65 and 204. Recoveries were assessed throughout the study to identify potential problems that might occur during routine sample processing. Incurred PCB concentrations and distributions over the entire data set were examined.

Development of SFE For Tissue Total Lipid Determinations: Comparison of SFE, Soxhlet Extraction and Column Elution

Total tissue lipid determinations are typically performed as part of most lipohillic contaminant extraction strategies to assess bioaccumulation potential. To accomplish this, a SFE-based method was developed that would allow accurate concomitant total lipid determinations in the 644 fish tissues samples collected for PCB determinations.

Aliquots (ca. 1 gm dry weight) of tissue VRM were extracted in 3 ml vessels at several CO_2 flow rates to determine optimal yields. No alumina was added to the vessel prior to SFE. The extraction vessels were weighed prior to extraction and again periodically thereafter, until a constant weight was achieved. Total extracted lipid was determined gravimetrically by the difference in weight of the vessel plus sample before and after extraction. Lipid extractions were performed using a sequential 10 minute static and 30 minute dynamic extraction at 150°C and 450 atm (ρ = 0.65 g/ml). No organic solvents were required.

SFE results were compared to two other common methods for total lipid determination. These were: 1) column elution, in which 1gm of lyophilized tissue was packed into a glass column and eluted with organic solvents. Both extraction with methylene chloride alone (10 ml) and methylene chloride (10 ml) followed by methanol (10 ml) were examined; and 2) Soxhlet extraction for 24 hours using 400 ml of methylene chloride. Total lipid contents were determined in both approaches by collecting the solubilized material and evaporating the extraction solvent to constant weight in tared pans at room temperature. Historically, the Soxhlet extraction approach has been convenient for total lipid analyses, as it is a starting point for many procedures for the determination of non-polar, lipophilic organic pollutants.

SFE Method Robustness: Application To Total Lipid Determinations in a Large Field Sample Set

Upon completion of the development and validation phase for lipid determination,

as with PCB extractions, it was desirable to establish the robustness of the SFE method over a large data set. To accomplish this, the newly-developed SFE method was applied to the determination of tissue total lipids in the 644 edible fish tissue samples collected previously and analyzed for PCBs. Method robustness was indicated by successful, sequential extraction of all 644 samples without restrictor plugging or other instrument component failure.

Correlation of PCB Concentrations With % Total Lipids

To determine whether lipid normalization of PCB concentrations in sub-samples (e.g. edible fillets) of fish tissue is a valid approach in understanding organic contaminant bioaccumulation, total PCB data from selected sampling sites were correlated with % total lipids. A freshwater riverine site (Clover, VA) and a fresh water lake site (Smith Mountain Lake, VA) were selected for comparison.

GC-ELCD Analysis

After addition of an internal quantitation standard (PCB 207, 209 or pentachlorobenzene), all SFE and purified Soxhlet extracts were analyzed on a Model 3400 gas chromatograph (Varian, Walnut Creek, CA), equipped with a 60m x 0.25mm i.d. DB-5 (J&W Scientific,) fused silica capillary column (25μm film thickness) and an OIC Model 4420 electrolytic conductivity detector (ELCD; Columbia, MO). The ELCD

was maintained at 850°C. Helium carrier gas flow rate was 1 ml/min. Injections (1μL) were made in the splitless mode (injector split vent opened after 2 minutes) by a Model 8100 autosampler (Varian). The injector was maintained at 300°C. The column temperature was held at 90°C for 2 minutes, programmed at 4°C/min to 320°C and held at that temperature for 10 minutes. Identification of PCB congeners was made using a halogen retention index (HRI; Mothershead et al., 1991).

Quantitation was accomplished with the use of relative response factors. These were obtained by comparison of the response of the internal standard to those of representative PCB congeners of varying degrees of chlorination. PCB standards were injected daily to verify GC system response and to confirm or recalculate response factors. Corrections were not made for recovery of surrogate PCB standards. The following PCB congeners were not completely resolved under the above GC conditions: 8/5, 17/8, 28/31, 90/101, 153/132, 138/158, 170/190, 195/208 and 196/203. Therefore, concentrations for these congeners are reported as sums of each pair of unresolved congeners.

MS Confirmation

Where required, analyte identity was confirmed using GC coupled to a negative chemical ionization mass spectrometer (GC/NCI-MS; Extrel, Pittsburgh, PA) via a heated transfer line. Methane was used as the moderator gas and the ion source temperature was maintained at 100°C under a constant 700 mtorr vacuum. PCB congeners were separated

and introduced to the MS with a Model 663 gas chromatograph (Hitachi, San Jose, CA). Chromatographic conditions were similar to those described for the GC/ELCD analysis.

Quality Assurance and Control (QA/QC)

Several quality control elements were monitored throughout the study. Blank matrices were interspaced between real samples to assess analyte carry over and laboratory contamination. Surrogate recoveries were monitored continuously in all samples. Spiked blanks (usually alumina oxide or sand) were also used to establish initial analyte solubility under a given set of extraction conditions. This approach further ensured that quantitative recoveries of surrogate compounds could be obtained in the absence of matrix effects before proceeding to "real-world" samples. Samples, blanks, VRMs and CRMs were extracted in replicate periodically during the study to establish accuracy and precision of the method. PCB standards containing congeners representing all degrees of chlorine substitution (e.g. mono-deca) at known concentrations were injected daily to verify GC-ELCD and GC/NCI-MS system response.

RESULTS

Sample Preparation: Lyophilization vs. Chemical Desiccation

Samples were dried initially via chemical desiccation. Wet samples were homogenized and mixed with either diatomaceous earth (1:1 w/w; Hydromatrix) or anhydrous sodium sulfate (1:10 w/w). Both approaches failed repeatedly. While it was possible to obtain a sample with a manageable powder-like consistency that appeared visually dry, when subjected to SFE, water was released from the sample and often plugged the restrictor and/or trap. This compromised PCB recoveries and water ultimately appeared in the final solvent extract. In contrast, sample lyophilization proved to be significantly more effective than addition of chemical desiccants. Freeze drying tissues also conserved considerable vessel volume by eliminating the need for inclusion of bulky desiccants.

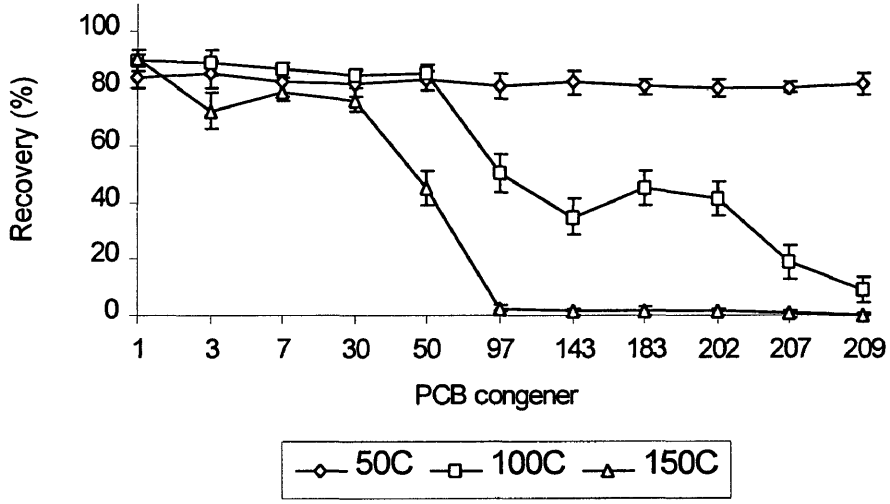
Extraction Temperature/Pressure

During initial method development on the PrepMaster unit, an initial 10 minute static equilibration period, followed by a 30 minute dynamic supercritical CO_2 extraction at 3 ml/min liquid flow, was sufficient to remove more than 99% of the PCBs available at 350 atm and 150°C (ρ = 0.56 g/ml). This was demonstrated by sequential re-extraction of fish tissues. Further method development on the AutoPrep 44 unit revealed that this

temperature and pressure regime provided the highest recoveries of surrogate and native PCB congeners from the tissues examined. Verification of these trends and assessment of the effects of increasing chlorine content and molecular mass were done by extracting tissue VRMs amended with a mixture of PCBs (PCB by-product standard) spanning the entire range of chlorine substitution.

It can be seen from the figures following (Figures 5-9) that the pressure/temperature regime of 350 atm/150°C was sufficient for quantitative removal of a mixture of PCB congeners spiked onto lyophilized fish tissue. At 100 atm no PCB extraction was apparent, regardless of temperature (50, 100 or 150°C; data not shown).

Figure 5. *Effects of SFE temperature (°C) at constant pressure (200 atm) on the extraction efficiency of amended PCB congeners from fish VRM (n = 3).*



When pressure was increased to 200 atm, PCB recoveries increased at all temperatures, particularly at 50°C. Recoveries were inversely related to temperature at 200 atm (Figure

5). A pronounced decrease in extraction efficiency was observed for PCBs with 4 or more chlorines at 200 atm and 100 and 150°C. At 300 atm, total PCB recoveries were nearly complete at all temperatures. On a congener-specific basis, maximum recoveries were realized by increasing pressure to 350 atm at 150°C (Figure 6). While total PCB recoveries increased when pressure was increased from 200 to 300 atm or 400 atm at 100 and 150°C, recoveries decreased modestly at 400 atm and 50°C. Also, at 400 atm, congener-specific recoveries were variable, regardless of temperature (Figure 7). Similar patterns emerged when a PCB mixture was extracted from a crab tissue VRM (Figure 8).

At 50°C, maximum total PCB recoveries were reached at 200 atm. At 100 and 150°C, a minimum pressure of 300 atm was required for complete PCB extraction. Total PCB recoveries from crab tissue remained essentially constant from 300 to 400 atm at 100 and 150°C (Figure 8). A decrease in total PCB recoveries was observed at 400 atm and 50°C.

Figure 6. *Effects of SFE temperature (°C) at constant pressure (300 atm) on the extraction efficiency of amended PCB congeners from fish VRM (n = 3). Extraction conditions considered optimum are indicated.*

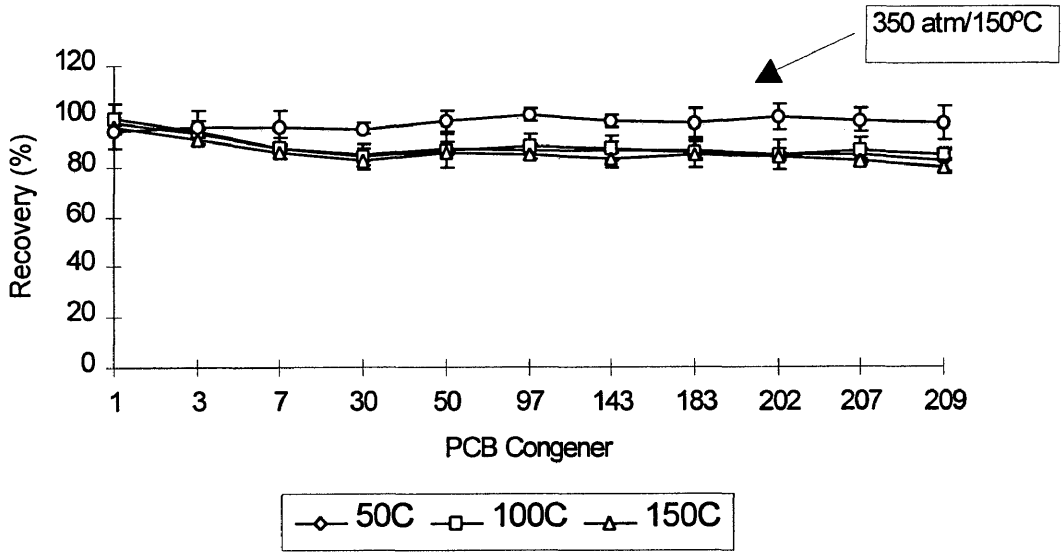
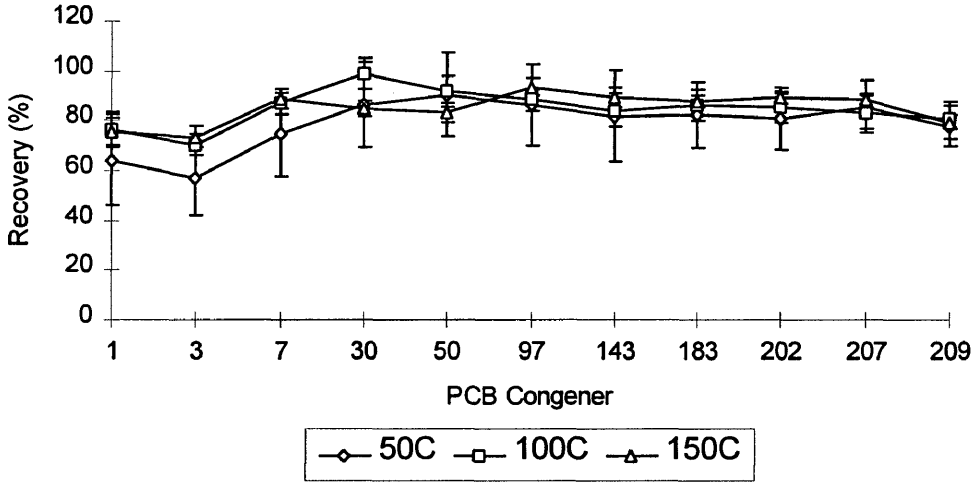


Figure 7. *Effects of SFE temperature (°C) at constant pressure (400 atm) on extraction efficiency of PCB congeners from fish VRM (n = 3).*



Total PCB extraction yields from alumina blanks (Figure 9) approximated those of PCB-amended crab tissue VRM (Figure 8). Monitoring PCB recoveries from blanks further allowed comparison of analyte-matrix interactions between laboratory adsorbents and real-world tissue samples. Interestingly, slightly higher amounts of total PCBs were obtained from alumina at 200 atm and 50 and 100°C (Figures 8 and 9). This trend is likely a result of stronger PCB binding to the surface of the tissue matrix, compared with the more polar alumina surface (matrix effect). Also interesting was the greater total PCB yields realized when extracting the crab VRM compared with alumina (Figures 8 and 9). Similar results were observed during PCB surrogate trapping optimization studies (see trap optimization results). All experiments were conducted with a dynamic liquid CO_2 flow rate of 3 ml/min. The restrictor was maintained at 100°C. PCBs were trapped on a 1:1 (w/w) mixture of C_{18}/Unibeads at 0°C and were desorbed at 90°C with 2 ml of

isooctane (1 ml/min desorption rate). Sample weights (1gm dry weight) and PCB spike concentrations (1μg/g) were kept constant throughout each experiment.

Figure 8. *SFE of total amended PCB congeners from crab VRM at different SFE pressure/temperature regimes (n = 3).*

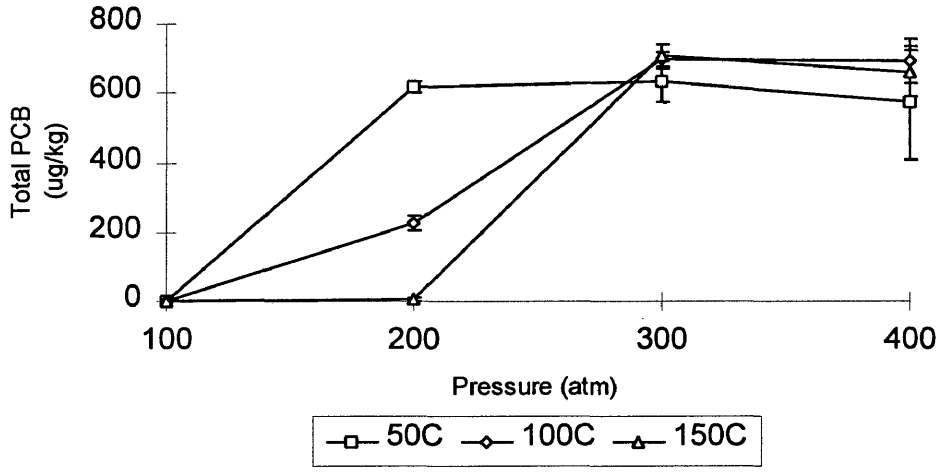
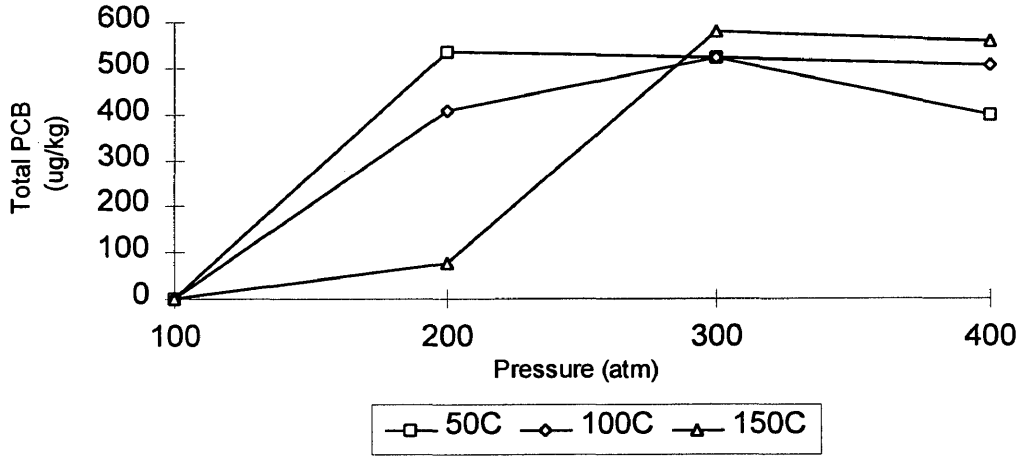


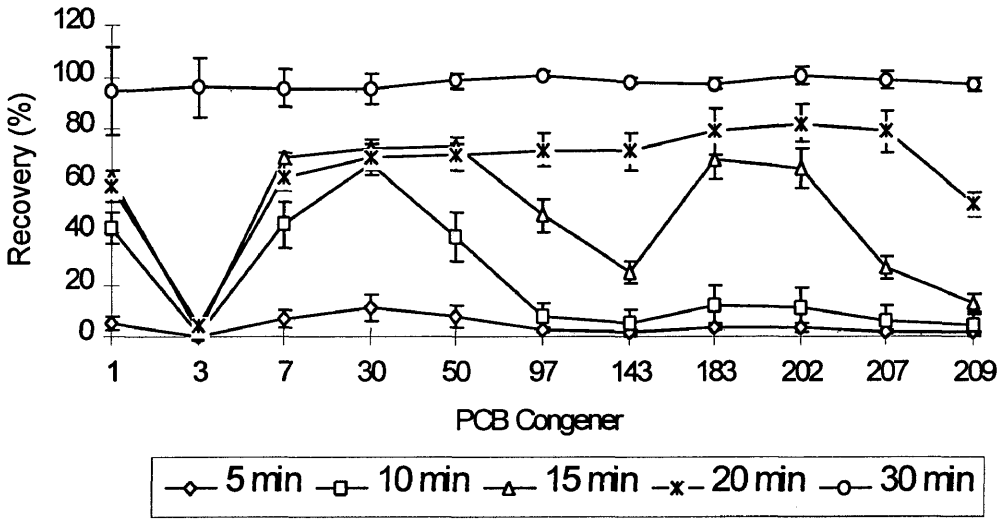
Figure 9. *SFE of total amended PCBs from alumina blanks at different temperature and pressure combinations. Data represent PCB recoveries from alumina QA/QC blanks interspaced between tissue samples during SFE (n = 1).*



SFE Kinetics

As noted previously, quantitative extraction was complete in 30 minutes. The general extraction kinetics profile (Figure 10) for a suite of PCB congeners was similar to that reported by other researchers (Hawthorne et al., 1993; Langenfeld et al., 1995). It is interesting to note the peculiar behavior of PCB 3 (4-chlorobiphenyl) during these experiments. This trend was not apparent with PCB 1 (2-chlorbiphenyl).

Figure 10. *Effects of extraction duration (kinetics) on SFE of amended PCB congeners from the crab VRM (n = 3).*

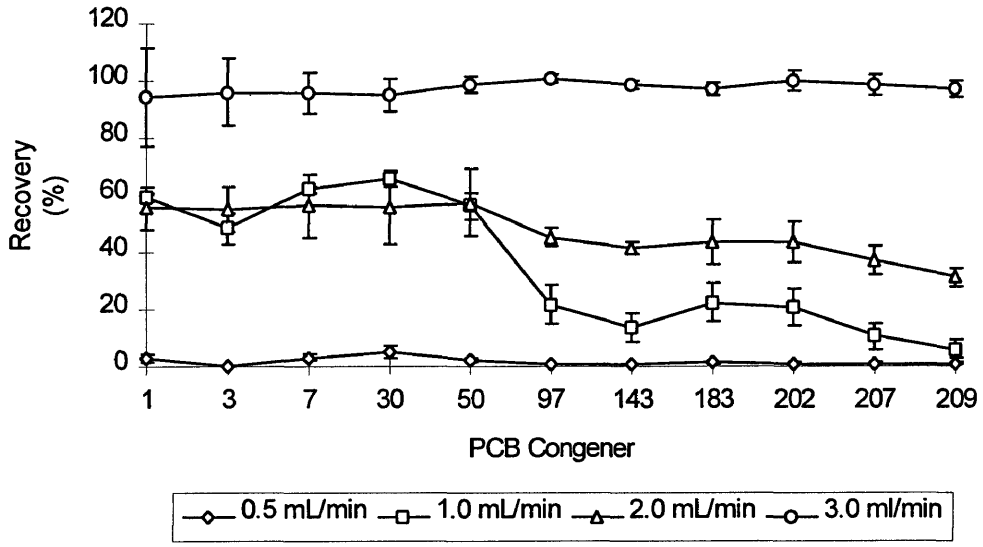


SF Flow Rate

Initial observations indicated that a 30 minute dynamic extraction at a liquid CO_2 flow rate of 3 ml/min was sufficient to achieve quantitative extraction of PCBs from tissue samples. This was confirmed during the SFE kinetic study. Therefore, 30 minutes was chosen as the benchmark extraction time for subsequent work. Recoveries of

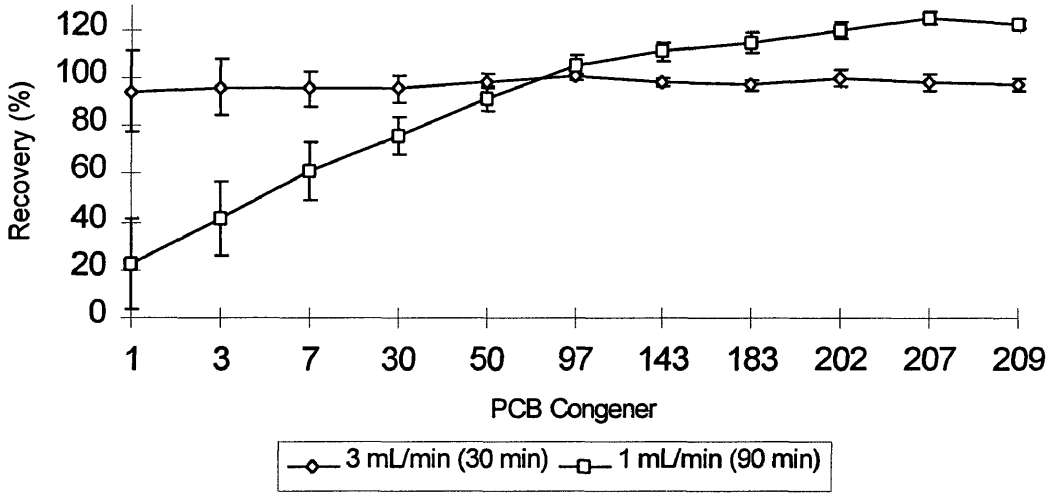
amended PCBs were lower at the lower CO_2 flow rates, particularly for the more chlorinated congeners (a breakpoint appeared between the tri- and tetrachlorobiphenyls; e.g. congeners 50 and 97), ranging from practically non-detectable amounts at 0.5 ml/min (1.5 vessel volumes) to quantitative extraction at 3.0 ml/min (9 vessel volumes; Figure 11). A similar trend was apparent in the temperature/pressure data (200 atm; Figure 5).

Figure 11. *CO_2 flow rate effects on SFE of amended PCB congeners from the fish VRM ($n = 3$).*



Dynamic extractions were performed for 30 minutes at 3 ml/min and 90 minutes at 1 ml/min. Thus, the same total volume of fluid was introduced to the sample during the extraction. The results indicate that flow rate is less important than the total fluid volume in quantitatively extracting amended PCB congeners from fish tissue (Figure 12).

Figure 12. *Comparison of CO_2 flow rate effects on SFE of amended PCB congeners from the fish VRM. Extractions were normalized for volume of SF CO_2 used (90 ml or 9 vessel volumes; n = 3).*

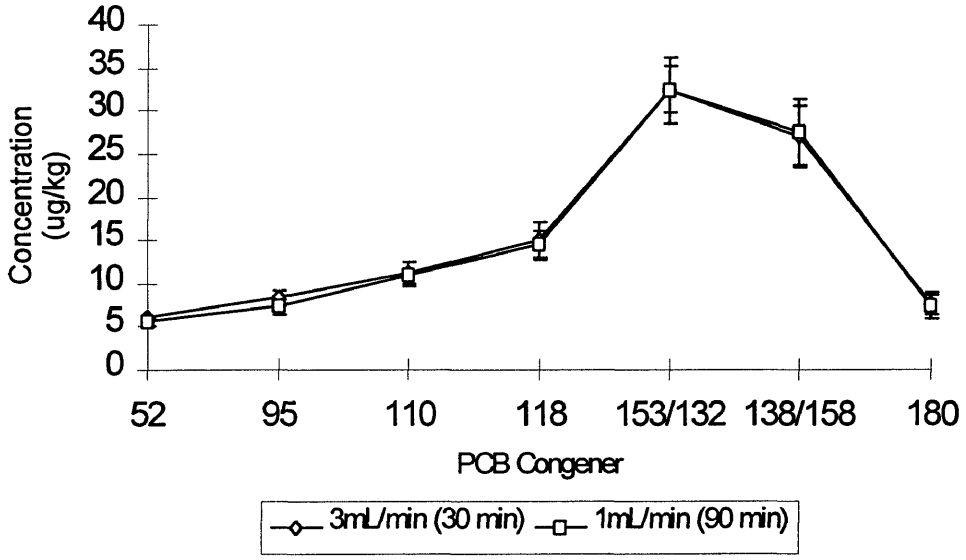


It is interesting to note the dramatic decrease in recovery and elevated standard deviations of PCBs 1, 3 and 7 (mono-and dichlorobiphenyls) and the apparent increase in recoveries of the more chlorinated congeners (e.g. 5 or more chlorines) during the 90 minute dynamic extraction. It is likely the lower chlorinated congeners were stripped from the solid phase trap during prolonged exposure to the decompressing SF stream during the 90 minute dynamic extraction. Increased recoveries of more chlorinated congeners during the 90 minute dynamic extraction suggest that longer extraction periods may be better for these congeners. It is also possible that elevated recoveries were due to quantitation errors. Regardless, these data demonstrate the inherent difficulty of trapping volatile chemical species using SFE with solid-phase analyte collection (see solid-phase

trapping results).

To assess the effects of CO_2 flow rate on field-incurred analytes, major native PCB congeners were extracted and quantified also. It can be seen (Figure 13) that recoveries are virtually identical when performing the extraction for 30 minutes at 3 ml/min or 90 minutes at 1 ml/min. It is also interesting to note the small standard deviations for the field-incurred PCBs. The data suggest that prolonged exposure to the decompressing SF stream is less important for PCB congeners substituted with four or more chlorine atoms.

Figure 13. *Comparison of the effect of CO_2 flow rate on SFE of native PCB congeners from the fish VRM. Selected congeners are major constituents of common Aroclor formulations. Extractions were normalized for volume of SF CO_2 used (90 ml or 9 vessel volumes; n = 3).*



Solvent Modifiers

Use of methanol as a CO_2 modifier failed to improve PCB recoveries from tissue VRMs. A combined 10 minute static and 30 minute dynamic extraction phase at 350 atm/150°C with unmodified CO_2 provided excellent recoveries of amended and native PCB congeners from all tissues examined.

Solid-Phase Trapping

SFE solid-phase trapping, as well as trap collection and elution temperatures, affected the recovery and reproducibility of surrogate PCB congeners from both alumina and fish tissues. Table 3 provides representative data for three trapping conditions established during preliminary development on the AutoPrep unit. Lowering the trap temperature to -30°C and eluting with isooctane from C_{18}/Unibeads provided the best surrogate PCB recoveries from blank alumina. Precision of surrogate PCB recoveries alone, as measured by percent relative standard deviation (%RSD) were approximately 20% under both trapping conditions with glass beads alone. Values represented include not only variations associated with the extraction step, but also those related to subsequent extract handling and GC analysis. Interestingly, mean recoveries of surrogate PCBs 30 and 65 from amended fish tissues were higher than from alumina alone under all trapping conditions (also shown in Table 3). When glass beads were used for trapping, mean PCB 204 recoveries from fish tissue were lower than from alumina alone. Use of C_{18}/Unibeads, lowering the collection temperature to -30°C and rinsing with isooctane at

an elevated temperature generally improved amended PCB recoveries from fish tissues. Mean recoveries under these conditions were excellent: 95.6, 92.0 and 102% for PCBs 30, 65 and 204, respectively. Precision of measurements also improved. %RSD dropped to 3.56, 4.13 and 6.56%, respectively.

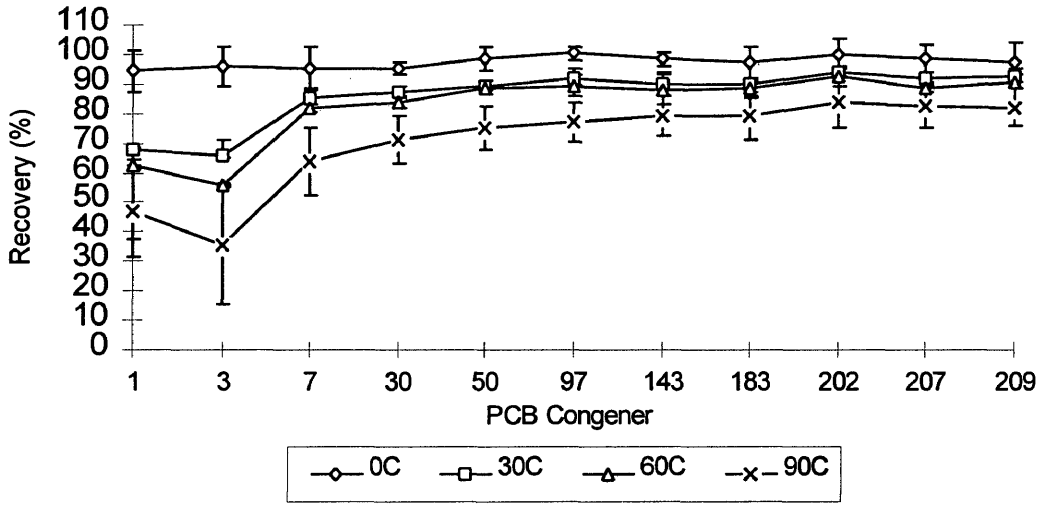
Table 3. *Effect of different trapping material, temperature and elution solvent/temperature regimes on the mean recoveries and precision of three surrogate PCB congeners from amended alumina and fish tissue (n=10). Values are expressed as a percent of PCB added to each matrix. %RSD = % relative standard deviation. 2 ml of solvent was used in each case.*

Material	**Glass Beads**	**Glass Beads**	**C_{18}/Unibeads**
Trap Temp (°C)	**20**	**-10**	**-30**
Elution Solvent	**Benzene**	**Isooctane**	**Isooctane**
Elution Temp (°C)	**40**	**80**	**90**
Alumina	**Mean (%RSD)**	**Mean (%RSD)**	**Mean (%RSD)**
PCB 30	65.6 (22.7)	71.3 (18.0)	89.2 (9.2)
PCB 65	69.8 (21.5)	73.3 (22.6)	84.4 (15.6)
PCB 204	79.2 (20.0)	81.5 (19.6)	97.3 (13.2)
Fish VRM	**Mean (%RSD)**	**Mean (%RSD)**	**Mean (%RSD)**
PCB 30	71.7 (8.1)	89.6 (3.9)	95.6 (3.6)
PCB 65	80.2 (23.8)	97.1 (6.2)	92.0 (4.1)
PCB 204	35.4 (29.4)	77.2 (16.3)	102 (6.6)

After assessing the impact of trapping/elution temperature and solvent regimes on recoveries of the three surrogate PCBs, the effects of these parameters on the recoveries of the PCB by-product mixture from a tissue VRM was examined to confirm that these conditions were optimum for all PCB homologues. The data indicated that excellent PCB recoveries can be achieved at a higher trapping temperature (e.g. 0°C). Trapping efficiency was examined at three other temperatures as well (30, 60 and 90°C; Figure 14).

The practical consequences of this finding were reduced: 1) coolant CO_2 required per sample; and 2) freezing of sample/atmospheric moisture in the trap during extraction. In light of these data, all subsequent SFE trapping was performed at 0°C. Mono- and dichlorobiphenyls were not trapped efficiently at any temperature other than 0°C. A curious result, however, is the unusual capacity of the C_{18}/Unibeads mixture to trap PCBs in relatively good yields even at the elevated temperatures. Higher chlorinated congeners were trapped more efficiently at all temperatures.

Figure 14. *Temperature (°C) effects on trapping efficiency of amended PCBs extracted from the crab VRM using SFE (n = 3).*

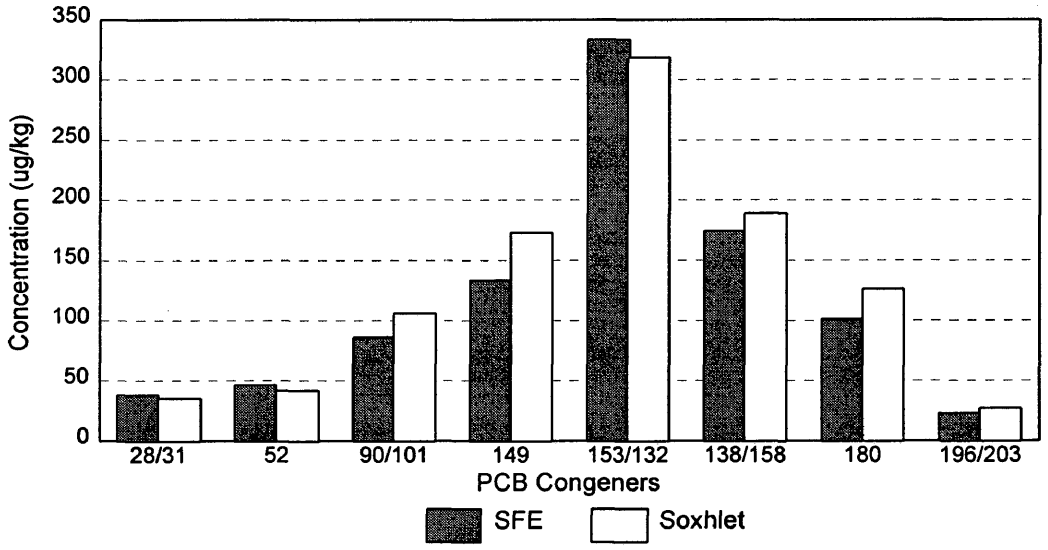


Comparison of SFE and Soxhlet

Preliminary validation of the entire method was accomplished initially by comparing the recoveries of selected PCB congeners from a goldfish tissue VRM using SFE and the widely-accepted Soxhlet approach. Mean recoveries from Soxhlet and SFE-

based methods for eight field-incurred PCB congeners present in fish were not significantly different (paired t-test, 0.05 level). A trend was apparent in that mean recoveries for lower chlorinated biphenyls (e.g. 28/31 and 52) with the SFE-based methodology were slightly higher than those determined using Soxhlet extraction (Figure 15). Conversely, mean concentrations of the more chlorinated biphenyls (e.g. 138/158, 180 and 196/203) appeared somewhat higher with Soxhlet extraction. Precision of the two methods, as determined by %RSD, were both approximately 10%.

Figure 15. *Comparison of mean concentrations (wet weight basis) of representative field-incurred PCBs, representing trichloro- through octachlorobiphenyls, determined in goldfish VRM using Soxhlet extraction and SFE (n = 3).*



SFE Method Validation

Final validation of the method was achieved by application of SFE to the extraction of field-incurred PCB congeners from a candidate fish tissue CRM. Results

were compared to those obtained from a recent multi-laboratory intercomparison exercise. Mean PCB recoveries obtained with the SFE method were not significantly different (paired t-test, 0.05 level) from the multi-laboratory consensus results, as a function of analyte and after elimination of statistical outliers by NIST, or from those obtained by NIST alone (Figure 16). PCB concentration values are expressed on a wet weight basis. The %RSDs for the consensus results were higher than those obtained with the SFE method and NIST itself. However, the consensus data incorporate interlaboratory differences. Extraction methods used by the labs participating in the comparison exercise included Soxhlet, sonication and column elution. None of the labs reported the use of SFE. PCB concentrations are included in Table 4 for comparison of %RSDS among the different laboratories and analytical techniques.

Figure 16. *Comparison of PCB yields in fish CRM by SFE (n = 3), NIST (n = 3) and consensus analysis (n = 16-21). Mean values are in µg/kg (wet weight).*

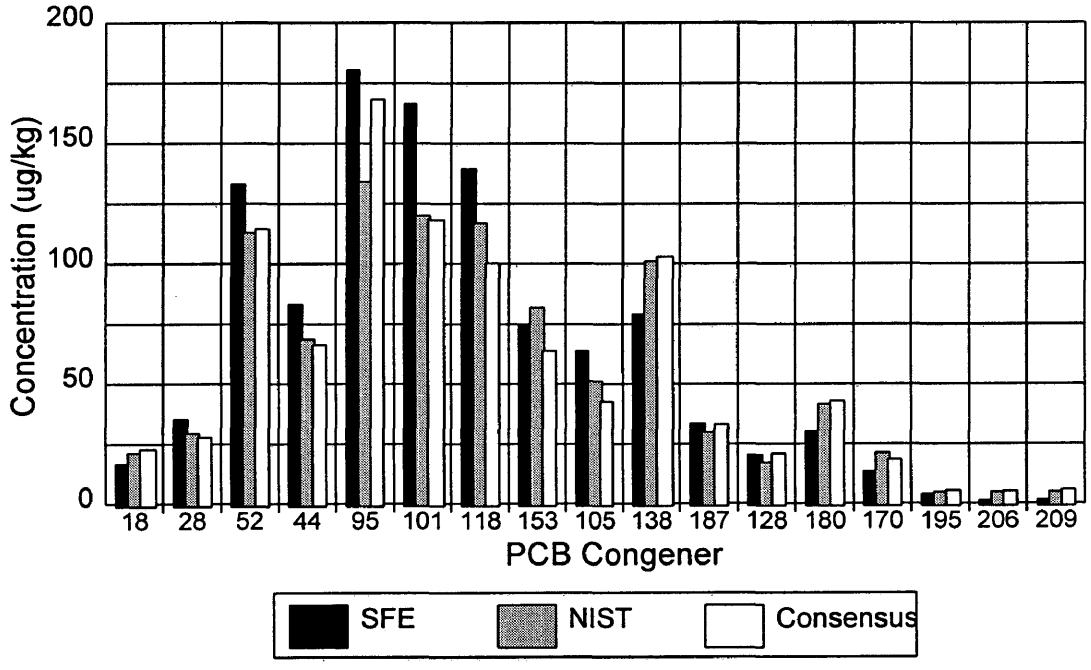


Table 4. *Comparison of PCB yields from a fish CRM using SFE with those obtained by NIST alone and by 16 different laboratories participating in an intercomparison exercise (consensus). Mean values are given in μg/kg (wet weight basis). %RSD = % relative standard deviation.*

	SFE		NIST		CONSENSUS	
PCB	mean	%RSD	mean	%RSD	mean	%RSD
17/18	16.7	6.41	22.7	10.7	21.3	23
28/31	35.3	8.1	27.7	18.4	29.3	28
52	133	8.67	115	8.91	113	28.3
44	83.1	9.69	66.3	7.15	68.4	26.9
95	180	8.52	168	3.96	134	30.6
90/101	166	10.3	118	5.63	120	15.8
118	139	12.2	100	4.07	117	27.4
153/132	74.4	11	63.7	4.27	81.8	25.2
105	63.6	13.3	42.5	4.13	51	30.8
138/158	78.9	14.3	103	6.37	101	25.7
187	33.3	13.7	32.9	12.8	29.6	25.7
128	20.4	16.3	20.7	10.1	16.9	29
180	30.1	14.6	42.9	7	41.6	26.4
170/190	13.3	16.2	18.5	16.5	21.1	27
195/208	3.92	25.4	5.26	9.1	4.5	31.1
206	1.15	9.41	4.97	3.44	4.6	30.4
209	1.4	2.06	5.54	6.05	4.7	27.7
TOTAL	1070		958		955	

Lipid contents of the goldfish and candidate CRM carp homogenates were determined by gravimetric measurement of extracts obtained from Soxhlet extraction with methylene chloride. Extractable lipids were high in these whole fish homogenates, 24% and 40%, respectively. SFE extracts obtained from these samples contained less than 0.1% extractable lipid, demonstrating the efficacy of alumina as an in-line adsorbent for retention of co-extracted lipids during the SFE process.

SFE Method Robustness: Application to PCB Determinations in a Large Field Sample Set and Results of Analyses

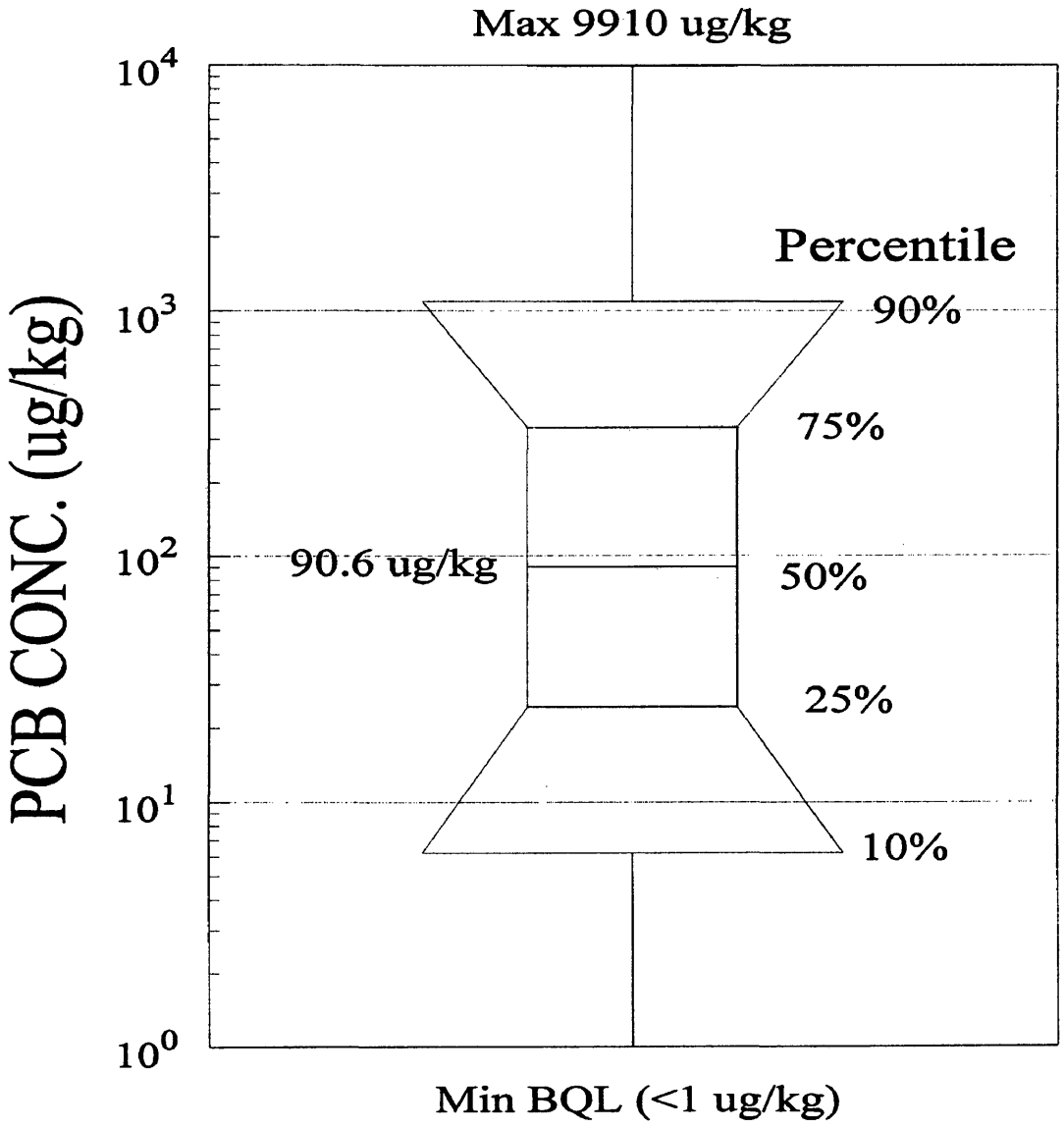
To assess robustness, the SFE method was applied to the determination of field-incurred PCB congeners in 644 edible fish tissue samples collected from fresh water systems in Virginia. Accuracy and precision of the method were monitored here by spiking all samples with PCB surrogates 30, 65 and 204, as before. The SFE-based approach provided good recovery of the three surrogate PCB congeners added to the samples. Good precision was also achieved, as determined by low standard deviations (%RSD). Mean recoveries of PCB surrogate congeners are given in Table 5. The robust capabilities of the AutoPrep 44 unit itself were further indicated during this study by the absence of restrictor plugging during the course of 1288 extractions (including concomitant lipid extractions of each sample).

Table 5. *Mean recoveries of PCB surrogate congeners from edible fish tissue samples (n=644) collected from the Roanoke River Basin, Virginia. SFE conditions: See text for SFE conditions. % RSD = % relative standard deviation.*

PCB	MEAN	% RSD
30	92.8	12.5
65	92.0	15.5
204	99.7	15.7

Total PCB concentrations in fish tissue were obtained by summing the individual congener concentrations. Given the large number of PCB congeners typically encountered in environmental samples, and the large size of the sample set, this was a convenient approach to representing PCB tissue burdens. Total PCB concentrations ranged from below the quantitation limit (approximately 1μg/kg with a final extract volume of 0.2 ml) to 9910 μg/kg (dry weight basis). However, the median PCB concentration was only 90.6 and the mean 414 (SD = 911) μg/kg. The distribution of total PCB concentrations in 644 fish fillets is shown in Figure 17.

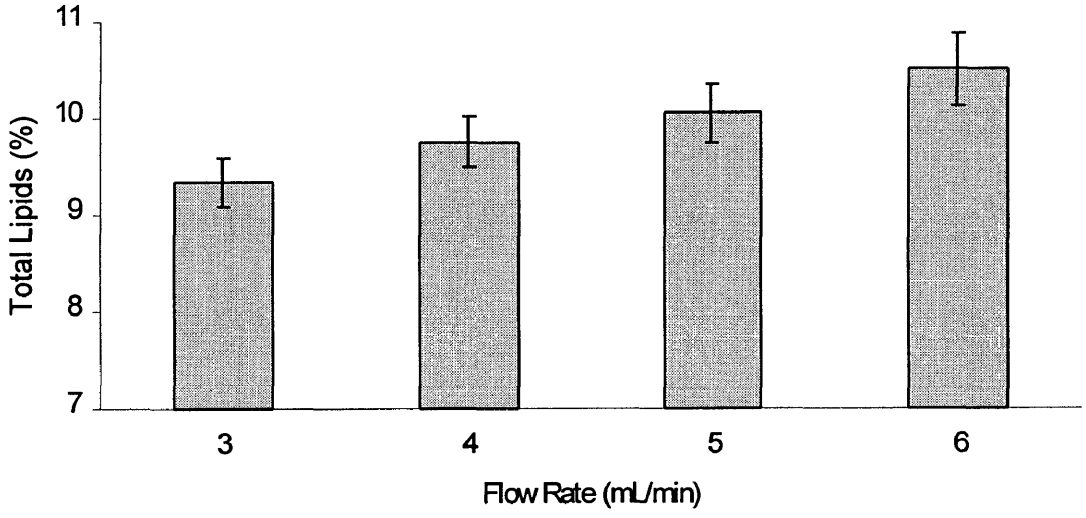
Figure 17. *Distribution of total PCB concentrations in edible fish fillets collected from the Roanoke River Basin. Values are expressed on a log scale. Noted are the maximum, minimum and various percentiles of the concentrations determined (n = 644).*



Development of SFE For Tissue Total Lipid Determinations and Application to a Large Field Sample Set

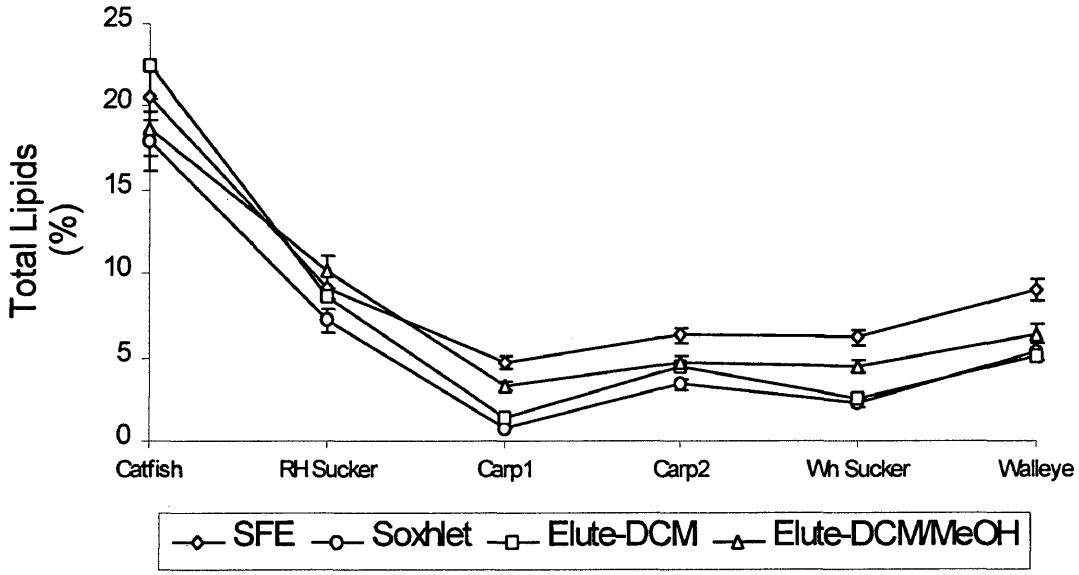
In addition to quantitative PCB determinations, SFE also facilitated a simple, straightforward no-solvent approach for determining % total extractable lipid in the tissue samples examined. Total lipid values comparable to those obtained by Soxhlet extraction were obtained using SFE at 450 atm, 150°C and 6 ml/min flow rate of unmodified CO_2. As with the method developed for PCB determinations, the lipid method incorporated a 10 minute static, followed by a 30 minute dynamic extraction step to achieve quantitative lipid yields. Use of organic solvents was eliminated completely. Amount of lipid extracted was a function of CO_2 flow rate (Figure 18). These SFE conditions produced lipid values comparable to Soxhlet extraction for several fish species with low %RSD (≤ 10%; Figure 19). It is evident from these data that Soxhlet extraction and SFE produced

Figure 18. *Effects of CO2 flow rate on SFE of total lipids from the fish VRM (n = 3).*



consistently higher lipid values than column elution with methylene chloride. Following column elution with methylene chloride by an additional aliquot of methanol and combining these fractions increased yields. However, these results generally remained lower than SFE- and Soxhlet-derived values. Method robustness was demonstrated by the successful extraction of the 644 fish tissue samples, previously collected for PCB determinations. The SFE approach also eliminated the need for collection and handling of the extract and required no additional glassware.

Figure 19. *Comparison of total lipids extracted from edible fish tissue by four different methods: SFE, Soxhlet and elution from a tissue-packed column using either methylene chloride (DCM) alone or DCM followed by methanol. Five different species were examined. RH = Red Horse; WH = White Horse. Values are expressed on a dry weight basis (n = 3).*



Correlation of Tissue PCB Concentrations with % Total Lipids

It is widely accepted that partitioning of non-polar organic pollutants into tissues is directly related to the tissue's total lipid content. To explore the issue further, data obtained on total PCB congener concentrations in representative fish tissue samples using the SFE-based extraction approach were lipid-normalized using the ratio approach (e.g. the ratio of PCB concentrations to total lipid content; note that PCB concentrations reported in Figure 17 were not normalized for tissue lipid content). During initial data reduction, it was evident that lipid content was highly variable spatially, temporally, interspecifically and, to a lesser degree, intraspecifically. It was observed that PCB concentrations generally did not follow lipid content uniformly, thus prompting more intensive scrutiny of the data set. When considering all individuals collected from two representative sampling sites encompassing a riverine and a fresh water lake system, there was generally poor correlation between the two parameters (Figures 20-21). Normalizing PCB concentrations to lipid values using the ratio approach resulted in negligible improvement of the correlation (Figures 22-23).

Figure 20. *Correlation of total PCB concentrations with % total lipids in edible fish tissue samples collected from the Roanoke River Basin (Clover).*

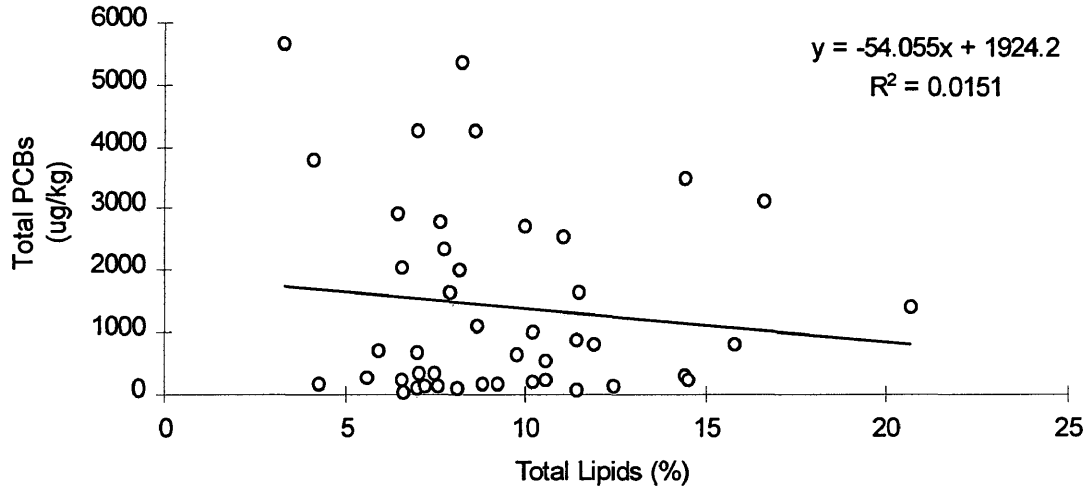
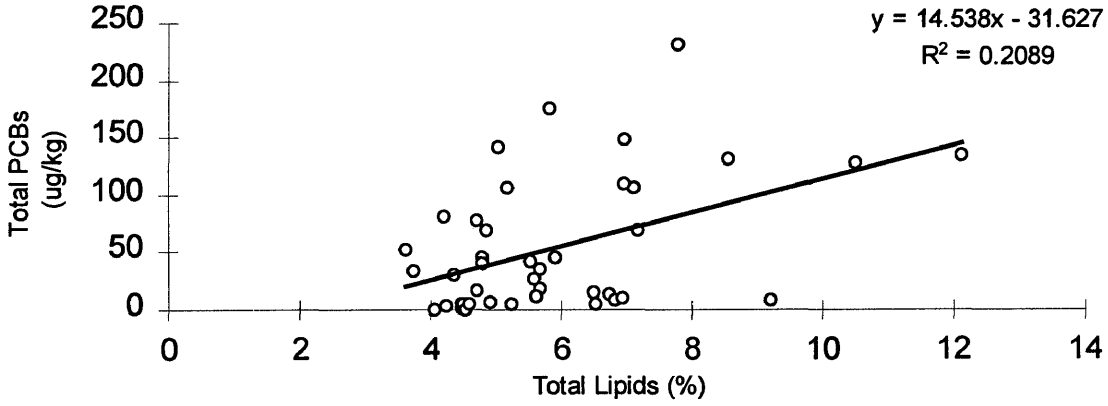


Figure 21. *Correlation of total PCB concentrations with % total lipids in edible fish tissue samples collected from the Roanoke River Basin (Smith Mountain Lake).*



The Clover site was interesting in that the correlation between the two parameters was very poor and actually negative (r^2 = 0.02 and 0.13; m = -54.1 and -32.1 for PCB and lipid-normalized PCB, respectively). The Smith Mountain Lake site showed some improvement in degree of correlation and slope (r^2 = 0.21 and 0.02; m = 14.5 and 0.56 for PCB and lipid-normalized PCB, respectively). Although these data represent all species collected at each sampling site, analysis of species-specific data revealed little improvement in the level of correlation between the two parameters. Further, the number of individuals of a given species collected at each site was often small (n typically ≤ 5).

Figure 22. *Correlation of lipid-normalized total PCB concentrations and total lipids in fish samples collected from the Roanoke River Basin (Clover).*

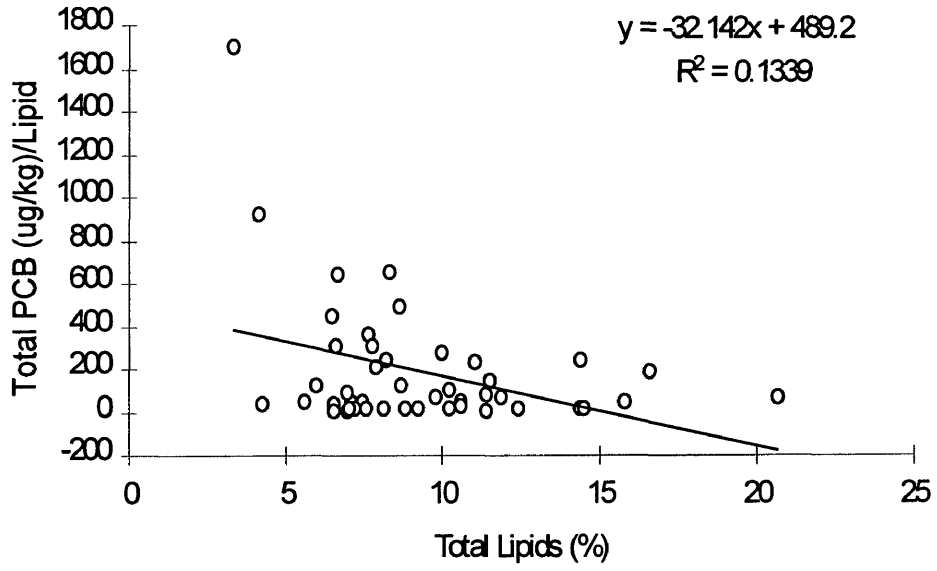
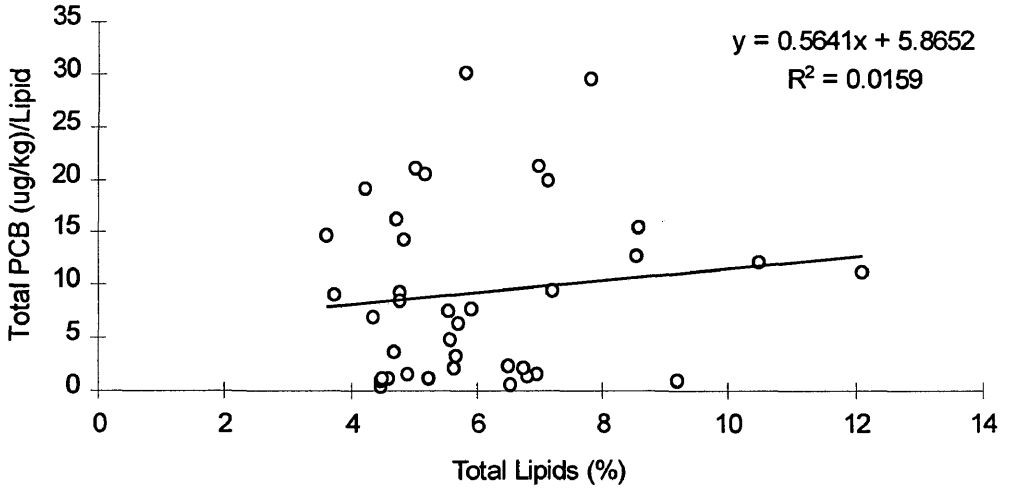


Figure 23. *Correlation of lipid-normalized total PCB concentrations with % total lipids in edible fish tissue samples collected from the Roanoke River Basin (Smith Mountain Lake).*



DISCUSSION

Recognition of the health and safety hazards of commonly used organic solvents in the laboratory and their increasing purchase and disposal costs have resulted in burgeoning interest in SFE. Despite the relatively large capital costs of SFE instrumentation, their presence in environmental/analytical laboratories is proliferating. Some evaluations of the cost effectiveness of commercially-available SFE instruments have been published (Lopez-Avila et.al., 1991). However, many estimates address only the sample preparation and extraction step, and thus underestimate the potential economic merits of SFE. Few have addressed the considerable costs of extensive post-extraction clean-up required in most environmental sample analysis procedures.

The initial sample extraction step is typically the most laborious and time consuming in any PCB analysis procedure. Unfortunately, it has also remained the least automated in the process, despite significant advances at other procedural levels (e.g. extract purification). Setup and cleaning of glassware such as Soxhlet extractors are particularly labor intensive and provide ample opportunity for glassware breakage, personal injury and contamination. However, with improvements in analytical-scale SFE, up to 44 samples may be extracted in series, greatly expediting the overall analysis of multiple samples. While extracting several samples in series with Soxhlet extraction is possible, comparable sample throughput cannot be achieved with the same minimal solvent expenditures that SFE permits. Only in recent years have commercially-produced

SFE instruments been widely available. Several are currently on the market that allow researchers to perform multiple extractions in series or parallel. These features are a hallmark of the AutoPrep 44 system and were exploited extensively during this work.

SFE of organic pollutants is more complicated than conventional liquid solvent-based methods, such as Soxhlet and sonication, as it is capable of a much higher degree of analyte selectivity. The solvating power of the extraction fluid, in this case CO_2, can be changed by altering pressure, temperature and the presence/concentration of liquid organic modifiers. Duration of extraction and flow rate can be manipulated to customize/optimize extraction efficiency. The manner and rate at which extracted analytes (and co-extracted materials) are delivered to the restrictor and trapping system strongly influences trapping success. While this may be considered an obstacle to overall extraction optimization, refinement of the analyte collection step may allow minimization (or elimination) of solvent concentration procedures required for other approaches. Use of a solid-phase trap also may permit on-line extract purification and class-selective separations to be performed (Van Bavel et al., 1996). If extracts are free of major co-extracted compounds, the opportunity for direct coupling to GC and liquid chromatography (LC) is also possible. This could drastically reduce detection limits. At present, less than 1% of the solvent extract is commonly injected onto the GC system. This fact imposes stringent demands on extract cleanliness.

When developing an SFE method, the type of analyte, sample matrix, matrix water content and co-extractive materials must all be considered. During this study,

emphasis on the behavior of PCB congeners in concert with actual tissue matrices was emphasized to increase applicability. Both lab-amended and field samples with incurred/native residues were utilized to ensure initial analyte solubility in the SF and account for potentially strong analyte-matrix interactions. Before SFE, the moisture content of the samples was considered. The water content of biological samples used here was high (75-90% total tissue weight). Therefore, drying of the sample prior to SFE was found to be highly desirable. While some authors have reported that small amounts of water may actually improve extraction efficiency (Ashraf-Khorassani et al., 1995; Schleussinger et al., 1996), most agree that, owing to its extreme polarity, water in the sample is problematic for efficient extraction of non-polar analytes such as PCBs (McNally, 1995). Under these circumstances initial removal of water from the sample is believed to be crucial because of the potential of co-extracted water to freeze and plug restrictors and traps. Water can also alter the critical parameters of the extraction solvent, leading to diminished extraction efficiency (Crowther and Henion, 1985).

Moisture content of biota samples is approximately twice that of aquatic sediments, making the drying process all the more important when considering SFE of biological samples. This fact may account in part for the for the small number of studies of biological matrices. Co-extracted sample water occasionally caused trap blocking during this work when sub-zero (e.g. -30°C) trapping temperatures were used. As a remedy, numerous pre-extraction chemical desiccants have been reported to be successful for biota and sediments, including diatomaceous earth (e.g. Hydromatrix), sodium sulfate,

calcium chloride, magnesium sulfate, alumina and florisil (Nam et al., 1990; Hopper and King, 1991; Johansen et al., 1992; Burford et al., 1993; Levy et al., 1993). However, these materials can occupy significant internal vessel volume leading to sample size reduction and concomitant increases in analyte detection limits. They may also solidify upon reaction with sample water and lead to undesirable effects such as plugged extraction vessels. The failure of chemical desiccants to retain moisture during SFE could be due in part to the elevated temperatures at which the extractions were conducted. For instance, Algaier and Tehrani (1993), reported that raising the extraction temperature from 25°C to 150°C released increasing amounts of water from cotton plugs using unmodified CO_2. Thus, it is possible that SFE at 150°C (optimum extraction temperature determined for PCBs) might have been responsible for the co-extraction of water observed during this work.

Attempts to dry tissue samples with chemical desiccants (e.g. Hydromatrix, anhydrous Na_2SO_4) were problematic. These materials were grossly ineffective at retaining sample moisture and consumed considerable extraction vessel volume. When subjected to SFE, water was released from the sample and often plugged the restrictor and/or trap. This compromised PCB recoveries and led to water in the final solvent extract. Sample capacity of extraction vessels compatible with automated SFE systems has been a major impediment to widespread acceptance of the technique among environmental chemists. Adequate sample size is needed to permit isolation of sufficient analyte for subsequent GC determination, as residues typically occur in the μg/g- to ng/g

range in environmental matrices (e.g. PCBs, PCDDs). Lyophilization proved to be more effective here than addition of chemical desiccants. Witter et al. (1995) also advocated freeze-drying to increase SFE reproducibility. Further, freeze drying tissues prior to extraction conserved extraction vessel volume by eliminating the need for inclusion of these bulky drying agents. A major concern when freeze-drying samples is loss of volatile analytes. However, recovery data obtained during this study on lower molecular weight PCB congeners (those most susceptible to loss during this process) were in excellent agreement with results obtained by labs participating in the NIST intercomparison exercise that did not lyophilize their fish homogenates (Hale and Gaylor, 1995; Figure 16 and Table 4). Further, during preliminary SFE trials, recoveries of surrogate PCB congener 30 (trichlorobiphenyl), added to alumina blanks before freeze drying, were good.

In an attempt to further streamline the SFE process, preliminary research was conducted to ascertain whether PCBs could be extracted from aquatic biota samples at lower temperatures (higher fluid density), without co-extraction of sample water. The data indicate this might be possible (Figure 9). However, it is not yet clear if, even at lower temperatures, this approach would completely eliminate sample moisture co-extraction during SFE. Progress has been made on the sample drying question in other laboratories. For example, a method has been developed recently by Capangpangan and Suffet (1996) to dry filtered, suspended solids from natural water samples prior to SFE. The technique has been modified slightly to allow drying of small quantities (1-2g) of

wet biota in trial SFE runs by Hale and Gaylor (unpublished). In this desiccation method, wet samples are applied to a glass fiber filter and suspended over a bed of activated $CaCl_2$ in a closed glass container for 24 hours. Larger wet sample sizes (e.g. $\geq$ 10 grams wet tissue), required to ensure reasonable detection limits for PCBs, have been problematic. Regardless of the desiccation technique chosen prior to SFE, it is extremely important that any water present in the extract be removed before GC.

Manipulation of extraction temperature and pressure were critical parameters in achieving quantitative PCB recoveries from tissue samples. Repetitive work on extraction of PCB congeners from alumina alone and various tissue VRMs supported the observation that CO_2 density was important. Density decreases with increasing temperature at a given pressure. Only a pressure of 200 atm was required to remove total PCBs from alumina and amended tissue matrices at 50°C (ρ = 0.8 g/ml; Figures 8-9). At higher temperatures, higher pressures were needed. However, elevated temperatures have been observed to be required for quantitative extraction of many environmentally-relevant analytes (e.g. PAH and PCBs) from sediments (Lee and Peart, 1994; Tong and Imagawa 1995; Langenfeld et al., 1995). Higher temperatures increase the solubility of some analytes more effectively than density. It may also increase desorption kinetics through physical disruption of the matrix and increasing analyte vapor pressure. Extraction temperatures near an analyte's melting point would likely be favorable, as liquids are generally more easily solubilized than solids.

A combined 10 minute static/30 minute dynamic extraction at pressure of 350

atm, temperature of 150°C (ρ = 0.57 g/ml) and liquid flow rate of 3 ml/min produced consistent and quantitative recoveries for a range of PCB congeners from alumina, tissue VRMs and the Carp CRM (Figures 8, 9 and 16). Extraction at 400 atm resulted in reduced recoveries and erratic standard deviations (Figure 7). It is unknown why SFE at a slightly higher pressure, at the same constant temperature, would lead to such inconsistent results. It is possible that greater amounts of lipid were co-extracted and interfered with chromatographic performance. However, this was not evident during data analysis. Similarly, Langenfeld et al. (1993), observed that increasing extraction temperature was more effective than increasing the pressure or SF density for extracting PCBs from sediments. They further showed that, at higher temperatures, variations in pressure had little or no effect on analyte recovery.

SFE temperature and pressure data suggests that matrix effects may exert considerable influence on extraction efficiency of PCBs from biota samples. The dominance of matrix-analyte interactions during SFE has been corroborated with other environmentally important analytes as well. For example, Langenfeld et al. (1995), showed that, while maintaining constant fluid density, higher temperatures were needed to quantitatively extract polychlorinated dibenzo-p-dioxins (PCDDs) and PAHs from sediments and soils using CO_2. These studies further support analyte desorption from the matrix (and more generally matrix-analyte interactions) as the rate-determining step. Once matrix-analyte interactions are effectively diminished, analyte solubility in the SF may then become the rate-limiting step (Langenfeld et al., 1993).

While solvent modifiers were not required to quantitatively extract PCBs from tissue samples here, this approach has been exploited historically to minimize matrix-analyte interactions during SFE of organic contaminants (Hawthorne, 1990; McNally, 1995). While supercritical CO_2 is a relatively good solvent for extracting non-polar compounds from most environmental matrices, these solvating interactions can be weak for certain analyte/matrix combinations. For example it has been shown that pure CO_2 often fails to extract persistent organic pollutants such as chlorinated dioxins from fly ash (Onuska et al., 1993), PAHs from urban air particulate matter (Langenfeld et al., 1993), PAHs and nitroaromatics from soil (Wujcik and Seiber, 1996) and PCBs from river sediments (Langenfeld et al., 1993). To enhance the relatively low polarity of SF-CO_2, liquid modifiers can be employed to increase CO_2 solvent strength. This has led to widespread adoption of CO_2-SFE as a major extraction technique in other fields, including the food and pharmaceutical industries, where the need for non-toxic solvents is evident (McHugh and Krukonis, 1994). The role of modifiers in the SFE process has been debated since the inception of analytical SFE in the early 1990's. However, it is generally agreed that liquid modifiers 1) enhance the SF solubility of some target analytes and 2) modify the matrix and disrupt matrix-analyte interactions. It is now believed that the latter mechanism is the most important (Hawthorne et al., 1993).

Few studies have been published aimed at elucidating PCB extraction kinetics from biological samples using SFE. To remedy this deficiency, a kinetic study was initiated. The general extraction kinetics profile (Figure 10) for a suite of PCB congeners

was similar to that reported by other researchers (Hawthorne et al., 1993; Langenfeld et al., 1995). It is interesting to note the peculiar behavior of PCB 3 (4-chlorobiphenyl) during these experiments. The behavior of this congener diverged substantially from that of other congeners, including PCB 1 (2-chlorobiphenyl) at 5, 10, 15 and 20 minute extraction time intervals. It is possible that the substitution pattern (para) of PCB 3 induces a more pronounced matrix effect and is retained more strongly than PCB 1 (ortho-substituted) or the other congeners. The observed behavior of PCB 3 could also be an experimental artifact. However, the trend is consistent at each time measurement, except the 30 minute extraction, and the %RSDs are reasonable. Like the temperature and pressure data, the kinetics data indicate that matrix effects must be carefully considered when developing SFE methods.

Numerous complications associated with SFE of biological materials are likely to blame for the paucity of published studies. One problem has been the high lipid contents of these organisms, which, when co-extracted, may plug SFE restrictor devices. The introduction of heated, adjustable restrictors have largely ameliorated this problem. However, the ability to perform selective extraction or on-line cleanup with SFE would be highly beneficial. Post-extraction purification steps, needed to remove co-extracted lipids, represent a significant portion of the effort required in most PCB determinations. To make matters worse, these steps also require the use of copious organic solvents and expensive analytical instrumentation such as GPC and/or HPLC.

The goldfish and carp homogenates used in this study contained significant

methylene chloride-extractable lipids (24 and 40%, respectively). As a consequence, Soxhlet extracts of these homogenates required extensive purification (GPC and florisil column treatment) prior to GC analysis. In contrast, inclusion of alumina in the SFE extraction vessel eliminated the need for any additional off-line purification. This was a momentous achievement in this research. The combination of selective SFE extraction and alumina retained more than 99% of the lipids. Recent studies have also demonstrated the feasibility of on-line retention of co-extracted lipids during SFE of biological samples by adding alumina oxide directly to the extraction vessel (France et al., 1991; Johansen et al., 1992; Hale and Gaylor, 1995; Hale and Gaylor, 1996). In addition to eliminating the need for extract purification, lipid-free sample extracts promote quality chromatographic separations and prolong the operating performance of GC injector ports and analytical columns. Obtaining extracts that are as free as possible of co-extracted lipids should, therefore, be a high priority when developing SFE methods for any biological matrix.

During inital method development with the PrepMaster SFE instrument, 5 ml extraction vessels were used and only 1-2 grams of alumina were added to a 1 gram tissue sample. This reduced the amount of co-extracted lipids to such a level that only a single florisil solid-phase extraction (SPE) column chromatography step was required prior to GC analysis. Similarly, Bowadt et al. (1994), used a single acid silica clean-up step in conjunction with milder SFE extraction conditions prior to on-column GC injection. While these simple clean-up steps represent significant improvement over post-extraction purification required following Soxhet extraction, translation of the approach to the

AutoPrep 44 and 10 ml extraction vessels eliminated entirely the need for florisil SPE column cleanup of the SFE extract. The use of 10 ml vessels also appeared to improve PCB recoveries slightly during preliminary trials. Successful translation to the larger (and longer) 10 ml extraction vessels here appears to contradict the observations of Furton and Rein (1991). These researchers observed that, when extracting PAHs from C_{18} sorbent materials, recoveries were improved by a factor of 2 when the diameter to length ratio of the extraction vessel was decreased from 1:20 to 1:1. Similarly, broadening extraction cell diameter led to improved recoveries of PCB congeners extracted from solid-phase chromatography packings using SFE (Furton and Lin, 1992). PCB congener recoveries were dependent upon chlorine substitution, with recoveries decreasing with increasing chlorine content. The results of these studies also illustrate the magnitude of the matrix effect.

Although, inclusion of alumina in the vessel downstream of the sample reduces sample capacity, the advantages of this step far outweigh this drawback. During the course of > 700 extractions, approximately 6-8 grams of alumina were used to ensure maximum lipid retention and prevent fouling of the analytical column. However, recent data suggest strongly that, if larger sample size is required, a 1:1 (w/w) ratio of alumina to sample is adequate to retain the bulk of co-extracted lipids, achieve quality GC separations and maintain quantitative PCB recoveries. In the interest of capillary GC column maintenance, however, it would be prudent to increase this ratio.

Method quantitation limits (with 1 gram dry tissue) using the GC-ELCD varied

from about 0.5 to 0.1 μg/kg (wet weight basis, with a final extract volume of 0.2 ml), as a function of analyte degree of chlorination. Substitution of an analyte detection method with greater sensitivity (e.g. electron capture detection or ECD) would reduce this limit further. However, the ECD is less analyte-specific than the ELCD, responding to virtually any electron-rich species (e.g. S, N, O and Cl). The ELCD response can be tailored exclusively to halogen-substituted organic compounds. Thus, current sensitivity of the ELCD method appears adequate without major modification for most regulatory and scientific studies of PCB congener burdens. Studies of low concentrations of specific highly toxic coplanar PCB congeners may require some additional modifications.

Quantitative post-extraction analyte collection was a major hurdle during SFE method development. Analyte collection during SF decompression is often one of the most crucial, and difficult to optimize, parameters during SFE method development. Poor analyte collection efficiency can be misinterpreted as inefficient sample extraction. Successful optimization of one SFE variable is frequently achieved at the expense of another. In a typical SFE scenario (Figure 3), analytes are removed from the matrix and swept from the extraction cell through a restriction device. A restrictor is used to permit SF decompression and flow rate control prior to introduction to the collection apparatus. Upon decompression, analytes precipitate out of the sub-critical fluid and must be quantitatively retained. In analytical SFE, dissolved species are trapped using three major techniques: 1) off-line, cryogenic trapping on solid phase materials such as glass beads (Levy et al, 1993), C_{18} and C_{18}/Unibeads mixtures (Mulcahey et al., 1991; Bowadt et al.,

1994; Hale and Gaylor, 1995), florisil (Bowadt et al., 1994), stainless steel balls (Levy et al., 1992) and glass fiber extraction disks (Ezzel, 1995), with subsequent desorption using small volumes (2-5 ml) of a suitable liquid organic solvent; 2) collection directly into a small volume of liquid organic solvent (Langenfeld et al., 1992; Yang et al., 1995), and 3) direct coupling to a gas or liquid chromatograph (Johansen et al., 1992; Johansen et al., 1994; Nam and King, 1994). The first two techniques are referred to as "off-line" collection, while the third is an "on-line" method. A major advantage of off-line collection is the ability to use the resulting extract for more than one type of analysis. Further, using solid-phase trapping, it is possible to perform selective separation/fractionation on the sorbed extract using different elution solvents (van Bavel et al., 1996).

Here, the emphasis was on development of solid-phase analyte collection, rather than liquid solvent trapping. Solid phase collection permits more efficient recovery of analytes at higher supercritical CO_2 flow rates (Ashraf-Khorassani et al., 1992; Ezzel, 1995). Important parameters were solid-phase composition, trapping temperature, elution solvent, elution temperature and volume and identity of elution solvent. It was also discovered very early in the method development stage that "upstream" variables (those related to the extraction process itself, e.g. CO_2 flow rate, duration, co-extractives and solvent modifiers) significantly impacted trapping performance/efficiency to a large extent.

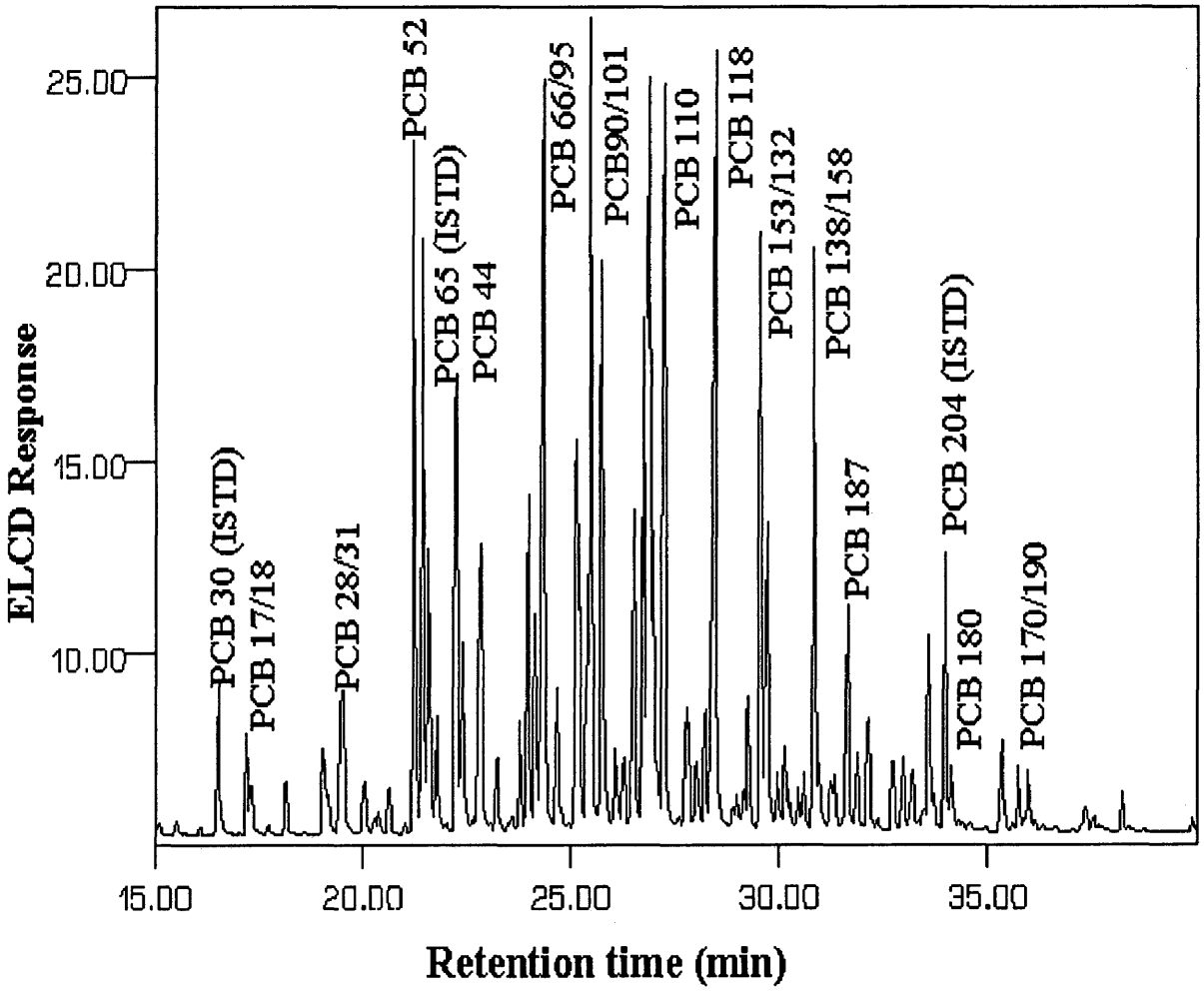
A number of researchers have investigated the use of different solid-phase

adsorbents for analyte collection during SFE. Here, a mixture of C_{18}-modified silica/Unibeads (1:1 w/w) and low (e.g. 0°C) trap temperatures provided excellent recoveries of surrogate PCB congeners from the tissue VRMs, the 644 edible fish tissue samples analyzed and alumina alone (Table 3 and 5; Figure 14). Johansen et al. (1992) also found that a C_{18}-based trap cooled to -30°C gave good results for PCBs extracted from fish tissue. In agreement with their findings, excellent PCB recoveries were initially obtained here by trapping at -30°C. However, it was later discovered, after translation to the AutoPrep 44 system, that quantitative PCB recoveries could also be obtained when the trap temperature was raised to 0°C using the C_{18}/Unibeads mixture (Figure 14). This finding drastically reduced trap freezing incidents and minimized the amount of coolant CO_2 required per extraction.

PCB elution from the solid-phase trap was straightforward, requiring only 2 ml of isooctane at a trap temperature of 90°C. This volume is several orders of magnitude less than the total required to complete Soxhlet and other conventional extraction approaches. Lee et al. (1994) have reported successful trapping of PCBs extracted from sediments on a C_{18} trap at 15°C. These were then eluted at 45°C using two 1 ml aliquots of isooctane/hexane. Bowadt et al. (1994) trapped PCBs extracted from lyophilized fish tissue on a florisil column, eluting with small aliquots of heptane and methylene chloride. While reports of successful solid-phase trapping are increasing, a majority of SFE investigators to date have trapped non-polar analytes in liquid solvent traps (Bowadt and Hawthorne, 1995).

Evaluation of PCB extraction/analysis methods should include an examination of their ability to produce accurate, precise and robust measurements of laboratory-amended and native PCB congeners in representative sample matrices. PCB values determined in freeze-dried fish tissue using SFE were similar to those obtained with the widely-used Soxhlet extraction approach. Further evaluation of the efficacy of the SFE method developed was conducted by applying it to the determination of native PCB congeners in a CRM (fish homogenate). Results of the SFE-based method compared favorably to those obtained by experienced external analytical laboratories using thoroughly validated methods (Figure 16 and Table 4). Somewhat lower concentrations of higher molecular weight PCB congeners were observed with the SFE methodology, as was seen during the Soxhlet extraction trials (Figure 15). PCB concentrations higher than consensus values were measured by the SFE method for some intermediate PCB congeners (e.g. PCB 95, 101, 118). This may be due to contributions by co-eluting pesticide residues or other halogenated compounds which would tend to inflate peak area counts during GC separation. A supplemental post-extraction column chromatography step to separate PCB congeners from pesticides was not included in this method. A sample GC-ELCD chromatogram of the CRM extract generated by SFE extraction is provided in Figure 24. It can be seen in this chromatogram that quality baseline resolution is achieved for a complicated mixture of native and surrogate PCBs, with no off-line purification of the SFE extract.

Figure 24. *ELCD chromatogram of a candidate SRM fish tissue extract obtained directly from the SFE with no further off-line purification. Surrogate and some representative native PCBs are identified by IUPAC congener number. See text for GC conditions.*



The robustness of the SFE-based method was validated by the successful sequential extraction of > 700 freeze-dried tissue samples collected from various fresh water and estuarine systems throughout Virginia. Another measure of method robustness (and reproducibility) was the consistent recoveries of surrogate PCB congeners 30, 65 and 204 obtained during routine extractions of 644 fish tissue samples (Table 6). The efficacy of the on-line retention of lipid by alumina inclusion in the vessel was supported by the observation that there was no appreciable GC resolution of column deterioration after injecting such a large number of biota sample extracts. As indicated previously, a highly detailed discussion of PCB congener composition and concentrations in the fish tissue data set are beyond the scope of this study. However, it is worthwhile to note that native PCB concentrations were generally low, with only a small percentage (< 10%) of samples containing elevated levels (Figure 17). PCB compositional patterns observed in the fish tissue samples were similar to those reported by other researchers (Maack and Sonzogni, 1988; data not shown). The range of concentrations were expressed on a log scale to highlight the relative maximum, minimum and various percentiles of the PCB concentrations determined.

Total tissue lipid content is of great concern in environmental chemistry studies since non-polar organic pollutants, such as PCBs, PAHs and polychlorinated terphenyls (PCTs) are lipophilic and partition into these biochemical compartments (Randall et al., 1991). Traditionally, the standard benchmark for the determination of total lipids in biological samples has been the method of Bligh and Dyer (1959). This procedure

involves the extraction of a fresh (wet) homogenized sample with a mixture of water, chloroform and methanol. The resulting extract is centrifuged, the chloroform layer decanted and the lipid content determined by evaporation of the chloroform to a constant weight (gravimetric). Lipid extraction is relatively time consuming and is typically done in addition to the extraction process conducted to determine concentrations of lipophilic contaminants in tissues.

Other researchers have successfully applied SFE to the determination of lipid content of biological tissues (Eldridge et al., 1986; King et al., 1989; Lembke and Engelhardt, 1993). A rapid and simple SFE-based procedure for determining total tissue lipid content was developed here also. Lipid measurements could be made by simply weighing the vessel plus sample before and after SFE. Total lipid values were comparable to those obtained with Soxhlet extraction, a benchmark also used by Levy et al. (1994). The Bligh and Dyer approach was previously compared to Soxhlet extraction using adipose, liver and muscle tissues derived from sea turtles (Rybitski et al., 1992). Comparable results for total lipids were obtained between the two methods. It was, therefore, reasonable to compare the SFE method directly to Soxhlet extraction, thus greatly simplifying the study. A concern with this comparative approach was that some degradation of complex lipids into fatty acid components was observed during Soxhlet extraction. This was likely due to continuous exposure to atmospheric oxygen. Oxidative degradation of lipids may not be critical, however, if the concern is primarily to determine total lipids and not individual lipid classes therein.

A common practice in environmental studies is to normalize contaminant concentrations in biological samples to tissue total lipid content. This has historically been done using the so-called "ratio approach (e.g. the ratio of pollutant concentration to total lipid, as determined by an appropriate extraction method) and is intended as a measure of bioaccumulation potential of the contaminant(s) being considered. However, the wisdom of applying this approach broadly across classes of non-polar organic pollutants and all biota types has recently been questioned. This is due to recognition of the great variation in extraction methods employed and the preferential partitioning of these compounds into specific lipid class pools (e.g. triglyceride, phospholipid, etc.; Kawai et al., 1988; Randall et al., 1991). Total lipid extractions are not intended to differentiate between lipid classes and thus do not facilitate detailed biochemical assessments of organic contaminant bioaccumulation on a class-specific basis. Although SFE shows great promise in performing class-selective extractions at low temperature (high fluid density), development of such a sophisticated lipid extraction method was not an objective here. Rather, a SFE-based extraction approach was desired that would eliminate organic solvent usage entirely and provide total lipid values concomitant with PCB data, comparable to established conventional extraction methods. It is generally believed that normalizing tissue contaminant burdens to total extractable lipids using the ratio approach is satisfactory, when it is evident from the raw data that contaminant concentration(s) vary in direct proportion to total lipid content (Hebert and Keenleyside, 1995). However, when a straightforward relationship does not exist, erroneous

conclusions may be reached regarding the importance of total lipid to bioaccumulation potential in the organism(s) under study. This appeared to be the case in the fish tissue data set examined in this study.

Considering individuals from the same species may lead to better correlations between PCB and total lipid values. However, as this was not an objective of the sample collection strategy, no controls were imposed to ensure that adequate numbers of individuals from a given species were collected from each sampling site. Furthermore, most studies aimed at identifying the influence of tissue lipid content on non-polar contaminant bioaccumulation have been predicated on aggregate comparisons of different fish species and/or fish species collected from different locations (Rasmussen et al., 1990; Rowan and Rasmussen, 1992). Having established precedence using this aggregate approach to lipid normalization, it was determined that treating the data set in this manner was reasonable.

Minimal published data exists examining the empirical relationship between tissue contaminant levels and tissue lipid content in large fish tissue data sets (Stow et al., 1997). This fact provided further impetus for at least a cursory examination of this relationship within the data set, albeit not the primary objective of the presented work. A possible solution to the problem of PCB-lipid correlation in organism sub-samples might be to normalize whole body contaminant burdens to lipid content using the ratio approach. A whole body lipid estimation method would likely be more meaningful in the context of bioaccumulation potential of organic contaminants in general and PCBs in

particular. There is no indication of such an approach being utilized in the published literature, however. If a rigorous analysis of PCB-lipid correlations in fish was the desired goal of a contaminant study, it might also be useful to examine fish tissue by imposition of stringent biological criteria, such as size class, sex and organism spawning condition. For instance, when examining the relationship between PCB levels and lipid content in a large fish tissue data set, Stow et al. (1997), observed greater positive correlations among samples collected during spawning, compared with non-spawning fishes. It is suggested that these results could be interpreted in view of lipid mobilization events during specific life stages of the organism.

CONCLUSIONS

In conclusion, SFE was shown to be effective in isolating amended and "native" PCB congeners from lipid-rich biological samples without the need for post-extraction purification prior to GC analysis. PCBs were quantitatively extracted from tissue samples with unmodified CO_2 using a combined 10 minute static and 30 minute dynamic extraction at 350 atm and 150°C (ρ = 0.56 g/ml) and a liquid flow rate of 3 ml/min. SFE extracts generated could be eluted from the trap, collected in GC autosampler vials with 2 ml isooctane and introduced directly to a gas chromatograph. Solid-phase trapping onto a 1:1 (w/w) mixture of C_{18}/Unibeads at 0°C was effective at quantitatively retaining PCB congeners representing all degrees of chlorination. Inclusion of neutral alumina in the extraction vessel was effective at retaining > 99% of total extractable lipids (defined versus Soxhlet extraction), with resulting extracts being separated with good resolution using GC-ELCD. The method was significantly less laborious, consumed minimal glassware and reduced hazardous organic solvent consumption by two orders of magnitude, compared with the conventional Soxhlet-based extraction approach. Total lipids were extracted from tissues with unmodified CO_2 using a combined 10 minute static and 30 minute dynamic extraction at 450 atm and 150°C (ρ = 0.65 g/ml) at a liquid flow rate of 6 ml/min. Use of organic solvents was eliminated completely. Weight of extracted lipid could be determined by the difference in vessel weight before and after SFE. Field-incurred tissue PCB concentrations were generally low and varied over a

large data set (n = 644). Recovery of amended surrogate PCBs from the fish were high and reproducible. Fillet tissue PCB concentrations did not correlate well with % total lipids in the fillets at the sites examined. Lipid normalized PCB data obtained from sub-samples of whole organisms (e.g. fish fillet tissue) should be interpreted with caution. Whole-organism lipid normalization would likely lead to more meaningful conclusions regarding bioaccumulation potential of non-polar organic contaminants. The robustness of the SFE-based method was confirmed by successful PCB and total lipid extractions of 644 lyophilized edible fish tissue samples collected from fresh water river systems in Virginia.

LITERATURE CITED

Albro, P.W. and Parker, C.E. *Journal of Chromatography*, 169 (1979) 161.

Alexandrou, N., Lawrence, M., Pawliszyn, J. *Analytical Chemistry*, 64 (1992) 301-311.

Algier, J. and Tehrani, J. *Pittsburgh Conference*, #395 (1993) 1-3.

Andersen, M.R., King, J.W. and Hawthorne, S.B. Analytical Supercritical Fluid Chromatography and Extraction. In: Lee, M.L. and Markides, K.E. (Editors), Provo, UT: Chromatography Conferences, 1990.

Ashraf-Khorassani, M., Combs, M.T. and Taylor, L.T. *Journal of High Resolution Chromatography*, 18 (1995) 709-712.

Ashraf-Khorassani, M., Houck, R.K. and Levy, J.M. *Science*, 30 (1992) 361-366.

Ashraf-Khorassani, M. and Taylor, L.T. *American Laboratory*, December (1995) 23-28.

Bayona, J.M. *LRA*, 5 (1993) 283-292.

Benner, B.A. *American Chemical Society*, 33 (1993) 324-326.

Bligh, E.G. and Dyer, W.J. *Canadian Journal of Biochemical Physiology*, 37 (1959) 911.

Bowadt, S. and Johannson, B. *Analytical Chemistry*, 66 (1994) 667-673.

Bowadt, S., Johansson, B., Fruekilde, P., Hansen, M., Zilli, D., Larsen, B. and de Boer, J. *Journal of Chromatography A*, 675 (1994) 189-204.

Bowadt, S., Johansson, B., Pelusio, F., Larsen, B.R. and Rovida, C. *Journal of Chromatography A*, 662 (1994) 424-433.

Bowadt, S., Johansson, B., Wunderli, S., Zennegg, M., de Alencastro, L.F. and

Grandjean, D. *Analytical Chemistry*, 67 (1995) 2424-2430.

Brinkman, U.A.Th. and de Kok, A. Halogenated Biphenyls, Terphenyls, Naphthalenes, Dibenzodioxins and Related Products. In: Kimbrough, R.D. (Editor), Amsterdam: Elsevier, 1980.

Burford, M.D., Hawthorne, S.B., Miller, D.J. *Journal of Chromatography A*, 657 (1993) 413-427.

Burford, M.D., Hawthorne, S.B., Miller, D.J. *Analytical Chemistry*, 65 (1993) 1497-1505.

Camel, V., Tambute, A. and Caude, M. *Journal of Chromatography*, 642 (1993) 263-281.

Capangpangan, M.B. and Suffet, I.H. *Journal of Chromatography A*, 738 (1996) 253-264.

Christie, W.W. Lipid Analysis: Isolation, Separation, Identification and Structural Analysis of Lipids. Second Edition, Oxford: Pergamon Press Ltd., 1982.

Crowther, J. B., and Henion, J. D. *Analytical Chem*istry, 57 (1995) 2711-2716.

David, F., Verschuere, M. and Sandra, P. *Fresenius Journal of Analytical Chemistry*, 344 (1992) 479-485.

de Boer, J. *Chemosphere*, 17 (1988) 1803-1810.

Eldridge, A.C., Friedrich, J.P., Warner, K. and Kwolek, W.F. *Journal of Food Science*, 51 (1986) 584-587.

Erickson, M.D. Analytical Chemistry of PCBs, Boca Rotan, Ann Arbor, London: Lewis

Publishers, 1992.

Ezzell, J.L. *Analytical Methods Instrumentation*, 2 (1995) 48-51.

Fahmy, T.M., Paulaitis, M.E., Johnoson, D.M. and McNally, M.E. *Analytical Chemistry*, 65 (1993) 1462.

France, J.E. and King, J.W. *Journal of the Association of Official Analytical Chemists*, 7 (1991) 1013-1016.

Friedrich, C., Cammann, K., Kleibohmer, W. *Fresenius Journal of Analytical Chemistry*, 352 (1995) 730-734.

Friedrich, J.P., List, G.R. and Heakin, A.J. *Journal of the American Oil Chemists Society*, 59 (1982) 288-292.

Furton, K.G. and Lin, Q. *Chromatographia*, 34 (1992) 185-187.

Furton, K.G. and Rein, J. *Chromatographia*, 31 (1991) 297-299.

Giddings, J.C., Myers, M.N. and King, J.W. *Journal of Chromatographic Science*, 7 (1969) 276-283.

Giddings, J.C., Myers, M.N., McLaren, L. and Keller, R.A. *Science*, 16 (1968) 67-73.

Greaves, J., Harvey, E. and Huggett, R.J. *Environmental Toxicology and Chemistry*, 10 (1991) 1391-1398.

Griest, W.H., Yeatts, L.B., Caton, J.E., *Analytical Chemistry*, 52 (1980) 199.

Hale, R.C. and Gaylor, M.O. *Environmental Science and Technology*, 29 (1995) 1043-1047.

Hale, R.C., Gaylor, M.O., Thames, J.F., Smith, C.L. and Mothershead, R.F.

International Journal of Environmental Analytical Chemistry, 64 (1996) 11-19.

Hale, R.C. and Greaves, J. *Journal of Chromatography*, 580 (1992) 257-278.

Hawthorne, S.B. Analytical-Scale Supercritical Fluid Extraction, *Analytical Chemistry*, 62 (1990) 633-642A.

Hawthorne, S.B., Galy, A.B, Schmitt, V.O. and Miller, D.J. *Analytical Chemistry*, 67 (1995) 2723-2732.

Hawthorne, S.B. and Miller, D.J. *Analytical Chemistry*, 66 (1994) 4005-4012.

Hawthorne, S.B., Miller, D.J., Langenfeld, J.J. and Burford, M.D. *ACS Symposium Series*, 508 (1992) 206-221.

Hawthorne, S.B., Miller, D.J., Langenfeld, J.J., Burford, M.D., Eckert-Tilotta, S. and Louie, K.P. *Journal of Chromatography*, 642 (1993) 301-317.

Hawthorne, S.B., Miller, D.J., Walker, D.D., Whittington, E.E. and Moore, B.L. *Journal of Chromatography*, 541 (1991) 185.

Hawthorne, S.B., Langenfeld, J.J., Miller, D.J. and Burford, M.D. *Analytical Chemistry*, 64 (1992) 1614.

Hebert, C.E. and Keenleyside, K.A. *Environmental Toxicology and Chemistry*, 14 (1995) 801-807.

Hildebrand, J.H. and Scott, R.L. The Solubility of Nonelectrolytes. New York: Reinhold, 1950.

Hooper, S.W., Pettigrew, C.A. and Sayler, G.S. *Environmental Toxicology and Chemistry*, 9 (1990) 655.

Hopper, M.L. and King, J.W. *Journal of the Association of Official Analytical Chemists*, 74 (1991) 661-666.

Howard, A.L. and Taylor, L.T. *Journal of High Resolution Chromatography*, 16 (1993) 39-45.

Huehnerfuss, H. and Kallenborn, R. *Journal of Chromatography*, 580 (1992) 580.

Johansen, R.H., Becher, G. and Greibrokk, T. *Fresenius Journal of Analytical Chemistry*, 344 (1992) 486-491.

Johansen, R.H., Becher, G. and Greibrokk, T. *Analytical Chemistry*, 66 (1994) 4068-4073.

Jones, G.R.N. *Lancet*, 2 (1989) 791.

Kawai, S., Fukushima, M., Miyazaki, N. and Tatsukawa, R. *Marine Pollution Bulletin*, 19 (1988) 129-133.

King, J.W. *Journal of Chromatographic Science*, 27 (1989) 355-364.

King, J.W. and France, J.E. Analysis with Supercritical Fluids: Extraction and Chromatography. Berlin, New York: Springer-Verlag, 1992.

Kuratsune, N. Halogenated Biphenyls, Terphenyls, Naphthalenes, Dibenzodioxins and Related Products. In: Kimbrough, R.D. (Editor), Amsterdam: Elsevier, 1980.

Lang, V. *Journal of Chromatography*, 595 (1992) 1-43.

Langenfeld, J.J., Burford, M.D., Hawthorne, S.B. and Miller, D.J. *Journal of Chromatography*, 594 (1992) 297-307.

Langenfeld, J.J., Hawthorne, S.B., Miller, D.J. and Pawliszyn, J. *Analytical Chemistry*,

65 (1993) 338-344.

Langenfeld, J.J., Hawthorne, S.B., Miller, D.J. and Pawliszyn, J. *Analytical Chemistry*, 67 (1995) 1727-1736.

Langenfeld, J.J., Hawthorne, S.B., Miller, D.J. and Pawliszyn, J. *Analytical Chemistry*, 66 (1994) 909-916.

Leblanc, G.A. *Environmental Science and Technology*, 29 (1995) 154-160.

Lee, H.B., Peart, T.E., Hong-You, R.L. and Gere, D.R. *Journal of Chromatography A*, 653 (1993) 83-91.

Lembke, P. and Engelhardt, H. *Chromatographia*, 35 (1993) 509-516.

Levy, J.M., Danielson, V., Ravey R.M. and Dolata, L.A. *LC-GC*, 12 (1994) 920-923.

Levy, J.M., Dolata, L.A. and Ravey, R.M. *Journal of Chromatographic Science*, 31 (1993) 349-352.

Levy, J.M., Dolata, L.A., Ravey, R.M., Storozynsky, E. and Holowczak, K.A. *Journal of High Resolution Chromatography*, 16 (1993) 368-371.

Levy, J.M., Ravey, R.M., Houck, R.K. and Ashraf-Khorassani, M. *Fresenius Journal of Analytical Chemistry*, 344 (1992) 517-520.

Lopez-Avila, V., Dodhiwala, N.S., Benedicto, J. and Beckert, B.F. *LC-GC*, 10 (1991) 762-769.

Lopez-Avila, V., Benedicto, J., Dodhiwala, N.S., Young, R. and Beckert, W.F. *Journal of Chromatographic Science*, 30 (1992) 335.

Maack, L. and Sonzogni, W.C. *Archives of Environmental Contamination and*

Toxicology, 17 (1988) 711-719.

McHugh, M. and Krukonis, V. Supercritical Fluid Extraction. Boston, London, Oxford, Singapore, Sydney, Tokyo, Toronto, Wellington: Butterworth-Heinemann, 1994.

McNally, M.P. *Analytical Chemistry*, 67 (1995) 308-315A.

McNally, M.P. *Journal of AOAC International*, 79 (1996) 380-387.

Meyer, A. and Kleibohmer, W. *Journal of Chromatography A*, 657 (1993) 1993.

Miller, D.J., Hawthorne, S.B. and McNally, M.P. *Analytical Chemistry*, 65 (1993) 1038-1042.

Moore, W.N. and Taylor, L.T. *Air Products Application Note*, Publication #320-9515, 1995.

Moriarty, F. and Walker, C.H. *Ecotoxicology and Environmental Safety*, 13 (1987) 208-215.

Mothershead, R.F., Hale, R.C. and Greaves, J. *Environmental Toxicology and Chemistry*, 10 (1991) 1341-1349.

Mulcahey, L.J., Hedrick, J.L. and Taylor, L.T. *Analytical Chemistry*, 63 (1991) 2225-2232.

Meyer, L.J.D., Damian, J.H., Liescheski, P.B. and Tehrani, J. Supercritical Fluid Extraction Versus Soxhlet Sample Preparation. In: Supercritical Fluid Technology: Theoretical and Applied Approaches to Analytical Chemistry. *American Chemical Society*, Series 488 (1992) 221-236.

McConnell, E.E. Halogenated Biphenyls, Terphenyls, Naphthalenes, Dibenzodioxins

and Related Products. In: Kimbrough, R.D. (Editor), Amsterdam: Elsevier, 1980.

Moore, J.W. and Ramamorthy, S. Organic Chemicals in Natural Waters. New York: Springer, 1984.

Nam, K.S., Kapila, S., Yanders, A.F. and Puri, R.K. *Chemosphere*, 20 (1990) 873-880.

Nam, K.S. and King, J.W. *Journal of High Resolution Chromatography*, 17 (1994) 577-582.

NIST/NOAA NS&T/EPA EMAP. Intercomparison Exercise Program for Organic Contaminants in the Marine Environment: Fish Homogenate I QA93FS: Draft Results from the NOAA NS&T Quality Assurance Workshop. Miami, Florida, 1993.

Onuska, F.I. and Terry, K.A. *Chemosphere*, 25 (1992) 17-20.

Onuska, F.I., Terry, K.A. and Wilkinson, R.J. *Journal of High Resolution Chromatography*, 16 (1993) 407-412.

Pawliszyn, J. *Journal of Chromatographic Science*, 31 (1993) 31-37.

Pipkin, W. *LC-GC*, 10 (1992) 14-19.

Prasunitz, J.M. Molecular Thermodynamics of Fluid-Phase Equilibria. New Jersey: Prentice-Hall, 1969.

Randall, R.C., Lee, H., Ozretich, R.J., Lake, J.L. and Pruell, R.J. *Environmental Toxicology and Chemistry*, 10 (1991) 1431-1436.

Rasmussen, J.B., Rowan, D.J., Lean, D.R.S. and Carey, J.H. *Canadian Journal of Fisheries and Aquatic Science*, 47 (1990) 2030-2038.

Riekkola, M.L. and Manninen, P. *Trends in Analytical Chemistry*, 12 (1993) 108-114.

Rowan, D.J. and Rasmussen, J.B. *Journal of Great Lakes Research*, 18 (1992) 724-741.

Rowlinson, J.S. and Richardson, M.J. *Advances in Chemical Physics*, 2 (1959) 85.

Rybitski, M.J., Hale, R.C. and Musick, J.A. *Copeia*, 2 (1995) 379-390.

Schleussinger, A., Ohlmeier, B., Reiss, I. and Schulz, S. *Environmental Science and Technology*, 30 (1996) 3199-3204.

Schmidt H. and Schulz G. *Justus Liebigs Annalytica Chemica.* 207 (1881) 320.

Schulz, D.E., Petrick, G. and Dulnker, J.C. *Environmental Science and Technology*, 23 (1989) 852-859.

Snyder, J.L., Grob, R.L., McNally, M.E. and Oostdyk, T.S. *Analytical Chemistry*, 64 (1992) 1940.

Stow, C.A., Jackson, L.J. and Amrhein, J.F. *Canadian Journal of Fisheries and Aquatic Science*, 54 (1997) 1031-1038.

Taylor, L.T. *Analytical Chemistry*, 67 (1995) 364-370A.

Taylor, L.T. Supercritical Fluid Extraction. New York, Chichester, Brisbane, Toronto, Singapore: John Wiley & Sons, 1996.

Tong, P. and Imagawa, T. *Analytica Chimica Acta*, 310 (1995) 93-100.

Van Bavel, B., Jaremo, M., Karisson, L. and Linstrom, G. *Analytical Chemistry*, 68 (1996) 1279-1283.

Wheeler, J.R. and McNally, M.E. *Journal of Chromatographic Science*, 27 (1989) 534-539.

Witter, B., Wittko, F. and Knauth, H. *Fresenius Journal of Analytical Chemistry*, 353 (1995) 107.

World Health Organization. Polychlorinated Biphenyls and Terphenyls: Environmental Health Criteria 2. World Health Organization, Geneva, 1976.

Wujcik, C.E. and Seiber, J.N. *Journal of Environmental Science and Health*, A31 (1996) 1361-1377.

Yang, Y., Hawthorne, S.B. and Miller, D.J. *Journal of Chromatography A*, 699 (1995) 265-276.

VITAE

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