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Modified Multiresidue Method for Chlordane, Toxaphene, and Polychlorinated Biphenyls in Fish

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As one of the original Community Studies Laboratories in 1965–79, we participated in the National Human Monitoring Program (Price 1972; Yobs 1972) for nearly ten years. During that period we adhered to the specified EPA methodology (Thompson 1974) for chlorinated pesticides in adipose tissue which requires extraction of fat from connective tissue, acetonitrile partitioning and cleanup by Florisil column chromatography prior to quantitation by electron-capture gas chromatography (ECD-GC). The liquid-liquid partitioning step is time-consuming and affords low recoveries of some residues such as HCB and mirex (PAM 1983). The simultaneous presence of polychlorinated biphenyls (PCB) and chlorinated pesticides, especially chlordane and toxaphene from lake fish (Tai et al. 1982; Ribick et al. 1982), in the 6% Florisil eluate complicates GC determinations and can cause misinterpretation of analytical data (Bevenue 1970) whether packed columns or capillary columns are used (Benecke et al. 1985).

For our studies of human exposure to environmental contaminants due to consumption of Lake Michigan sport fish, we developed a semimicro analytical procedure that eliminates the need for acetonitrile partitioning for cleanup of fish extracts. The modifications of the usual procedure are described in the text, including silica gel column chromatography (Picer and Ahel 1978) to achieve additional cleanup of the 6% Florisil fraction and separation of PCBs from chlorinated pesticides.

MATERIALS AND METHODS

Distilled-in-glass iso-octane and ethyl ether (2% ethanol as preservative) were purchased from Burdick and Jackson. All other solvents were either Baker Analyzed® or MCB OmniSolv® reagents. Anhydrous, granular Na_2SO_4 (Mallinckrodt #8024), 60–100 mesh Florisil (PR grade, Floridin Co.) and 70–230 mesh Silica Gel 60 (E. Merck #7734) were stored at least 24 hrs at 130°C.

The U.S. Environmental Protection Agency (Health Effects Research Laboratory, Research Triangle Park, NC) supplied the following analytical reference standards: Aroclor 1254 (PRL-FDA lot 1543), Aroclor 1260 (PRL-FDA lot 1544), Aroclor 1016 (lot D413), β-BHC, *cis*- and *trans*-chlordane, *cis*-and *trans*-nonachlor, oxychlordane, p,p´-DDT, p,p´-DDE, p,p´-DDD, heptachlor epoxide, dieldrin, HCB, mirex and toxaphene. Chemical names of these compounds are given in Analytical Reference Standards (Watts 1981). Quality control chicken fat (SRM #74) also was supplied by US EPA Health Effects Research Laboratory.

The following glassware equipment was purchased from Kontes of Illinois, Evanston, IL: chromatography column (K-420540-0233), 300 mm x 22 mm i.d., with Teflon stopcock and coarse fritted glass plate; chromatography column (K-420290), 400 mm x 19 mm i.d., with Teflon stopcock, coarse fritted glass plate and 250-mL top reser-

voir; chromatography column (K-420100–0023), 200 mm x 9 mm i.d., with 50-mL solvent reservoir; Kuderna-Danish evaporative concentrator assembly (K-570000–0250), fitted with 25-mL concentrator tube (K-570050–2525).

The gas chromatograph/data system used was a Varian 3700 equipped with constant current ^{63}Ni electron capture detectors and Varian 8000 AutoSamplers. The following columns were used: a) 183 cm x 2 mm i.d. glass packed with 3% SE-30 on 80–100 mesh Gas-Chrom Q; b) 183 cm x 2 mm i.d. glass packed with 1.5%OV-17/1.95%OV-210 on 80–100 mesh Gas-Chrom Q; c) 183 cm x 2 mm i.d., glass packed with 4% SE-30/6% OV-210 on 80–100 mesh Gas-Chrom Q (Applied Science Laboratories, Inc., State College, PA).

The following procedure provided laboratory support for the evaluation of human exposure to toxaphene-contaminated Great Lakes fish. Standardize sampling of fish, e.g., coho salmon, chinook salmon and lake trout from Lake Michigan, for skin-on fillet as defined by the Food and Drug Administration (PAM 1982). Section the edible portion into small pieces, preferably 1/2-in. cubes and freeze. Grind frozen pieces with a slight excess of dry ice in a blender (Waring EP-1) as described by Benville and Tindle (1970). Transfer the pulverized mixture to a suitable container, loosely covered, and store in a freezer until all the dry ice is sublimed. It is essential that the sample be completely homogenized. The fish homogenate should be dry and free-flowing for rapid, quantitative extraction of pesticides and PCBs when using the column technique (Hesselberg and Johnson 1972). Thoroughly mix 20g fish homogenate with 80g anhydrous Na_2SO_4 in a 250-mL beaker until the sample is dry. Allow mixture to stand several additional minutes to acquire a sandy texture. Lightly pack the dry mixture into a 400 mm x 19 mm chromatography column. Rinse beaker with 10 mL of 50% ethyl ether/petroleum ether (v/v), quantitatively transfer rinse to column and follow with remaining 190 mL of extracting solvent at an adjusted flow rate of 3–5 mL/min. Collect extract in a tared 250-mL beaker and evaporate to dryness on top of a moderately heated water bath under a gentle stream of nitrogen. Determine the lipid weight.

Dissolve 0.5 g fish lipid in 5 mL hexane. Prepare a 22-mm i.d. chromatographic column containing 102 mm (after settling) of activated Florisil topped with 1.5 cm of anhydrous Na_2SO_4. Prewet column with 40–50 mL petroleum ether. Place 250-mL Kuderna-Danish assembly with 25-mL graduated concentrator tube under column to receive eluate. (Note: From this point do not allow the solvent level to go below Na_2SO_4 layer.) Pipette lipid solution onto the column. After bulk of the extract passes into the column, rinse container with 2 x 5 mL petroleum ether and transfer to column. Elute column with 200 mL of 6% ethyl ether/petroleum ether eluant at about 5 mL/min (Fraction F-1). When the last of the eluant reaches the top of the Na_2SO_4 layer, change Kuderna-Danish receivers and continue elution with 200 mL of 20% ethyl ether/petroleum ether (Fraction F-2). Add two carborundum boiling chips to each K-D assembly and concentrate eluates to a small volume on a water bath. Continue final evaporation to less than 1.0 mL after attaching modified micro-Snyder columns to collection tubes. After cooling to ambient temperature, disconnect tubes and rinse joints with a little hexane. Adjust first eluate (F-1) to 1.0 mL and proceed with silica gel fractionation. The second eluate (F-2) contains dieldrin and may require additional cleanup.

Pesticides and PCBs are separated on the Silica Gel 60 column. Prepare 9-mm i.d. chromatography column by layering with prewashed glass wool plug, 5.0 g Silica Gel 60 and 1.5 cm anhydrous Na_2SO_4. After prewetting column, pipette first eluate (F-1) onto top of column, follow with 3 x 1 mL hexane rinses of container and discard this eluate. Elute column with 15 mL of hexane as last rinse enters Na_2SO_4 and collect as Fraction SG-1 (ca. 15 mL) in 25-mL Mills tube. As last of first eluant enters Na_2SO_4, elute column with additional 20 mL of hexane, change collection tube and

collect Fraction SG-2. Add 20 mL of benzene to column as last of hexane enters sodium sulfate and continue collection of Fraction SG-2 (ca. 28 mL). Benzene causes Silica Gel 60 to become translucent. When translucency front reaches within 1.5 cm of glass wool, change collection tube and collect remaining benzene as Fraction SG-3 (ca. 12 mL). Add a boiling chip to each tube, attach micro Snyder columns, and condense to ca. 0.5 mL on a water bath. After cooling, detach micro-Snyder column slightly and rinse joints with iso-octane dropwise (ca. 0.2 mL). Adjust volume to exactly 2 mL with iso-octane, mix by vortex, and transfer 1.0 mL of sample to the autosampler vial. Inject 5 μL of sample into the gas chromatograph operated under the following conditions: temperature of injector and detector are 240 and 320^{0}C, respectively; other parameters are listed in Table 1.

Table 1. Analytical parameters for pesticides and PCBs

Eluate	Analytes	G.C. Column	mL N_2/min.	Oven temperature/ Program rate
SG-1 SG-3	HCB, Mirex *cis*–Nonachlor Oxychlordane Hept. epoxide	1.5% OV-17/ 1.95% OV-210	20	198^{0}C isothermal
SG-2	PCB p,p´DDE	3% SE-30	30	5 min 160^{0}C, 17 min at 3^{0}C/min, 10 min final hold at 210^{0}C
SG-3	p,p´DDT	4% SE-30/ 6% OV-210	20	198^{0}C isothermal
SG-3	p,p´DDE β-BHC Toxaphene Chlordane *trans*–Nonachlor	3% SE-30	30	5 min 160^{0}C, 17 min at 3^{0}C/min, 10 min final hold at 210^{0}C

Table 2. Retention profile of porcine fat[1] on Florisil and silica gel columns

Adsorbent	Florisil (18–20g)	Silica Gel 60 (5g)		
Fraction	1	1	2	3
Eluent(mL)[2]	6% E.ether/ pet. ether(200)	Hexane(12)	Hexane(28)	Benzene(12)
Lipids eluted:				
Mean	96.5mg	<0.01mg	<0.01mg	<0.1mg
Range	24–137mg	–	–	–

[1] Ten replicate samples of 0.5 g fat
[2] Volume of eluate collected

RESULTS AND DISCUSSION

In order to eliminate the liquid-liquid partitioning step of the usual multiresidue method (AOAC 1980), we reduced the amount of extractable fat to avoid exceeding the capacity of cleanup columns. Our method combines the cleanup capacity of two adsorbents, Florisil and Silica Gel 60. Ten replications of 500 mg porcine fat were eluted through the macro Florisil column with 200 mL ethyl ether/petroleum ether (6+94), and eluates were evaporated finally to dryness in preweighed aluminum pans. The amount of fat retained on Florisil varied from 363 to 476 mg. When the eluted fat residues were redissolved in hexane and transferred to Silica Gel 60 columns, no measurable amount of fat was observed in the three silica gel eluates (Table 2). This and subsequent investigations confirmed that the combination of Florisil and Silica Gel 60 columns can retain at least 500 mg of fat under conditions previously presented. The second Florisil eluate, however, required additional cleanup before determination of dieldrin in the final extract by ECD-GC.

Polychlorinated biphenyls, chlordane and toxaphene currently found in lake fish are complex mixtures of chlorinated compounds that coelute on most GC packed columns and complicate qualitative and quantitative determinations of these residues as well as organochlorine pesticides. Unless a separation of PCBs is undertaken, PCBs will mask toxaphene and chlordane, which are likely to be quantitated as PCBs (Table 3 and Figures 1–4). For example, 2,2′3,4,4′,5′-hexachlorobiphenyl (peak 174, Webb and McCall 1973) and one of the toxaphene components coelute with p,p′-DDT on the 3% SE-30 column so that results for either analyte would tend to be higher prior to silica gel chromatography. Even after separation on silica gel, the 4% SE-30/6% OV-210 GC column (Table 1) is recommended for quantification of p,p′-DDT (Fig. 5). Quantification of p,p′-DDE, on the other hand, appears to be straightforward and uncomplicated. It is usually the largest GC peak and elutes in both SG-2 (PCBs) and SG-3 (pesticides) fractions. The proportion of p,p′-DDE (10–50%) appearing in SG-2 fraction depends on conditions such as compactness of silica gel in the column, room humidity and the particular lot of Silica Gel 60. Coelution of chlordane and toxaphene with p,p′-DDE on the 3% SE-30 column does not occur. In biological samples, PCB congeners 98 and 104 (Webb and McCall 1973) are usually minor constituents as compared to p,p′-DDE (RRT=100).

Table 3. Quantification[1] of PCB, p,p′-DDE and p,p′-DDT in the presence of chlordane and toxaphene in fish extracts before and after Silica Gel 60 chromatography

Sample	PCB		p,p′-DDT		p,p′-DDE		Chlordane	Toxaphene
	Before	After	Before	After	Before	After		
E	5.0	3.9	0.60	0.23	1.10	1.20	0.39	1.16
F	4.2	3.4	0.71	0.27	1.40	1.30	0.49	1.78
G	8.4	6.3	1.30	0.52	2.50	2.40	0.91	0.24
H	7.5	5.6	1.10	0.73	2.60	2.50	0.99	2.93
I	3.1	1.8	0.38	0.26	0.75	0.78	0.45	1.34
J	7.2	5.8	0.91	0.50	2.50	2.40	0.74	2.82

[1] Parts per million

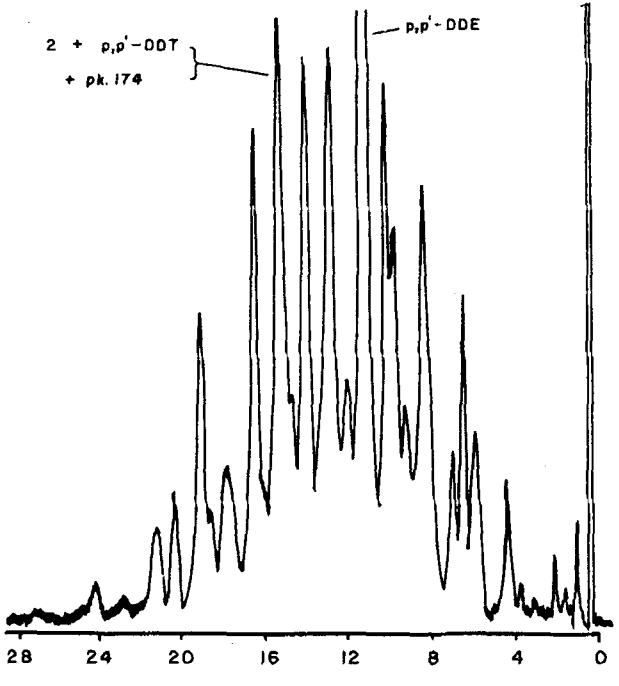

Figure 1. Combined silica gel fractions SG-2 (PCBs) and SG-3 (pesticides) on 3% SE-30 column. Number 2 corresponds to toxaphene peak. GC parameters given in Table 1.

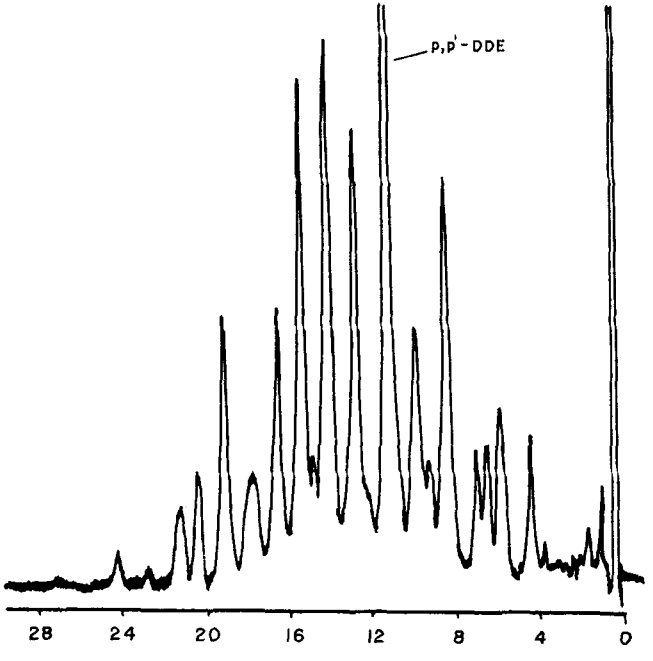

Figure 2. Silica gel fraction SG-2 showing PCBs and p,p'−DDE on 3% SE-30 column. See Table 1 for GC parameters.

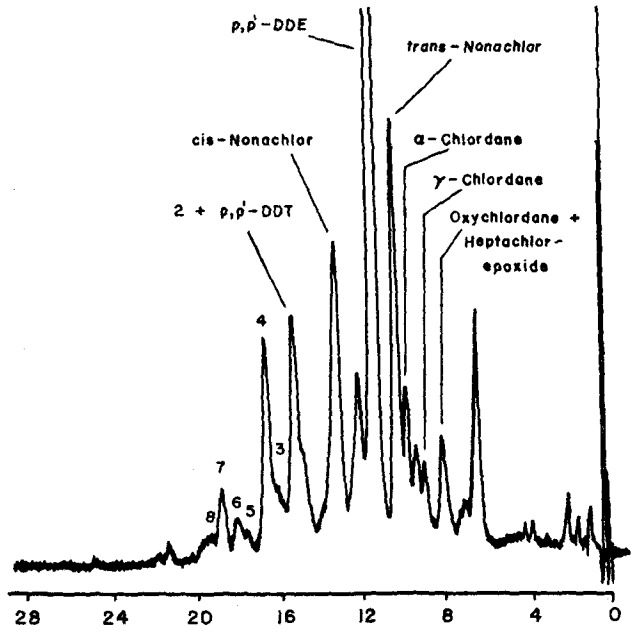

Figure 3. Silica gel fraction SG-3 on 3% SE-30 column. Numbers correspond to peaks of toxaphene in Figure 4. See Table 1 for GC parameters.

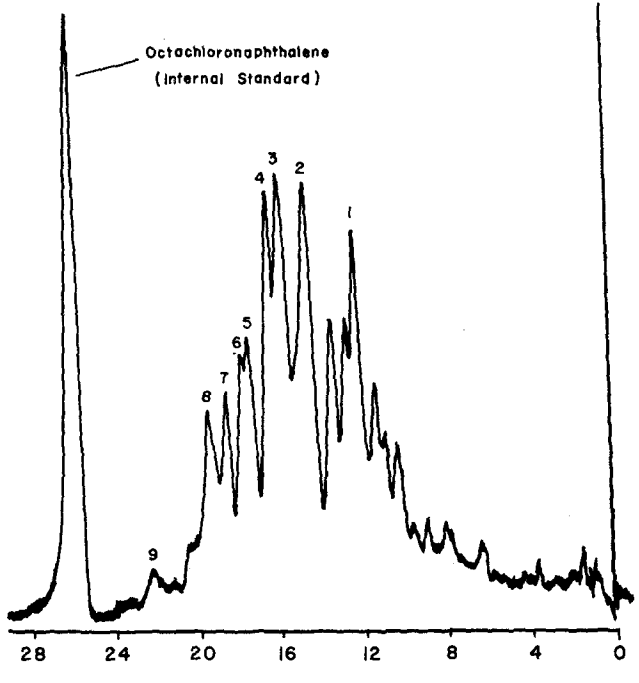

Figure 4. Toxaphene reference standard on 3% SE-30 column. See Table 1 for GC parameters.

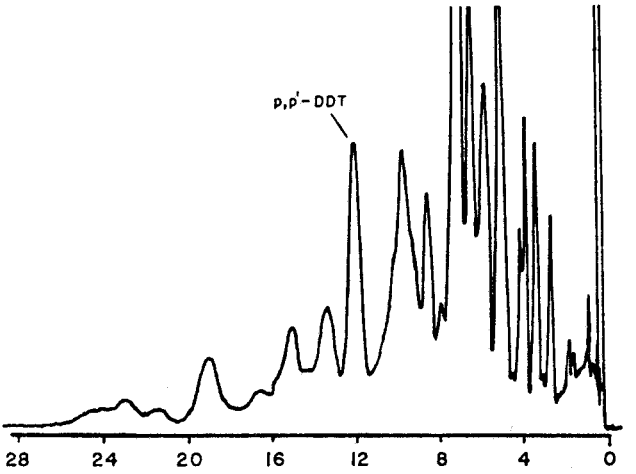

Figure 5. Silica gel fraction SG-3 on 4% SE-30/6% OV-210 column showing p,p'−DDT without interference. See Table 1 for GC parameters.

An interlaboratory control sample was prepared by thorough blending of ground skin-on fillets of three lake trout from Lake Michigan. Our analytical data are compiled in Table 4. The narrow range of extractable lipid values is indicative of the homogeneous condition of the sample matrix as well as consistency of the column extraction procedure. In terms of low relative standard deviations, except in the case of dieldrin, the method is highly reproducible. Attempts to clean up Florisil F-2 fraction by gel permeation chromatography resulted in low recoveries ($\leq$70%).

A unique feature of this method is the visual demarcation point between SG-2 (PCBs) and SG-3 (pesticides) silica gel fractions, and that is the translucency effect on silica gel as benzene moves down the column. By changing collection tubes when translucency front reaches a designated point in the column, separation of PCBs from chlorinated pesticides other than p,p´-DDE can be consistent from sample to sample. Although some laboratories refrain from the use of benzene, we resolved the problem of potential health hazard by performing column chromatography adjacent to a bench top exhaust system.

The analytical performance on an EPA quality assurance sample of spiked chicken fat is illustrated in Table 5. Recoveries of PCBs and chlorinated pesticides are excellent under conditions described previously. Elution of HCB in SG-1 silica gel fraction provides a greater recovery than the usual procedure (PAM 1983).

Gas chromatography/mass spectrometry with negative ion detection (GC/NI-MS) capability was used to confirm the presence of chlordane, toxaphene, other chlorinated pesticides and PCBs in fish homogenates (Scheel 1984). In addition, GC/NI-MS confirmations demonstrated the effectiveness of Silica Gel 60 column chromatography in its ability to successfully separate PCBs (SG-2), namely, Aroclors 1254 and 1260, from chlorinated pesticides, in this case the chlordane compounds and toxaphene (Table 6). Generally, only the higher molecular weight or late eluting isomers of toxaphene and technical chlordane are found in fish.

The method described for the analysis of fish samples exhibits high recoveries of chlorinated pesticides and PCBs without requiring liquid-liquid partitioning. One disadvantage is that low molecular weight PCBs such as Aroclor 1016 (1242) do not

Table 4. Replicate analysis of fish homogenate.[1]

Analyte	Mean[2] (N)	Range[2]	Standard Deviation	%RSD
Oxychlordane	0.025 (10)	0.023–0.027	0.0014	5.5
γ-Chlordane	0.035 (10)	0.033–0.036	0.0013	3.7
α-Chlordane	0.080 (10)	0.077–0.084	0.0023	2.9
trans–Nonachlor	0.134 (10)	0.128–0.142	0.0039	2.9
cis–Nonachlor	0.068 (10)	0.058–0.071	0.0039	5.8
Toxaphene[3]	0.949 (10)	0.794–1.178	0.1150	12.1
Hept.Epoxide	0.038 (9)	0.036–0.039	0.0084	2.2
p,p′-DDE	0.628 (10)	0.594–0.640	0.0151	2.4
p,p′-DDT	0.094 (10)	0.087–0.098	0.0031	3.3
p,p′-DDD	0.120 (9)	0.108–0.127	0.0057	4.8
HCB	0.010 (10)	0.009–0.011	0.0008	8.2
Dieldrin	0.061 (10)	0.042–0.078	0.0145	23.6
PCB (Aroclor 1254)[4]	1.194 (10)	1.146–1.225	0.0289	2.4
%Lipid	17.1 (10)	16.95–17.25	0.084	0.5

[1] Consists of skin-on fillets of three Lake Michigan lake trout.
[2] Parts per million whole weight basis.
[3] Toxaphene quantified by area of peaks eluting after *trans*–nonachlor.
[4] Determined according to Webb and McCall (1973).

Table 5. Replicate analysis of fortified chicken fat (EPA-SRM#74)

Analyte	Formulation	Mean[1]	Range[1]	Std. Dev.	%RSD	%Mean Recovery
Oxychlordane	150	145.3	142–149	2.00	1.4	96.9
trans–nonachlor	200	195.5	191–203	4.28	2.2	97.8
Hept.Epoxide	75	75.6	74–78	1.08	1.4	100.8
p,p′-DDE	3750	3722.0	3536–3832	86.2	2.3	99.3
p,p′-DDT	400	382.5	378–390	4.60	1.2	95.6
β-BHC	200	190.7	183–199	4.85	2.5	95.4
HCB	40	35.5	34.5–36.4	0.63	1.8	88.8
Dieldrin[2]	100	107.9	101–128	7.88	7.3	107.9
PCB as 1254	1500	1634.0	1599–1685	30.6	1.9	108.9

[1] Parts per billion. Ten replications.
[2] Separate sample through GPC prior to Florisil cleanup column.

separate sharply and another is the presence of p,p′-DDE in two silica gel fractions.

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Table 6. Elution profile[1] and recoveries of reference standards after Florisil and Silica Gel 60 column chromatography.

Analyte	Amount[2](ng)	SG-2		SG-3	
		Mean Recovery(%)	RSD(%)	Mean Recovery(%)	RSD(%)
Toxaphene[3]	682	0.4	23.9	96.2	2.1
γ-Chlordane	20	–		95.5	2.7
α-Chlordane	20	–		95.4	2.3
trans-Nonachlor	40	–		95.0	2.8
cis-Nonachlor	40	–		97.1	2.1
Aroclor 1016	3200	56.0	3.8	45.8	4.2
Aroclor 1260	3200	100.9	1.2	–	–

[1] None of this group eluted in SG-1 fraction.
[2] Number of replications = 10
[3] Quantitated on the basis of peak heights of 10 major peaks.

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