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Macro-analytical Methods Used to Analyze Tissues of the Hawaiian Monk Seal, *Monachus schauinslandi*, for Organochlorine Pesticides, Polychlorobiphenyls, and Pentachlorophenol

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Among the specimens which were processed by the Hawaii Epidemiologic Studies Program (HESP) laboratory as poisoning incidents, were tissues of an adult male Hawaiian Monk Seal, Monachus schauinslandi, which had died while in captivity without known cause. Various tissues of the animal were brought to the laboratory for analyses by the veterinary pathologist, State of Hawaii, Department of Agriculture, to determine whether the death of the monk seal had any connection with exposures to environmental pesticides or pollutants, particularly to organochlorine compounds. The specimens included urine, parts of liver, lung and blubber, an entire testis, and an entire kidney.

The monk seal, an endangered species in Hawaii, is a relatively large animal, the adult male averaging 175 kg (380 lb), TOMICH (1969), and consequently solid tissues from the animal were from correspondingly large organs. Macro methods for representative analyses of these tissues for multiresidues of organochlorine pesticides, polychlorobiphenyls (PCBs) and pentachlorophenol (PCP) were devised. The methods utilize portions of reported analytical protocols for the determination of organochlorines in human and animal tissues and combines them with other techniques associated with pesticide residue analyses. Generally, the tissues with the aid of distilled water and drops of concentrated sulfuric acid are first extracted with hexane or benzene, the separated extractants are then cleaned and fractionated by passage through Florisil columns, and the resulting eluates are analyzed by gas-liquid chromatography (GLC) with electron capture (EC) detection.

METHODS AND MATERIALS

Organochlorine Pesticides and PCBs

Extractions in the devised methods are similar to the extraction of organochlorines from blood serum with hexane according to

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the method by DALE et al. (1966), which is the multiresidue method outlined in the EPA, Manual of Analytical Methods (THOMPSON, ed. 1977). The solid tissues, however, are minced well before extracting them, and larger sample sizes, 5 - 12 g, are used with correspondingly larger amounts of hexane. An extraction is also conducted in a larger culture tube, 25 OD x 180 mm, with teflon-lined screw-cap, and with ca. 5 mL, distilled water, and ca. 10 drops of concentrated sulfuric acid. The minced sample is weighed and placed into the culture tube. Distilled water is added while rinsing the tissue down the culture tube. Acid is then added, followed by hexane. Both water and acid facilitate the extraction with hexane and assist in separating a relatively clean extract. About 15-20 mL hexane are suitable for sample sizes ranging from 5 to 7 g. The amount of hexane used for an extraction may vary and depends on the quantity and consistency of the tissue sample being analyzed, but the total amount used per sample must be accurately measured. Larger quantities of both water and hexane may be used to ensure efficient mixing of the extractant and tissues during slow rotation (ca. 50 rpm) on a Roto Rack^{R}. The extraction mixture is rotated for a period of about 4 h and is allowed to stand, usually overnight. The tube is then spun in a large capacity centrifuge at 2,000 rpm, and the upper hexane layer is collected by means of a disposable pipet. As in the analysis of blood serum, an accurately measured amount of most of the total volume of hexane is collected but not necessarily the entire amount. The extractant is then concentrated to ca. 0.5 mL with a gentle stream of nitrogen gas.

A Chromaflex column, 9 OD x 200 mm long, with a 50-mL reservoir (Kontes 420100, size 22) is packed with Florisil of chromatographically reproducible quality. Florisil, which is purged overnight in an oven at 135°C, allowed to cool while exposed to ambient laboratory temperature, and stored in reagent bottles with screw-caps lined with aluminum foil, is suitable. The column is filled with Florisil, which is retained in the column with a small wad of glass wool to a height of 100 mm after gentle tapping, and the Florisil is topped with 20 mm of anhydrous granular sodium sulfate. Adhering to chromatographic techniques, the column is first washed with 10 mL hexane, the concentrated extract is then placed on the column with washes kept to a minimum 2 mL, and the sample is eluted off the column by the following protocol with each fraction being collected separately: Fraction 1 ..... 10 mL, hexane; Fraction 2 ..... 20 mL, 20% benzene in hexane; Fraction 3 ..... 20 mL, 40% benzene in hexane; Fraction 4 ..... 10 mL, 80% benzene in hexane.

The concentrations of the fractions are adjusted for GLC analysis, EC detection. The quantities of residues are determined by including in the calculations peak heights, sample weights, dilution or concentration factors, and the fractions of total extractants analyzed, as in routine residue analyses of biological extracts by GLC.

PCBs are collected in fraction 1 with the organochlorine pesticides, HCB, aldrin, oxychlordane, trans-nonachlor, p,p'-DDE,

and p,p'-DDT. These compounds usually elute off the column completely in this fraction, particularly PCB's, but may carry over into fraction 2. β-HCH, heptachlor epoxide, dieldrin, and p,p'-DDD are collected in fraction 2, and may carry over into fraction 3 for their complete elution. Fraction 4 is required to elute dieldrin completely off the column. The elution pattern was determined and monitored with recovery runs of some mixed standards of organochlorine pesticides and PCBs.

Five μL of standards and samples were usually injected into the GLC instrument which operated with the following conditions. (1) Tracor Micro-Tek 220 Gas Chromatograph, ^{63}Ni detectors; (2) 2, borosilicate glass columns, 1.8 m x 4.0 mm ID; one column packed with 4% SE-30/6% OV-210 on 80-100 mesh Gas Chrom Q; second column packed with 1.50% OV-17/1.95% QF-1 on 80-100 mesh Gas Chrom Q; nitrogen carrier gas, 70 mL/min; (3) temperatures: column, 203°C; inlet, 233°C; detectors, 280°C; transfer, 250°C. Each fraction was analyzed by GLC on both columns having different resolving characteristics for organochlorine compounds and PCP. All solvents, reagents, and materials were of the quality or were processed for pesticide residue analyses, and standard laboratory glassware were cleaned accordingly.

The macro analytical method for organochlorines in animal tissues was applied without difficulty to all of the solid monk seal tissues except blubber and urine. With blubber, nearly all of the macro samples dissolved in the hexane fractions during the extraction phase and the extracts that resulted were diluted lipid samples. Therefore, the continued processing of these extracts through the Florisil column could not be conducted with the desired results obtained with the other solid tissues. The lipid content of these extracts were first approximated to be 74.7% by evaporating aliquots to constant weight with nitrogen gas and by measuring the weights. 2.8 - 3.0 g Portions of the lipid were then analyzed according to the method for the analysis of organochlorine pesticides and PCBs in adipose tissue by de FAUBERT MAUNDER et al. (1964), MILLS et al. (1963), and as outlined in the EPA Manual of Analytical Methods (THOMPSON, ed. 1977). For urine, 2.00 mL samples were analyzed by conducting the extractions with 6.00 mL hexane and 2 drops of concentrated sulfuric acid in proportionately smaller culture tubes, 16 x 125mm.

Commercial beef and calf liver samples were fortified with a mixed standard of organochlorine pesticides ranging in levels of 15 to 240 pg of HCB, β-HCH, aldrin, oxychlordane, heptachlor epoxide, trans-nonachlor, p,p'-DDE, dieldrin, p,p'-DDD, and p,p'-DDT, and with 3 ng of Aroclor 1260 for recovery runs. The mixture of organochlorine pesticides and Aroclor 1260 were added to control liver simultaneously in some trials and separately in other trials. The quantities of liver used ranged from ca. 5 - 12 g and approximated the actual quantities of monk seal tissues analyzed. For the organochlorine pesticides, the averages of recoveries by the macro method ranged from a low 73% for aldrin up to greater than 100% for β-HCH, and the averages for the other pesticides were 80 to 90%.

For Aroclor 1260, the averages of the recoveries of each of the components corresponding to 7 GLC peaks quantitated, ranged from 80 to 99%.

Pentachlorophenol

The analysis of PCP in solid tissues was conducted by the macro method described for the organochlorines except that benzene was used as the extractant in place of hexane. Also, derivatization (alkylation) of the chlorinated phenol in the benzene extract, an additional step, was conducted with diazomethane as in the analysis of PCP in urine and blood, CRANMER & FREAL (1970), RIVERS (1972), and the EPA Manual of Analytical Methods (THOMPSON, ed. 1977). The derivatization is also outlined in a modification of the analysis of PCP in urine and blood by the HESP laboratory, HESP Annual Report No. 8 (1975). Briefly, benzene extracts are first adjusted with a stream of nitrogen gas (or concentrated by other means without loss of PCP) to ca. 5 mL. Derivatization is then conducted in a high draft-fume hood by adding diazomethane reagent prepared in basic hexane from N-methyl-N'nitro-N- nitrosoguanidine, the reaction mixture is cleaned by passage through a column of Florisil, and the methyl ether of PCP is analyzed by GLC. The derivative is eluted off the Florisil column with 20 mL of 20% benzene in hexane, followed by an additional 20 mL of 40% benzene in hexane to ensure complete elution. Monk seal urine was analyzed using 2.00 mL samples extracted with 6.00 mL benzene and 2 drops of concentrated sulfuric acid, as in routine urine analyses, HESP Annual Report, No. 8 (1975).

The PCP determinations were monitored by concurrent recovery runs with 5-12 g, commercial liver samples fortified with solutions of the sodium salt of PCP (prepared in 0.01 N NaOH). The level of fortification was 100 ng for the solid tissues. For urine, recoveries of 50 and 100 ng levels of PCP (Na salt) from 2.00 mL control samples were conducted and analytical quality control requirements for the laboratory's routine urine analyses were maintained. The recoveries of PCP from these tissues after blank and sample corrections were 70 to 96%.

Blubber extracts in the PCP analyses also required additional cleanup processes before analyzing by GLC. With an original 5 g sample, a benzene fraction readily separated after extraction by slow rotation. Methylation of the separated benzene extract with diazomethane was conducted without diffficulty. However, when the derivatized extract was concentrated, ca. 3 mL of viscous oil, which could not be reduced in volume any further, resulted. This was too much lipid to be cleaned by passage through a microcolumn of Florisil. Therefore, the extract was first processed by liquid-liquid partitioning using large volumes of acetonitrile, hexane and 2% sodium sulfate solution, a modification of the acetonitrile partitioning by TESSARI & SAVAGE (1980), and then passed through a macro Florisil column. With ca. 100 mL, acetonitrile, and hexane washes totalling ca. 20 mL, the oily extract was transferred to a 1-L separatory funnel containing 550 mL 2% sodium sulfate solution and 100 mL hexane. The funnel was then shaken for 2 min and

allowed to stand until the aqueous and hexane layers clearly separated. The lower aqueous layer was drained into another 1-L separatory funnel containing another 100 mL hexane and the liquid-liquid extraction repeated. The aqueous layer which was extracted a second time with hexane was then discarded and the hexane fractions were combined and concentrated to 5 mL by low temperature, rotary flash evaporation. The hexane concentrate was then chromatographically passed through a 25 x 300 mm glass column which was prepared by packing gently with Florisil to a height of 100 mm, topping with 25 mm anhydrous granular sodium sulfate, and prewashing with ca. 100 mL hexane. The methylated PCP was eluted off the column with 100 mL hexane. The eluate was concentrated and adjusted without difficulty for GLC analysis and gave rise to suitable chromatograms.

The devised methods are simple, relatively fast, flexible, and may be modified to improve the analyses or to accommodate new and problem situations. Fractions eluted off the Florisil column may be concentrated again and rechromatographed through freshly prepared Florisil columns to reduce materials which cause interferences and high backgrounds in gas chromatograms. Background materials may also be reduced by using 2% sodium sulfate solution rather than distilled H_2O during extractions of tissues with hexane or benzene. Smaller amounts of original lipid samples may be used to preclude additional cleanup processes. Finally, elution patterns of organochlorines off Florisil columns should be determined for different batches of Florisil and modified accordingly during column chromatography to account for differences in batches.

RESULTS AND DISCUSSION

The results of the analyses of monk seal tissues for organochlorine pesticides and PCBs are shown in Table 1. p,p'-DDE and Aroclor 1260 were the only organochlorines detected in the tissues analyzed. Blubber had the highest levels of both residues, followed by liver and kidney, respectively. Lung and testis had approximately similar but even lower levels. There was no detection in urine. Seven components of Aroclor 1260 which gave rise to the more consistent and prominent GLC peaks were quantitated. Separation of p,p'-DDE which was in the same fraction off the Florisil column as Aroclor 1260 (fraction 1) was not necessary since its GLC peak did not interfere with the 7 Aroclor 1260 peaks, and quantitations with the two materials in the same fraction was not a problem. Figure 1 shows typical chromatograms of fraction 1 and is one of the liver samples. The relative amounts of p,p'-DDE and Aroclor 1260 present were so different that fraction 1 had to be greatly diluted for the quantitation of p,p'-DDE (see Figure 1). The same situation was experienced with the other solid tissues. The results listed in Table 1 are based on total weights of samples analyzed. The results for p,p'-DDE are from the SE-30/OV-210 column and the results for Aroclor 1260 are from the OV-17/QF-1 column. Use of larger amounts of urine in an analysis may have detected very low levels of p,p'-DDE and Aroclor 1260. However, the small quantity of original specimen limited the analyses to 2 mL sample sizes.

TABLE 1. Results of analyses of Hawaiian Monk Seal samples by the macro method, multiresidue screen for organochlorine pesticides and PCBs.

Sample	p,p'-DDE	Aroclor 1260, ppm							
	ppm	Peak: 4	5	6	7	8	11	12	ave.
Liver-1	0.67	0.68	1.2	0.33	1.1	0.62	0.50	0.39	0.69
Liver-2	0.94	0.89	1.4	0.41	1.4	0.82	0.72	0.56	0.88
Liver-3	0.44	0.49	0.79	0.55	0.98	0.38	0.33	---	0.59
Lung-1	0.065	0.058	0.086	0.014	0.086	0.053	0.036	0.035	0.052
Lung-2	0.061	0.084	0.13	0.027	0.13	0.080	0.056	0.048	0.079
Lung-3	0.040	0.043	0.055	0.012	0.057	0.033	0.023	0.016	0.034
Kidney-1	0.058	0.079	0.13	0.04	0.12	0.058	0.050	0.037	0.074
Kidney-2	0.14	0.19	0.28	0.094	0.28	0.11	0.12	0.089	0.17
Testis-1	0.051	0.083	0.12	0.041	0.12	0.057	0.054	0.041	0.074
Testis-2	0.063	0.076	0.11	0.035	0.11	0.055	0.051	0.035	0.068
Blubber-1	13	17	24	9.1	23	8.6	9.0	7.6	14
Blubber-2	13	15	21	7.9	20	7.9	8.5	6.3	12
Urine	N.D.[a]				N.D.				

[a] Not detected

Table 1 includes the results of repeated analyses for each of the solid specimens. Each analysis was conducted with a composite tissue sample prepared by combining samplings cut from different regions of a specimen. The samplings were minced and mixed together well, and the composite which resulted was considered to be representative of the entire specimen or organ being analyzed. For each repetition a separate composite sample was prepared in the same manner and analyzed. An analysis therefore, covered different regions of a specimen and the apparent variation in results for a particular specimen is due to the actual variation in levels of organochlorines in different regions of the specimen and not due to variation in the method of analysis. By following such techniques, an overall analysis of a large specimen was believed to have been possible without having to macerate and sacrifice the entire specimen or organ. For the larger specimens, liver and lung, a second repetition was run.

The monk seal tissues were also analyzed by an alternate micro method for organochlorine pesticides in human or animal tissue and human milk according to the procedure by ENOS et al. (1967), which is outlined in the EPA Manual of Analytical Methods (THOMPSON, ed., 1977). In this method, duplicate 0.5 g samples were analyzed. As in the macro method tissues were first sampled from different

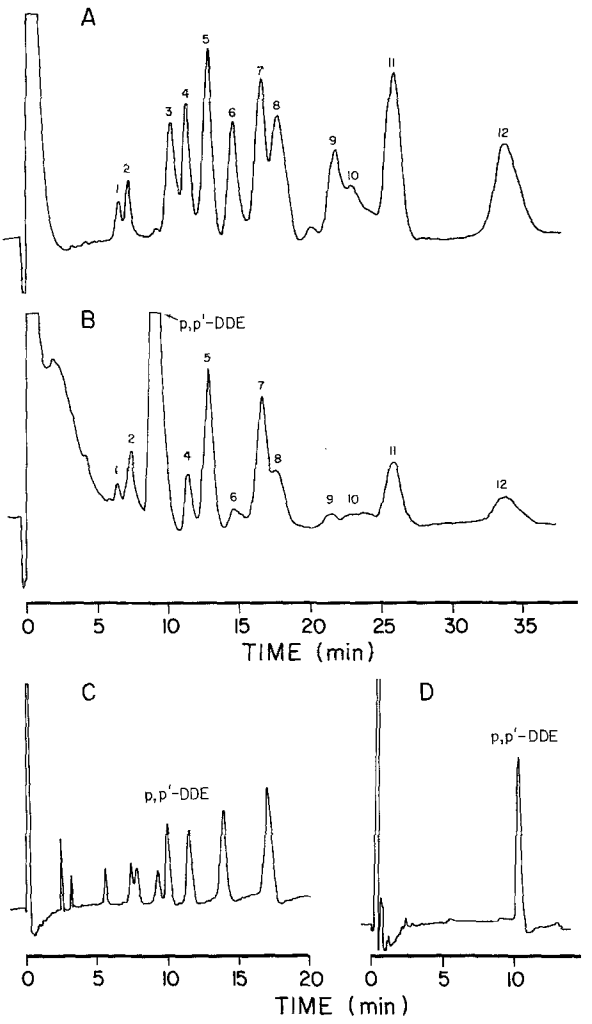

Figure. 1. Gas Chromatograms for standards and for an extract, fraction 1, off Florisil column of a Hawaiian Monk Seal liver sample with p,p'-DDE and Aroclor 1260. A - Aroclor 1260 standard showing 12 characteristic peaks, 5 ng. B - liver extract, fraction 1, concentrated to 5 mL, 5 µL injection; p,p'-DDE present with Aroclor 1260. C - mixture of 10 organochlorine pesticide standards; peaks, left to right, HCB, β-HCH, aldrin, oxychlordane, heptachlor epoxide, trans-nonachlor, p,p'-DDE, dieldrin, p,p'-DDD, and p,p'-DDT; p,p'-DDE, 100 pg. D - same liver extract as B, but diluted 10 x to 50 mL; 5 µL injection.

regions of a specimen. Then the samples were minced and mixed together. From this one composite, two 0.5 g samples were weighed and analyzed. Table 2 shows the results of the duplicate analyses for each solid specimen. The results are consistent with those obtained by the laboratory's multi-residue macro method, considering the variations between samplings of large specimens, and p,p'-DDE and Aroclor 1260 were the only organochlorines detected. As expected, the precisions of the results for a specimen by the micro method are much better than the precisions of the results by the macro method. However, the results in the micro method are for composites of relatively small amounts of tissues, only a little more than 1 g being needed for duplicate analyses, and are consequently representatives of small parts of the specimens and not of the entire specimens.

Further confirmation of the presence of p,p'-DDE and Aroclor 1260 in monk seal tissues

Table 2. Results of analyses by the micro method for organochlorine pesticides and PCBs in tissues of the Hawaiian Monk Seal

Sample	p,p'-DDE ppm	PCB 1260, ppm Peak: 4	5	6	7	8	11	12	ave.
Liver-1	1.2	1.6	2.9	1.0	2.7	1.4	1.2	1.1	1.7
Liver-2	1.3	1.6	2.9	0.98	2.8	1.4	1.3	1.1	1.7
Lung-1	0.072	0.10	0.14	0.064	0.15	0.066	0.063	0.050	0.092
Lung-2	0.075	0.091	0.12	0.048	0.12	0.055	0.051	0.046	0.075
Kidney-1	0.38	0.52	0.81	0.26	0.74	0.31	0.34	0.27	0.46
Kidney-2	0.32	0.44	0.67	0.23	0.62	0.27	0.28	0.22	0.39
Testis-1	0.20	0.35	0.51	0.17	0.48	0.22	0.24	0.18	0.31
Testis-2	0.20	0.34	0.48	0.18	0.46	0.22	0.23	0.18	0.30
Blubber-1	11	10	14	5.8	14	5.6	5.6	4.7	8.6
Blubber-2	9.9	9.2	12	4.8	13	5.9	4.9	4.3	7.8

was conducted by GC/Mass Spectrometry (MS). One liver extract and one blubber extract were selected for the confirmation. p,p'-DDE was separated from Aroclor 1260 by passing the extracts through a Silica Gel 60 column according to the protocol outlined by the Texas Epidemiologic Studies Program, EPA, (unpublished) for the separation of PCBs from organochlorine pesticides, a modification in the analysis of human milk by TESSARI & SAVAGE (1980). The Finnigan Model 3000 operating at 70 electron volts was the instrument used in the GC/MS analyses.

The results for the determination of pentachlorophenol in monk seal tissues, which were conducted separately from the analyses of the organochlorine pesticides and PCBs are shown in Table 3. PCP was detected in all specimens. Urine and blubber contained the greatest amount of PCP, while the solid tissues had very low but nearly equal amounts. Figure 2, are representative gas chromatograms for the PCP analyses.

From this study, we cannot attribute the death of the Hawaiian Monk Seal to the organochlorines and pentachlorophenol which were found in tissues of this mammal. Baseline (normal) data of residue levels for these pollutants in animal tissues in Hawaii are so limited that pathogenic levels in our fauna cannot be predicted. Moreover, it is difficult to even speculate whether the residues detected in the monk seal tissues were due to exposures of earth's background (ambient) atmosphere or to exposures of the mammal's immediate (specific) environment. The possibility still remains

Table 3. Results of the analysis of pentachlorophenol in Hawaiian Monk Seal Specimens

Specimen	PCP, ppb
Liver	1.0
Lung	1.5
Kidney	Trace (0.8)
Testis	Trace (0.9)
Blubber	77
Urine	66

though that these residues or their parent forms may be implicated as contributing factors toward endangering the monk seal which is native to the Hawaiian Archipelago. With the exception of PCP, the levels of the residues found in monk seal appear to be higher than what would be expected as normal, particularly in blubber where results are also based closer to lipid content. Interestingly though, the detection of PCP in animal tissues other than in urine and blood after the review by BEVENUE & BECKMAN (1967) and the work by BARTHEL et al.(1969) is believed not to have been extensively studied, except for an isolated determination of PCP in human adipose tissue by SHAFIK (1973).

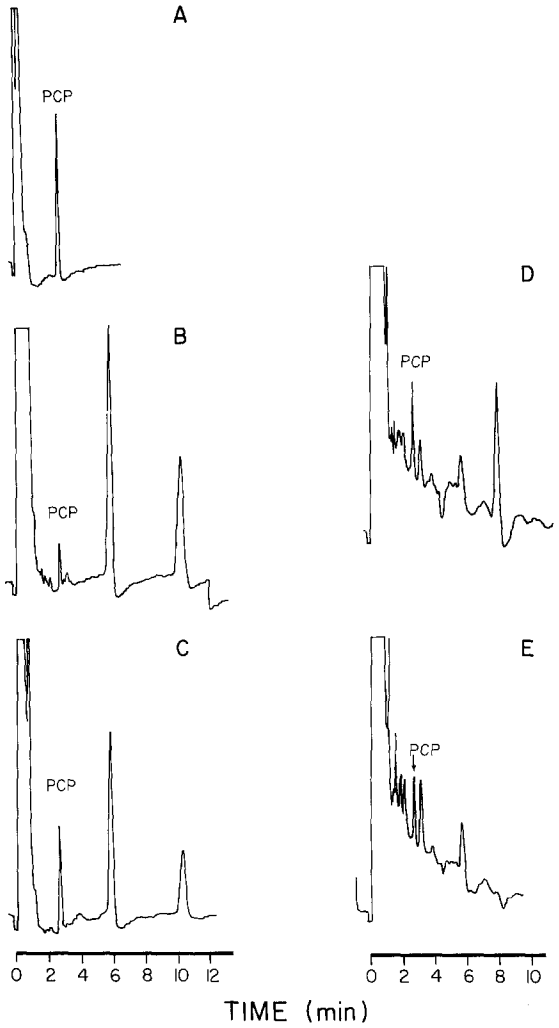

Figure 2. Typical gas chromatograms, A-E, for the analysis of pentachlorophenol in Hawaiian Monk Seal tissues. A - PCP standard, 30 pg; B - lung extract; C - same lung extract as B, but fortified with PCP standard; D - kidney extract; E - testis extract.

Although PCP is water soluble and easily excreted from animals, it apparently is translocated throughout the system and most likely stored, over a period, in the lipids of tissues.

The macro methods followed in this study, however, are streamlined without loss of low detection limits and accuracy, and are convenient in handling great numbers of variable specimens. Large specimens which are difficult to sacrifice entirely for analysis

due to size or specimens of tissues which must be preserved, such as those from endangered species, are particularly suitable. Since the monk seal analyses, the macro methods have been applied to human tissues, mainly liver, kidney, fat, and brain of infants, with reproducible and confirmed results for multiresidues of organochlorine compounds and PCP. Also, these recent analyses have been carried out with an abbreviated elution protocol for the Florisil column and without additional cleanup.

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