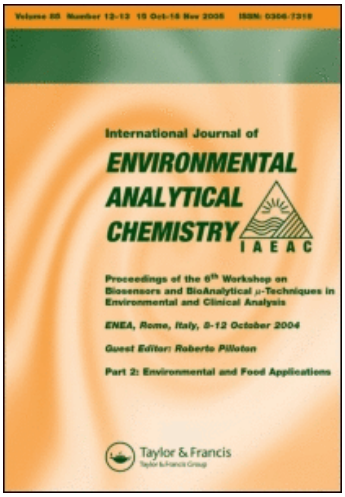

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Robustness of Supercritical Fluid Extraction (SFE) in Environmental Studies: Analysis of Chlorinated Pollutants in Tissues from the Osprey *(PANDION HALIAETUS)* and Several Fish Species

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ROBUSTNESS OF SUPERCRITICAL FLUID EXTRACTION (SFE) IN ENVIRONMENTAL STUDIES: ANALYSIS OF CHLORINATED POLLUTANTS IN TISSUES FROM THE OSPREY (*PANDION HALIAETUS*) AND SEVERAL FISH SPECIES

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Most commonly used methods for the extraction and subsequent determination of chlorinated pollutants in biotic tissues are time-consuming and require large volumes of hazardous organic solvents. Supercritical fluid extraction (SFE) has recently emerged as a potentially rapid, selective and environmentally-safe alternative. However, its robustness has not been widely evaluated for biological matrices. A SFE-based approach, using CO_2 and solid-phase trapping, was successfully applied to the analysis of 644 fish fillets for chlorinated pesticides and PCBs. Resulting extracts required no pre-GC purification steps. Eight mesentery samples, obtained from osprey (*Pandion haliaetus*) carcasses associated with a mass mortality event in southeast Virginia, were also examined. Bird extracts required simple Florisil column purification prior to GC. Mean total PCB concentrations were much higher in osprey than in fish, 30,400 (SD 12,700) and 414 (SD 911) ug/kg, respectively. Dominant PCBs in the osprey possessed higher degrees of chlorination than those in the fish. Comparison of chromatographic patterns suggested preferential reductions in the concentrations of specific PCBs in the birds. 4,4-DDE was the major pesticide in both birds and fish. Mean concentrations were 8500 (SD 2380) and 81.2 (SD 127) ug/kg, respectively.

KEY WORDS: Supercritical fluid extraction (SFE), PCB, DDT, fish, osprey.

INTRODUCTION

Supercritical fluid extraction (SFE) is finding increasing use in the analysis of trace organic environmental pollutants[1,2]. Readily available materials such as CO_2, above their critical pressure and temperature, are excellent solvents for extracting nonpolar analytes. CO_2 itself is nontoxic and, following the extraction step, reverts back to a harmless gas at room temperature and atmospheric pressure. Thus it presents no waste disposal problems and solvent concentration steps are minimized.

Polychlorinated biphenyls (PCBs) and chlorinated pesticides, such as DDT and its breakdown products DDE and DDD, are persistent, toxic and ubiquitous environmental pollutants. Due to their lipophilic nature, they concentrate in the fatty tissues of aquatic organisms such as fish[3]. Piscivorous birds may be exposed to elevated contaminant

burdens after consuming these fish[4]. PCBs were originally manufactured and released to the environment as complex mixtures. A total of 209 possible individual PCB congeners exist. Each possesses different physical and chemical properties, as well as toxicities. Thus knowledge of the concentrations of individual congeners in different biological compartments is critical to assessing deleterious impacts.

To date only a limited number of reports are available detailing the application of SFE for the removal of chlorinated organic pollutants from biological tissues. The majority of these examined either laboratory-amended matrices or only a limited number of samples with field-incurred residues. Thus, the robustness of SFE for these types of samples is largely unproven. This is critical if SFE is to supplant other techniques in widespread use in the environmental field. The ability of a SFE-based approach to selectively and efficiently remove chlorinated pollutants from a large number of biological tissue samples, i.e. its robustness, was examined here. Differences in the concentrations and patterns of individual congeners in fish and osprey tissue were then examined by high resolution capillary gas chromatography (GC).

METHODS

Glassware and utensils were thoroughly cleaned with detergent and rinsed with Milli-Q water and acetone. Nonvolumetric glassware was then baked at 400°C. Prior to use, hardware contacting the samples was rinsed with toluene, acetone, methanol and methylene chloride. All organic solvents used were residue grade or equivalent (Burdick & Jackson).

A total of 644 individual fish from 19 species were collected from 15 freshwater locations in the Commonwealth of Virginia (USA). Representative fillets, consisting of body musculature, were removed from these and lyophilized in an FTS Systems freeze-drier.

In addition, subsamples of mesenteric material were removed from the body cavities of eight ospreys. The osprey, *Pandion haliaetus*, is a widely distributed piscivorous raptor. All birds were found dead in a small Virginia salt marsh. Their proximity to each other suggested human involvement in their deaths. Carcasses were desiccated and partially decomposed when discovered. Mesenteric material obtainable from each bird was quite limited and was heterogeneous in composition (see discussion below on analysis of replicates). Avian material was extracted without further drying.

Supercritical extractions were conducted on a Suprex AutoPrep 44™ unit. The associated restrictor system was of a variable-flow, heated design. Samples were homogenized and aliquots (ca. 1 g dry weight) loaded into 10 ml stainless steel extraction vessels. In the case of the ospreys, fatty material was preferentially chosen for extraction from each sample of mesenteric tissue obtained. The exit end of each extraction vessel was filled with approximately 6 g of 150 mesh neutral alumina (Aldrich), activated at 150°C, to retain lipids. Typically a batch of 10–12 samples was loaded into the autosampler at one time. An extraction vessel containing alumina was placed at the end of each queue. No interfering compounds were detected greater than 1% of the concentration of the target analytes. PCB surrogate congener standards 30, 65 and 204 (IUPAC numbering) were added to all vessels prior to extraction. These congeners are reported to be absent from common Aroclor formulations[5].

Samples were subjected to an initial 10 minute static equilibration period at 150°C and 355 bar. They were then extracted at a rate of 3 ml/min (compressed fluid flow rate) with unmodified supercritical CO_2 (SFE-SFC Grade, Air Products) for 30 min. The SFE restrictor was maintained at 100°C during extraction. The solid-phase trap

was packed with 20–30 um C_{18}-modified silica (Aldrich), mixed 1/1 (w/ w) with 80/100 mesh Unibeads^R 2S (Alltech). It was maintained at –30°C during extraction. After extraction the trap was heated to 90°C and chlorinated pollutants were eluted with 2 ml of isooctane. This extract was concentrated to ca 0.2 ml under a stream of purified nitrogen.

Resulting fish extracts were suitable for GC injection without additional purification. Bird mesentery extracts required modest cleanup due to the presence of co-extracted material. These extracts were eluted with 12 ml of benzene from a column containing 2.5 g of Florisil, previously activated at 150°C.

After addition of an internal quantitation standard (pentachlorobenzene), all extracts were analyzed on a Varian 3400 GC, equipped with a 60 m × 0.25 mm I.D. DB-5 (J&W Scientific) fused silica column (25 um film thickness) and an OIC Model 4420 electrolytic conductivity detector (ELCD). The ELCD was maintained at 850°C. Helium carrier gas flow was ca. 1 ml/min. Injections (1 ul) were made in the splitless mode (injector split vent opened after 2 min.) by a Varian 8100 Autosampler. The injector was maintained at 300°C. The column temperature was held at 130°C for two minutes, programmed at 4°C/min to 320°C and held there for 15 min. Identification of PCBs was made using a halogen retention index[6] . Quantification was accomplished with the use of relative response factors, obtained by comparison of the response of the internal standard to those of representative analytes. When coelution of PCB congeners occurred, quantitation was done with the response factor of the dominant congener, as determined by Schulz *et al.*[5]. Standards were injected daily to verify GC system response. Corrections were not made for recovery of the surrogate standards. The following PCB congeners were quantified and a total PCB concentration calculated for each sample (those separated by a "/" were not completely resolved under the above GC conditions): 1, 2, 3, 4/10, 6, 7/9, 8/5, 15, 17/18, 16/32, 19, 22/51, 25, 26, 27/24, 28/31, 29, 33/20, 34, 35, 37/42/59, 40, 41/64, 44, 45, 46, 47/48/75, 49, 52, 53, 60/56, 63, 66/95, 67/100, 69, 70, 74, 77/110, 82/151, 83, 84, 85, 87/115, 90/101, 91, 92, 97, 99, 105, 107, 118, 119/150, 121, 122/131, 128, 129/178, 130, 134/143, 135, 136, 138/158, 146, 149, 153/132, 157/201/173, 167, 170/190, 171/156, 172, 174, 175, 176/137, 177, 178/129, 179/141, 180, 183, 185, 187, 189, 193, 194, 195/208, 196/203, 199, 205, 206, 207, 208/195 and 209. Note that not all of the above congeners were detected in each sample.

Effectiveness of the SFE-based procedure was initially confirmed by the analysis of "native" PCBs and 4,4'-DDT and its breakdown products (4,4'-DDD and 4,4'-DDE) in replicates of a reference material carp homogenate (*Cyprinus carpio*, Reference Material Carp-1, National Research Council of Canada). In addition, material from Osprey #6, remaining after initial analysis, was homogenized. This was split into duplicates and reanalyzed to estimate method precision on this matrix.

RESULTS AND DISCUSSION

Reports describing the application of SFE to the isolation of chlorinated pollutants from fish tissues are limited[7–10]. Plugging of extractor restrictors by lipids or ice formation has been one obstacle in commercially manufactured SFE[11]. High lipid loads in extracts may also degrade GC performance. Off-line acid treatment, adsorption/size exclusion column chromatography and other techniques have been widely used to purify extracts, prior to GC analysis. The SFE instrument used here employed a combination of restrictor heating and a controllable aperture to minimize plugging. We experienced no restrictor plugging during the course of over 700 extractions.

The supercritical CO_2-based approach provided good recovery of the three surrogate PCB congeners added to samples. Representative mean recoveries were 92.8% (SD = 11.6) for PCB 30, 92.0% (SD = 14.3) for PCB 65 and 99.7% (15.2) for PCB 204 (n = 165).

While monitoring recoveries of spiked standards is useful for tracking general SFE operating performance and subsequent extract handling, it does not adequately measure extraction efficiency of contaminants originally present in the matrix[12]. The use of appropriate reference materials which contain field-incurred analytes is a better assessment tool than spiked samples or blanks. Figure 1 compares results for the analysis of 4,4-DDT, 4,4-DDE and 4,4-DDD in a reference material fish homogenate using the SFE-based methodology. Concentrations are compared to mean values (consensus values) obtained by laboratories participating in a recent interlaboratory comparison exercise[13]. These laboratories used several different conventional solvent extraction-based approaches. Laboratory results which were deemed to be statistical outliers by that study's authors were eliminated from mean calculations. Consensus results for a range of PCB congeners were also similar to those obtained by the SFE-approach and have been published previously[10].

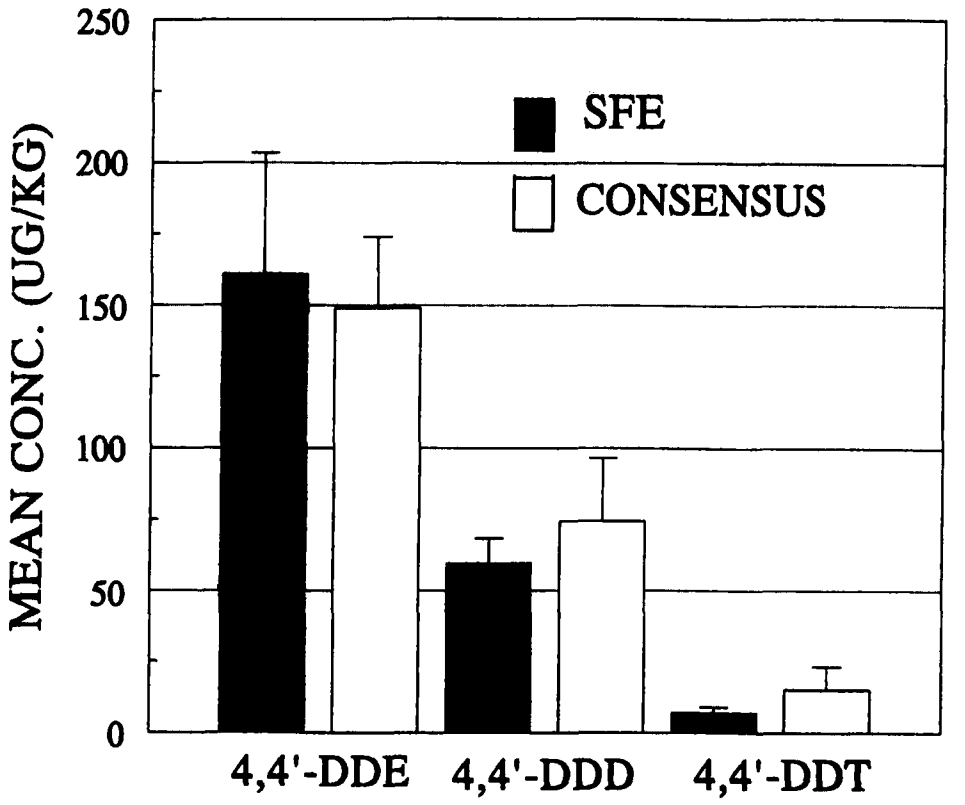

Figure 1 Mean 4,4'-DDE, 4,4'-DDD and 4,4'-DDT concentrations determined by SFE (n = 3) in a candidate fish reference homogenate compared to consensus values determined during an interlaboratory comparison exercise using organic solvent-based methods (n = 17, 15 and 8 for the three analytes, respectively). Bars represent one standard deviation.

Table 1 Concentrations (ug/kg) of several organochlorine pollutants determined in mesenteric materials from the carcasses of eight ospreys found dead in a southeast Virginia saltmarch. Duplicates obtained from remaining Osprey #6 were analyzed. These contained less fatty material than the original.

Osprey	*Total PCBs*	*4,4-DDT*	*4,4-DDE*	*4,4-DDD*
1	37,700	297	8540	175
2	24,500	194	8360	103
3	33,800	337	8600	182
4	11,300	73.0	5320	37.5
5	13,800	113	7380	67.1
6	45,300	385	12,900	115
7	43,100	359	6300	73.7
8	33,700	268	10,600	58.0
Duplicates				
6A	21,200	196	7790	38.2
6B	19,600	191	7940	88.9

Results from the analysis of duplicates obtained from Osprey #6 agreed well (6A and 6B, Table 1). These values differed substantially from those obtained from the original analysis of Osprey #6, due to the small amount of mesenteric material available and its heterogeneity. Sample remaining following initial analysis was somewhat depleted in fat-rich material, as this was preferentially chosen and extracted previously. Initial results were used for statistical calculations.

The SFE temperature and pressure conditions used here were relatively rigorous. Nonetheless, the fish extracts produced required no further purification prior to GC analysis with splitless injection. Addition of activated alumina to the exit end of the vessels was instrumental in retarding lipid co-extraction. Some reduction of chromatographic resolution was noted after injection of the osprey mesentery extracts. Purification of the extracts with a miniature Florisil column was sufficient to remove these interferences. Other researchers have found addition of alumina useful for on-line retention of lipids[14].

The SFE process itself was rapid, requiring only 40 min per sample. The SFE unit used had an automated, sequential sample introduction system, allowing unattended extractions to be performed and improving sample throughput compared to manual instruments.

Inclusion of C_{18}-modified silica in the trap and subambient collection temperatures provided improved analyte recoveries over, for example, glass beads alone[10]. Method quantitation limits using the ELCD were about 1 ug/kg (with a final extract volume of 0.2 ml) and varied according to the degree of chlorination of the analyte.

Total PCB concentrations in the fish fillets, obtained by summing individual congeners, ranged from below the quantitation limit to 9910 ug/kg (dry weight basis). However, the median PCB concentration was only 90.6 and the mean 414 (SD 911) ug/kg. A highly detailed discussion of the pesticide and PCB congener composition and concentrations in the fish is beyond the scope of this paper due to the large number of species and sites examined.

Total PCB concentrations in mesentaric tissues from the eight ospreys were higher and ranged from 11,300 to 43,100 ug/kg. Total PCB and DDT data for each of the eight ospreys are given in Table 1. The median total PCB concentration was 33,700

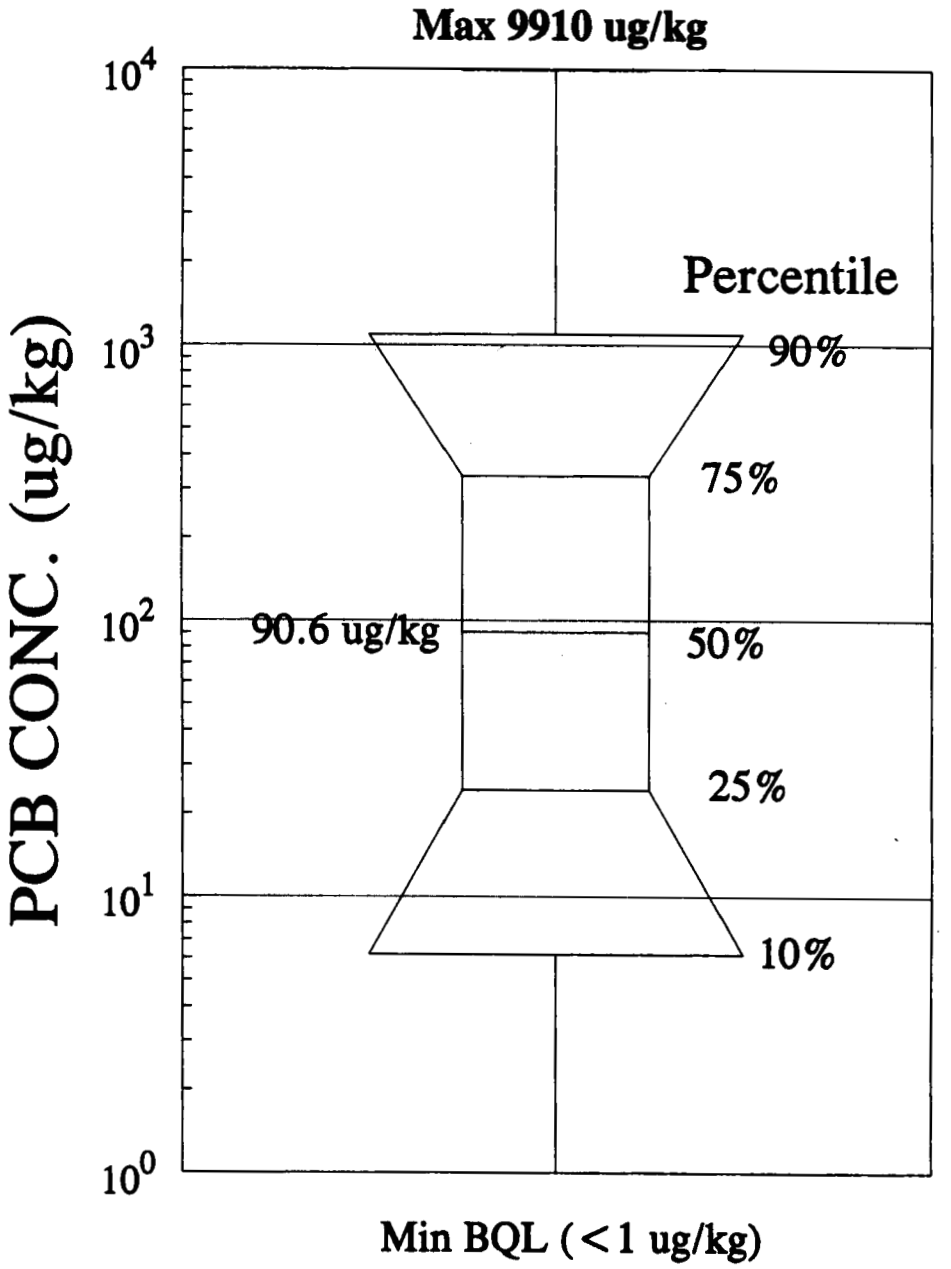

Figure 2 Distribution of total PCB concentrations in 644 fish fillets collected from Virginia waters expressed on a log scale. Noted are the maximum, minimum and various percentiles of the concentrations determined.

and the mean 30,400 (SD 12,700) ug/kg. The mean value is in the range of values reported for other birds[4]. Contributing to the concentration differences are the tissue types examined, i.e. muscle in the fish and mesenteric material in the birds. Boumphrey *et al.*[15], working with a variety of species in Great Britain, reported that total PCB

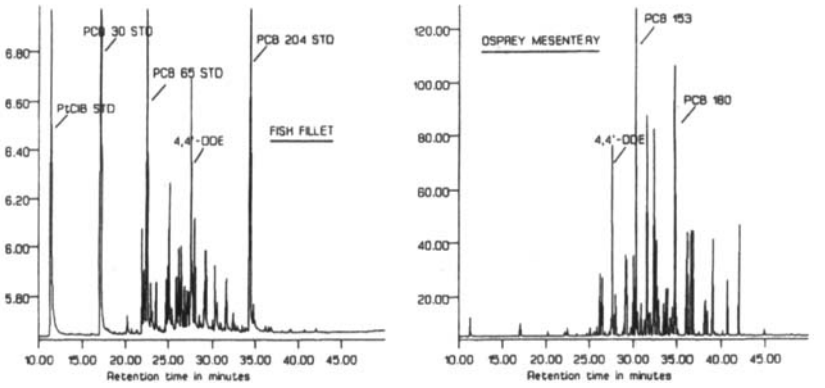

Figure 3 ELCD chromatograms of SFE extracts from a fish fillet and osprey mesentery sample. Several compounds are identified for reference. The osprey contained higher concentrations of most analytes. Distributions of the contaminants were shifted towards more highly chlorinated PCBs in the ospreys.

concentrations, on a wet weight basis, were one to two orders of magnitude higher in fat compared to muscle tissue in the same birds. Due to the condition of the ospreys, it is difficult to convert these values to a fresh weight basis for direct comparison with other studies or to assess potential health implications. Likewise, accurate determination of the lipid content of the mesenteric material was not possible due to its degraded condition. However, mesentary typically contain significant fat concentrations.

In terms of congener pattern, the PCBs in the osprey mesenteric tissues were shifted towards those with higher degrees of chlorination compared to the fish (Figure 3). Major PCB congeners in the osprey included: 138/158, 149, 153/132, 170/190, 180, 187 and 196/203. Other authors have reported similar distributions for piscivorous birds[3,15]. In general agreement with published research, major congeners in the fish included: 66/95, 90/101, 99, 110, 118, 138/158 and 153/132[16,17]. Preferential reductions of some tetrachlorobiphenyl PCBs (e.g. PCB 52 [2,2',5,5'], 44 [2,2',3,5'] and 70 [2,3',4',5]) were apparent (Figure 4). Birds appear to possess higher capacities to breakdown xenobiotics

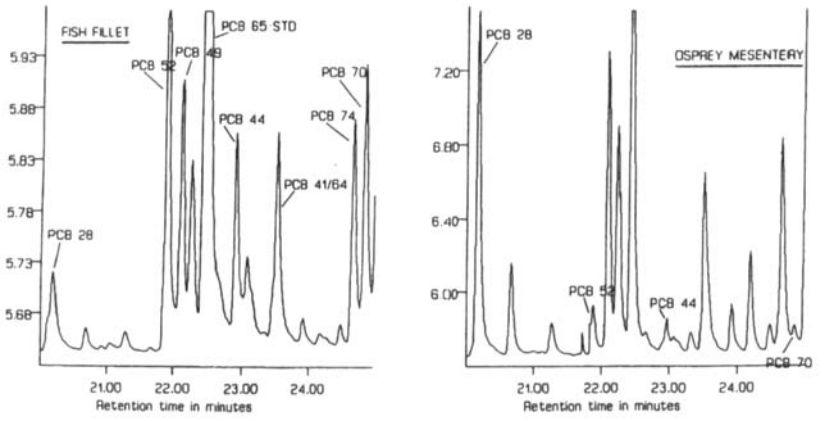

Figure 4 ELCD chromatograms of SFE extracts of a fish fillet and osprey mesentery sample showing the relative reduction of PCB congeners 52, 44 and 70 in the osprey.

than fish and reduction of these PCBs may be linked to the presence of unchlorinated *meta-* and *para-* ring positions[18]. However, it could not be confirmed whether the reductions observed for specific congeners and the shift towards higher chlorinated congeners in these birds were due to the ospreys' metabolic processes or to microbial or physical processes following their deaths.

4,4-DDE was the predominant DDT breakdown product, ranging from less than 1.0 to 1180 ug/kg in the fish and 5320 to 12,900 ug/kg in the osprey mesentery (Figure 5 and

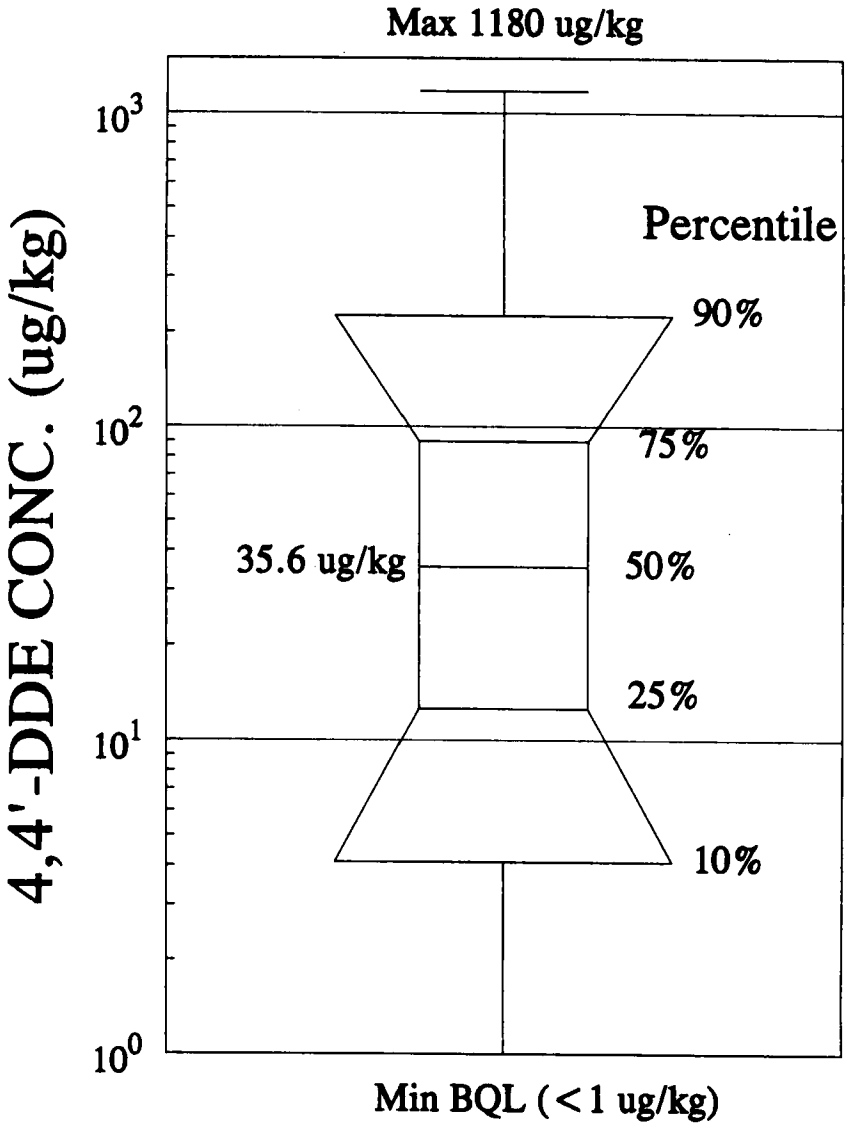

Figure 5 4,4-DDE concentration distribution in 644 fish fillets collected from Virginia waters expressed on a log scale. The maximum, minimum and various percentiles are provided.

Table 1). Median concentrations were 35.6 and 8450 ug/kg, respectively. Mean values were 81.2 (SD 127) and 8500 (2380) ug/kg, respectively. 4,4-DDT concentrations were generally an order of magnitude less than 4,4'-DDE values. 4,4'-DDD concentrations were generally less than one-half those of 4,4'-DDT.

CONCLUSIONS

This paper illustrates the utility of SFE for the analysis of large numbers of tissue samples for nonpolar chlorinated pesticides and PCBs, evidence of the maturation of the technique. The SFE method used was accurate and reproducible. Pre-GC purification of SFE-generated fish extracts to remove co-extracted lipids was not required, thus decreasing sample preparation effort considerably.

Mesenteric tissue obtained from dead ospreys contained much higher concentrations of total PCBs and DDTs than fish fillets. The degraded condition of the osprey tissues prevented assessment of the toxicological significance of these concentrations. Osprey samples also showed evidence of selective elimination of specific PCB congeners.

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