Exhibit #
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Aroclor® 1016: Toxicity to and Uptake by Estuarine Animals[1/]

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proportion of peaks in liver

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ABSTRACT

Bioassays were conducted to determine the acute toxicities of the polychlorinated biphenyl (PCB) Aroclor 1016 in flowing sea water to American oysters (Crassostrea virginica), brown shrimp (Penaeus aztecus), grass shrimp (Palaemonetes pugio) and pinfish (Lagodon rhomboides) and to determine its chronic toxicity to and uptake and retention by pinfish. Acute 96-hour EC50/s were: oysters, 10.2 µg/ℓ; brown shrimp, 10.5 µg/ℓ; grass shrimp, 12.5 µg/ℓ. The PCB was not toxic to pinfish at 100 µg/ℓ for 96 hours but significant mortality occurred when pinfish were exposed to 32 µg/ℓ of Aroclor 1016 for 42 days. Pinfish exposed to 1 µg/ℓ for 56 days accumulated the chemical with maximum concentrations attained in whole-fish by 21 to 28 days. Maximum whole-body residue (wet weight) was 17,000 x the nominal concentration in test water. Tissue alterations such as severe vacuolation in the pancreatic exocrine tissue surrounding the portal veins occurred in pinfish exposed to 32 µg/ℓ of Aroclor 1016 for 42 days.

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Polychlorinated biphenyls (PCB's) have been used industrially for over forty years (Broadhurst, 1972), and recently there has been concern about their environmental impact. Because of this concern, manufacture and sale of most PCB's was discontinued and sales restricted for uses that are not likely to produce environmental contamination. A new PCB, Aroclor 1016, is now manufactured in the United States for sale to capacitor manufacturers as a substitute for all other PCB's. This new PCB is similar to Aroclor 1242, except that amounts of isomers containing five or more chlorine atoms per biphenyl group have been considerably reduced. Domestic sales of Aroclor 1016 increased from about 3.3×10^6 pounds in 1971 to 20.9×10^6 pounds in 1972 (W.B. Papageorge, personal communication[2/]).

Our study was conducted to determine the acute toxicity of Aroclor 1016 to the American oyster (Crassostrea virginica), brown shrimp (Penaeus aztecus), grass shrimp (Palaemonetes pugio), and pinfish (Lagodon rhomboides) and to determine its chronic toxicity to and uptake and retention by pinfish.

2/ W. P. Papageorge, Monsanto Industrial Chemicals Company. 800 N. Lindbergh Boulevard, St. Louis, MO 63166, USA.

MATERIALS AND METHODS

Test animals

Test animals were collected near the Gulf Breeze Laboratory and acclimated to laboratory conditions for at least seven days before exposure. If mortality exceeded 1% in the 48 hours immediately preceding the test or if abnormal behavior was observed during acclimation, the animals were not used. Oysters tested were from 35 to 55 mm in height; brown shrimp, 11 to 26 mm rostrum-telson length; grass shrimp, 20 to 32 mm rostrum-telson length; and pinfish 27 to 84 mm standard length. Animals were not fed during acute toxicity tests but they could obtain plankton from the unfiltered sea water. In the chronic exposures and the uptake and retention test, pinfish were fed commercial fish food that contained no detectable PCB (<.01 µg/g).

Acute and chronic tests

Acute toxicity to Aroclor 1016 was determined by exposing ten individual animals to 1, 10 or 100 µg/ℓ for 96 hours in each of two 20ℓ aquaria. Each experiment was conducted twice. The PCB was dissolved in acetone or polyethylene glycol 200 and metered at 30 or 0.1 ml/hr, respectively, into unfiltered sea water that entered each aquarium at 75 ℓ/hr. Two control aquaria received the same quantities of water and solvent. Temperature and salinity of the water flowing into aquaria in replicated tests were similar (± 10%).

Chronic toxicity of Aroclor 1016 to pinfish was determined in three experiments, each lasting 42 days. In each experiment, 50 fish were

placed in each 90ℓ aquarium that received 140 ℓ/hr of sea water. The PCB, dissolved in polyethylene glycol, was metered into the water at 0.083 ml/hr in the first two experiments and 1.04 ml/hr in the third experiment. Control aquaria received the same quantity of water and solvent.

The same exposure techniques that were used in the first two chronic toxicity experiments were used to determine: (1) the rate of uptake and retention of Aroclor 1016 in pinfish exposed to 1 μg/ℓ for 56 days and (2) the rate of depuration of Aroclor 1016 by pinfish in PCB-free water for 56 days.

Effect of Aroclor 1016 was assessed by measuring percentage reduction in shell growth of exposed oysters as compared to control oysters (Butler, 1962), by determining mortality in shrimps and fish, and by pathological examination of chronically exposed fish.

Histopathological examination

Dr. J. A. Couch, pathobiologist at this laboratory, examined viscera from live pinfish from the third 42-day exposure. Viscera were fixed either in 10% neutral buffered formalin or in Davidson's fixative. Those fixed in Davidson's were stored in 70% ethyl alcohol until processed for paraffin sections (7μ) and stained with Harris' hematoxylin and eosin (HHE) or Periodic Acid Schiff's (PAS). Viscera fixed in 10% neutral buffered formalin were processed for frozen sections (12μ) and stained with oil Red O and hematoxylin.

Chemical analyses

Concentrations of Aroclor 1016 in water and animals were determined by electron capture gas chromatography. Unfiltered water samples from each concentration were analyzed once during the 96-hour exposures and weekly during longer exposures. Concentrations in animals that survived the 96-hour exposures were determined as whole-body residues. At the conclusion of each chronic exposure, surviving pinfish were dissected and PCB residues in flesh, flesh and scaleless skin, and remaining tissue determined. Residues in all tissues were summed to compute concentrations of Aroclor 1016 in whole fish. The same procedure was followed in the uptake and retention study, except fish were removed for analysis at selected intervals during exposure and depuration. Also, at the end of the 56-day exposure, brain, gills, heart and liver were removed from exposed fish for residue analysis. All fish samples were composites of 10 individuals.

Tissue samples that weighed more than 5 g were prepared for analysis by mixing them with anhydrous sodium sulfate in a blender. The mixture was extracted for 4 hours with petroleum ether in a Soxhlet apparatus. Extracts were concentrated to approximately 10 ml and transferred in 3- to 4-ml portions to a 400 x 20 mm chromatographic column that contained 76 ml of unactiviated Florisil. After each portion settled in the column, vacuum was applied until all solvent was evaporated. This was repeated with three 5 ml

rinses. The residue was eluted from the column with 70 ml of a 9:1 mixture (v/v) of acetonitrile and distilled water. The eluate was evaporated to dryness and the residue transferred to a Florisil column (Mills, et al., 1963) with petroleum ether. Aroclor 1016 was eluted with 6 percent ethyl ether in petroleum ether.

Tissue samples that weighed less than 5 g were analyzed by a modification of the micro method described in the Pesticide Analytical Manual, Volume III (U.S. Food and Drug Administration, 1970). The samples were weighed into a size 23 Duall tissue grinder and extracted three times with 5 ml portions of acetonitrile. The acetonitrile was flooded with 15 ml of 2% (w/v) sodium sulfate in distilled water and extracted with three 5 ml portions of hexane. The hexane was evaporated to approximately one ml and transferred to a 9 x 200 mm Chromaflex column with a 50 ml reservoir that contained 3.3 g of Florisil topped with 3.3 g of anhydrous sodium sulfate. Aroclor 1016 was eluted with 20 ml of 5% ethyl ether in hexane and adjusted to an appropriate volume for analysis.

Water samples were extracted with petroleum ether, dried with anhydrous sodium sulfate and adjusted to an appropriate volume for analysis.

All samples were analyzed by electron capture gas chromatography using a 15 x 3.2 mm glass column packed with 2% OV-101 on 100-120 Gas Chrom Q. Nitrogen flow rate was 25 ml/min, the oven temperature

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was 190 C, and the injector and detector temperature was 210 C.

Aroclor 1016 was quantitated by comparing the total height of all peaks in the sample with the total height of all peaks in a standard of known concentration. Recoveries were greater than 80%; data were not adjusted for recovery. All tissue residues were determined on a wet weight basis.

RESULTS AND DISCUSSION

Acute (96-hr) exposure

Aroclor 1016 was acutely toxic to the estuarine organisms tested (Tables 1 and 2). Shell growth in oysters was inhibited greatly by exposure to 100 µg/ℓ for 96 hours. Sensitivities of brown shrimp and grass shrimp were similar and pinfish was the least sensitive species. Acute toxicities of Aroclor 1016 to oysters, brown shrimp and pinfish were similar to that of Aroclor 1242 to these species[3/] and Aroclor 1254 to oysters, pink shrimp (Penaeus duorarum) and pinfish (Duke et al., 1970).

All animals accumulated Aroclor 1016 (Table 1). The quantities accumulated depended on the exposure concentrations and not on species. Whole-body concentrations in live animals ranged from 440 to 4,200 x the nominal concentration in test water and 1,200 to 6,700 x the measured concentration in test water.

3/ P. R. Parrish, unpublished data.

Chronic (42-day) exposure

Toxicity of Aroclor 1016 to juvenile pinfish was greater in tests lasting six weeks than in 96-hour exposures. Pinfish seemed unaffected by 10 µg/ℓ or less, but died in concentrations of 32 and 100 µg/ℓ (Table 3). Mortality began in the second week of exposure. Fifty-percent mortality did not occur in the third exposure, for example, until the 33rd day at 32 µg/ℓ and the 18th day at 100 µg/ℓ. Delayed mortality of pinfish was also observed with Aroclor 1254 (Hansen et al., 1971).

Most of the fish that died in the 42-day exposure exhibited symptoms of poisoning, such as changed appearance and behavior. Initially, their color darkened, they stopped feeding, and they swam erratically with bodies inclined downward. Difficulty in swimming progressed until the fish swam with their tails and dorsal fins breaking the water surface. Finally, the fish lost equilibrium, swam upside down, and died. Affected fish became vulnerable to attack by other pinfish in the tank. In the third experiment, the majority of dying fish exposed to 32 and 100 µg/ℓ lost scales, skin and, finally, flesh in front of the dorsal fin forming lesions sometimes as deep as the neural spine. This condition did not occur in fish exposed to 32 µg/ℓ in the second experiment.

Hepatocytes in liver sections of thirteen control fish showed no unusual characteristics when stained with HHE and PAS or oil Red O. The hepatocytes from six fish demonstrated only a moderate PAS-positive

reaction, indicating moderate-to-light glycogen reserves. In seven other control fish, liver sections stained with oil Red O also showed a broad range of lipid patterns. Structure of livers of control fish was normal, being tubulosinusoidal in nature, with disseminated pancreas prominent along the course of the portal vein (Fig. 1).

Eight fish exposed to 10 µg/ℓ of Aroclor 1016 for 42 days showed no pathologic microscopic visceral characteristics distinguishable from control fish. Half of the fish samples were paraffin-processed; half were prepared for frozen sections.

Pinfish exposed to 32 µg/ℓ of Aroclor 1016 had several liver and pancreatic alterations that distinguished them from control fish and fish exposed to 10 µg/ℓ. Tissues from eight fish were examined; half were paraffin-processed and half were studied as frozen sections. Hepatocytes appeared slightly enlarged and more basophilic than in the control fish (Fig. 2). Normal liver cord orientation was somewhat altered and PAS-positive granules accumulated at the edge of the pancreatic acinar tissue in the liver (Fig. 3). Less lipid material existed in the livers of fish exposed to 32 µg/ℓ than in control fish. This contrasts with the heavy, abnormal accumulation of lipid in the livers of spot exposed to 6 µg/ℓ of Aroclor 1254 for 30 days (Couch, 1973). The most remarkable alteration in the pinfish was the occurrence of severe vacuolation in the pancreatic exocrine tissue surrounding the portal veins (Figs. 2,3). This

vacuolation was distinguishable from normal secretory vacuoles and deposits in pancreatic tissue from control fish because those in exposed fish were small, abundant, and contained no secretory granules (Fig. 1).

After the first exposure, 16 pinfish from each control or Aroclor 1016-contaminated aquarium were held in PCB-free water and the salinity reduced to determine if ability of pinfish to survive osmotic stress had been impaired by exposure to sublethal concentrations of Aroclor 1016. (Aroclor 1254 reduced the ability of pink shrimp, Penaeus duorarum, to withstand stress of decreased salinity--D. R. Nimmo, personal communication.[4/]) Salinity was lowered from an initial salinity of 28 o/oo by 50% on each of four consecutive days (14, 7, 3.5 and 1.8 o/oo). None of the fish died until the salinity fell below 2 o/oo. At that salinity, mortalities of control and PCB-exposed fish were similar.

Pinfish exposed for 42 days to concentrations of Aroclor 1016 between 0.1 and 32 µg/ℓ stored the chemical in proportion to the concentration in test water (Table 3). Concentrations in whole fish ranged from 11,000 to 24,000 x the nominal concentration in the test water and 14,000 to 55,000 x the measured concentration in test water, whereas

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4/ D. R. Nimmo, Gulf Breeze Environmental Research Laboratory, Gulf Breeze, Florida 32561.

in PCB-free water (Table 4). After 56 days of depuration, concentrations in whole fish decreased by 51%. In an earlier study, spot which had accumulated Aroclor 1254 lost 66% after 56 days in PCB-free water (Hansen et al., 1971).

We examined chromatograms to compare the proportions of nine peaks of Aroclor 1016 in reference standards (Fig. 4) with those peaks from water and pinfish tissue samples (Table 5). Chromatograms of standards, tissue spikes and water samples were similar, while chromatograms of standards and tissue samples were dissimilar. Chromatograms of Aroclor 1016 from different pinfish tissues (flesh, flesh and skin, and rest) were similar. Chromatograms from all tissues had smaller early eluting peaks, numbers 1, 2 and 3. Reduction in early eluting peaks of Aroclor 1254 from shrimp and fish tissue was noted by Nimmo et al. (1971). The relative proportions of the nine peaks found in chromatograms from pinfish tissues early in the 56-day exposure, late in the exposure and throughout the 56-day depuration period were similar. We do not know whether differences between chromatograms from reference standards and chromatograms from tissue samples reflect alterations in Aroclor 1016 molecules, differential solubility, or other factors. It is unlikely, however, that the PCB molecules were altered because no change in relative proportions of peaks was noted throughout the 112-day experiment.

The potential environmental hazard of a chemical is dependent upon its likelihood of entering the environment and its potential

hazard to organisms. Aroclor 1016 is similar to other PCB's in its toxicity to and uptake and retention by estuarine animals. Therefore, its substitution for other PCB's reduces environmental hazard only if the policy of restricting sales for uses not likely to produce environmental contamination is continued.

ACKNOWLEDGEMENTS

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TABLE 1

Acute toxicity to and uptake of Aroclor 1016 by American oysters (Crassostrea virginica), brown shrimp (Penaeus aztecus), grass shrimp (Palaemonetes pugio), and pinfish (Lagodon rhomboides) in 96-hour exposures. Effect is expressed as percent reduction in shell growth in oysters and death in shrimps and fish. Whole body residues are from animals alive at end of exposure period.

SPECIES	TEST CONCENTRATION (µg/ℓ) Nominal	Measured	EFFECT (%)	WHOLE-BODY RESIDUE (wet weight, µg/g)
C. virginica	Control	ND*	0	ND*
	1	0.6	10	4.0
	10	7.2	38	32.
	100	58.	93	95
P. aztecus	Control	ND	0	ND
	1	0.9	8	3.8
	10	8.9	43	42.
	100	33.	100	-
P. pugio	Control	ND	8	ND
	1	0.4	33	1.1
	10	9.4	38	22.
	100	38.	93	44.
L. rhomboides	Control	ND	2	ND
	1	0.8	5	2.2
	10	6.9	0	21
	100	56.	18	65.

*ND - not detectable: <0.05 µg/ℓ in water; <0.1 µg/g in tissue.

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TABLE 2

Acute toxicity of Aroclor 1016 to American oysters (Crassostrea virginica), brown shrimp (Penaeus aztecus) and grass shrimp (Palaemonetes pugio). Effect is expressed as percent reduction in shell growth in oysters and death in shrimps.

	96-HOUR EC50 (µg/ℓ)	TEMPERATURE (C)		SALINITY °/oo	
	Nominal	Mean	Range	Mean	Range
C. virginica	10.2	30	25-32	29	26-31
P. aztecus	10.5	31	29-32	29	28-30
P. pugio	12.5	30	29-32	29	25-30

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TABLE 3

Toxicity and uptake of Aroclor 1016 by pinfish (Lagodon rhomboides) exposed for 42 days in three separate experiments.

TEST CONCENTRATION (µg/ℓ)		MORTALITY %	CONCENTRATION IN FISH (µg/g, wet weight)		
Nominal	Measured		Flesh	Flesh & skin	Whole fish
Control	ND[1]	36	ND	ND	ND
0.1	0.1	16	0.7	0.8	2.4
1.0	0.8	48	5.1	6.3	11.
10.0	3.0	38	60.	90.	166.
Control	ND	12	0.5	0.6	0.5
1.0	0.9	16	4.0	6.0	17.
3.2	2.5	16	34.	39.	65.
10.0	7.0	28	63.	76.	170.
32.0	13.	44[2]	140.	180.	620.
Control	ND	6	ND	ND	ND
10.0	6.8	6[2]	23.	49.	111.
32.0[3]	21.	50[2]	30.	48.	106.
100.0[3]	59.	50[2]	38.	72.	205.

[1] ND = not detectable: <0.05 µg/ℓ in water; <0.1 µg/g in tissue.

[2] Mortality significantly greater than in control fish, $\alpha = 0.01$.

[3] Exposure terminated and tissues analyzed when 50% of the fish died: 33 days at 32 µg/ℓ and 18 days at 100 µg/ℓ.

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TABLE 4

Concentrations of Aroclor 1016 (µg/g wet weight) in pinfish (Lagodon rhomboides) exposed to 1 µg/ℓ of this PCB. Each sample consisted of tissues from ten fish.

DAYS OF EXPOSURE	CONCENTRATION		
	Flesh	Flesh and skin	Whole fish
0	ND[1/]	ND	ND
3	0.8	0.9	1.6
7	1.6	3.4	3.9
14	2.3	3.3	6.5
21	3.2	4.3	9.7
28	3.0	6.8	25.
42	4.0	6.0	17.
56	5.9	8.3	17.
Depuration			
14	4.1	7.8	13.5
28	3.5	6.6	9.3
56	2.2	3.9	6.6

1/ ND - not detectable; <0.1 µg/g.

TABLE 5

Percentages of the nine measured peaks from chromatograms of Aroclor 1016 reference standards and from tissues of pinfish exposed to 1 g/ of Aroclor 1016 for 56 days and then held in PCB-free water for 56 days.

	PERCENTAGE OF PEAK[1]									CHROMATO-GRAMS
	1	2	3	4	5	6	7	8	9	
Reference standard	12.4	11.3	10.0	23.5	13.3	6.4	8.1	8.8	6.2	8
All tissues through 14 days of exposure	2.4	5.3	1.8	39.6	7.7	10.3	12.1	9.4	11.4	7
All tissues, days 21-56 of exposure	2.5	5.0	2.1	33.2	7.8	11.9	11.3	10.3	15.9	12
All tissues during 56-day depuration	1.0	1.7	0.3	38.2	2.8	15.0	12.4	11.9	16.7	7

[1] Determined as $\frac{\text{peak height}}{\text{sum of nine peaks}} \times 100$.

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LEGEND FOR FIGURES

FIG. 1. Section of normal liver from control pinfish. The vacoluas in hepatocytes are results of extraction of lipid and/or glycogen during paraffin processing. Note the normal pancreatic exocrine tissue containing normal secretory deposits (arrows). (X450).

FIG. 2. Section of liver from pinfish exposed to 32 µg/ℓ Aroclor 1016 for 42 days. The hepatocytes appear relatively dense with more prominent nuclei than in Fig. 1. Note the lack of lipid or glycogen vacuoles in hepatocytes. Small, abnormal vacuoles in pancreatic exocrine tissue (arrows) distinguish fish exposed to 32 µg/ℓ from control fish. (X450).

FIG. 3. Pigment deposition (PAS-positive) between pancreatic exocrine tissue and live parenchyma (arrow). Note nature of small vacuoles in pancreatic tissue. This tissue is from fish exposed to 32 µg/ℓ Aroclor 1016. (X1000).

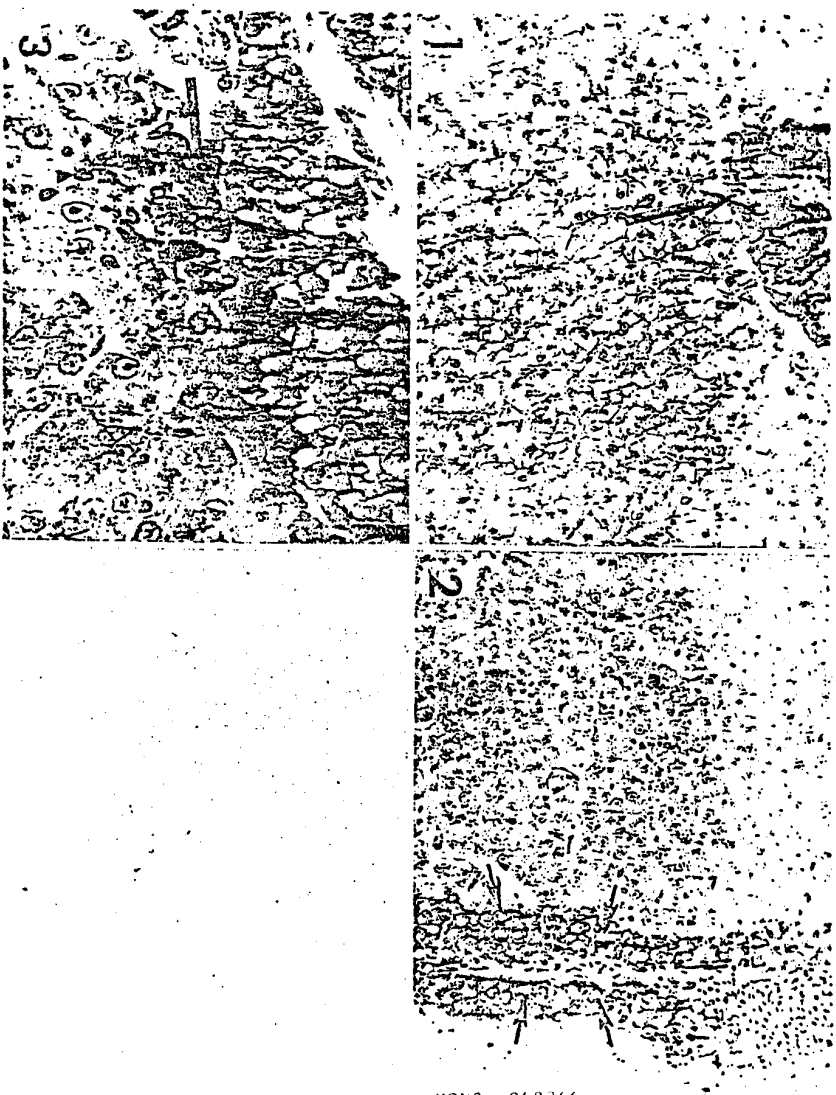

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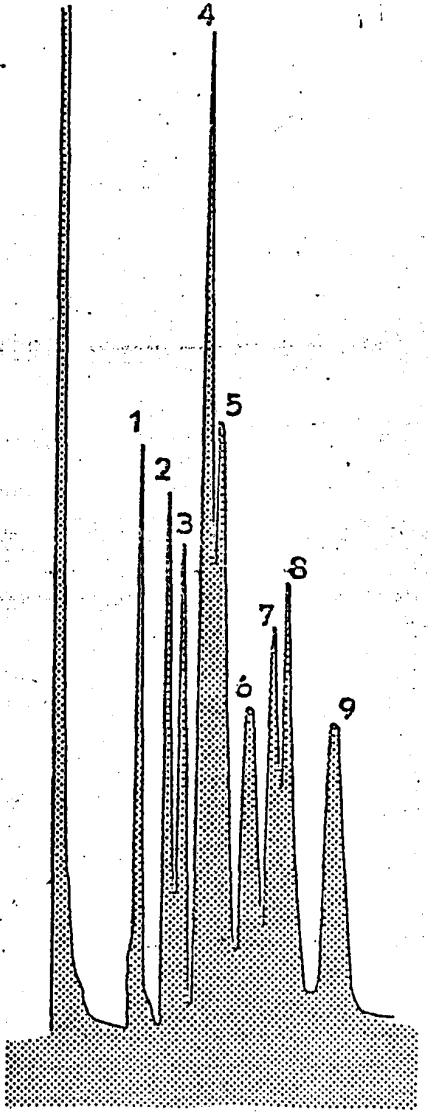
FIGURE 4

Chromatogram of nine peaks of Aroclor 1016 reference standard. Operating conditions: gas flow nitrogen 25 ml/min; injection and detector temperature 210 C; oven temperature 190 C; H^3 electron capture detector; 152.4 x 0.32 cm glass column packed with 2% OV-101 on 100-120 Gas Chrom Q.



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