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MEASURING THE OXYGENATED METABOLITES OF CHLORINATED HYDROCARBONS

Oxygenated or secondary metabolites of synthetic chlorinated hydrocarbons including DDTs and PCBs have been correlated with chronic toxicity in sea animals (Brown et al. 1982). However, the kinds and amounts of these compounds present in samples are difficult to determine when the usual procedures are used. We have added to our extraction procedure a heat-catalyzed base-hydrolysis step that makes it possible to extract oxygenated metabolites for gas chromatographic analysis, and the validity of these measurements has been confirmed by GC/MS.

Using this extraction procedure and an electron-capture gas chromatograph, we have found previously unreported compounds in sediments and animals from the southern California Bight. The oxy-metabolites of DDTs in marine organisms and sediments collected both from (relatively) clean and contaminated regions of coastal southern California made 35 to 99% of the total chlorinated hydrocarbons (parent compounds + oxy-metabolites); PCB oxy-metabolites comprised 89 to 99% of this total. Determination of the concentrations of oxy-metabolites in monitoring programs may be important because these are the major form of xenobiotic hydrocarbons present in the environment and also the form which can result in chronic toxicity.

BACKGROUND

Enzymes of the mixed-function oxidase (MFO) system catalyze the oxidation of a variety of substrates in organisms. Laboratory studies have shown these substrates to include xenobiotic compounds such as polychlorinated biphenyls (PCBs) and benzo(a)pyrene (Bend et al. 1977; Jerina and Daly 1974; Den Tonkelaar and Van Esch 1974). As a consequence of MFO catalysis, nucleophilic positions on substrate

molecules are oxygenated to highly reactive epoxide intermediates which can conjugate spontaneously or enzymatically with cellular molecules, including detoxifying agents such as glutathione or glucuronic acid (Jerina and Daly 1974). If these detoxifying agents are depleted, the highly reactive intermediates may conjugate with macromolecules such as enzymes, DNA, RNA, or metal-binding proteins, with resultant chronic toxicity (Gillette et al. 1974). It has been suggested that such conjugation with genetic material could be central to the etiology of cancer (Bend et al. 1977; Thakker et al. 1979).

Recent laboratory studies (Roubal et al. 1978; Collier et al. 1980; Varanasi et al. 1979; Varanasi and Gmur 1980) have indicated that oxygenated metabolites may not be excreted to the extent previously thought, but may actually accumulate within organisms. These laboratory studies have shown that at equilibrium, oxygenated metabolites are present at concentrations up to ten times higher than their parent compounds. However, there is a lack of information available on the concentrations of these metabolites in samples collected directly from contaminated environments.

METHODS

Surface sediments (0-2 cm) were collected at a depth of 60 m from two stations: Station PV 7-3, approximately 2 km offshore of the Palos Verdes Peninsula adjacent to the Joint Water Pollution Control Plant (JWPCP) outfall, and Station SMB 2-3, a less contaminated area located 20 km NW of the Palos Verdes outfall in Santa Monica Bay. Shrimp *(Sicyonia ingentis)* and California spotted scorpionfish *(Scorpaena guttata)* were collected by otter trawl at the same sample locations as the sediments. Animals were dissected under clean laboratory conditions immediately after collection, and all samples were kept frozen at -20°C until analysis.

Approximately 1-5 g of sample was homogenized in a 20-ml aliquot of pesticide-quality acetone using a high-speed homogenizer. The homogenizer blades were then rinsed twice with 20-ml aliquots of acetone. The aliquots were combined, and 60 ml of 2% NaOH solution was added to the homogenate. We then extracted the sample three times for the base extractable parent compounds by adding 50 ml of pesticide quality n-hexane and vigorously shaking for 2 min, following with centrifugation at approximately 1000 X g to separate the layers. The top (n-hexane) layers were collected, and the bottom (aqueous) layer was saved for the next step. The hexane layers were combined and cleaned up on activated Florisil (heated at 750°C for 4 hours) and analyzed for parent compounds.

The aqueous fraction was then processed through a heat-catalyzed

base-hydrolysis step to hydrolyze the conjugated metabolites. This was accomplished by heating the samples in a 90°C water bath for 30 min. Once the aqueous fraction had cooled, the pH was adjusted to approximately 8 by the addition of hydrochloric acid (NOTE: in the samples used for comparing the extraction with and without hydrolysis (Tables 1 and 2), the pH was adjusted to <2). We then extracted the sample with 30% ethyl ether in n-hexane by shaking vigorously for 2 min and separating the layers by centrifugation, followed by similar extractions with 15% ethyl ether in n-hexane and pure n-hexane. Finally, the sample was condensed using roto-evaporation to approximately 50 ml and analyzed for oxy-metabolites--except for p,p'-DDA which is only extractable at a pH <2. For the results shown in Tables 1 and 2, the p,p'-DDA was derivitized by roto-evaporating the samples to dryness and then adding 5 ml of methylating agent (5 mg of 3-methyl-1-p-tolyltriazene/1 ml of ethyl ether). The ether was removed using a stream of nitrogen, and the extract was dissolved in 30% ethyl ether in n-hexane.

Analysis for DDT and PCB parent compounds and p,p'DDA was accomplished using a Tracor MT220 GC equipped with an electron capture detector and a 1.8-m X 2-mm-ID glass column packed with 1.5% OV17 + 1.95% QF1 on 80-100 mesh Gaschrom Q. The column temperature was held at 200°C with a nitrogen flow of 20 ml/min. Analysis for the oxy-metabolites p,p'-DDOH; 2,2',5'-trichloro-4- biphenylol; 2',3',4',5'-tetrachloro-4-biphenylol; 2',3,3',4',5'-pentachloro-2-biphenylol and 2',3',4',5,5'pentachloro-2-biphenylol (purchased from Ultra Scientific Corp.) was accomplished using a Varian 4600 GC equipped with an electron capture detector and a 30-m DB-5 fused silica capillary column with a 0.25-mm ID (J & W Scientific) and a helium carrier with a flow velocity of 25 cm/sec. The temperature was programmed from 150 to 250°C at 4°/min. Confirmation of the oxygenated metabolites was accomplished using a Dupont DP1 gas chromatograph/mass spectrometer (GC/MS) equipped with a capillary column identical to that described above and a MX-Series Hewlett Packard computer with an NBS library which contains the spectra of 30,000 compounds.

It is important when attempting to confirm the presence of a compound to analyze as pure a sample as possible. In the extraction used in determining the results in Tables 1 and 2, the samples were extracted for oxy-metabolites at a pH of <2 according to Gold et al.(1980). The natural fatty acids that were also present in the samples due to the pH of <2 during this extraction interfered with the analysis and overshadowed the spectra of the oxygenated metabolites (Figure 1). To alleviate this problem, additional samples were extracted for oxy-metabolites at a pH of approximately 8. This change in the technique was very successful at removing the fatty acid components from our extracts; however, it failed to extract the metabolite p,p'-DDA, which is only extractable at a pH <2.

Table 1. Mean (± 1 standard deviation; n = 5) concentrations of DDT parent compounds and their oxygenated metabolites comparing samples extracted with and without hydrolysis.

Sample Description	Station	Mean Concentration (mg/wet kg) Parent DDT Compounds[a]	Oxygenated DDT Metabolites[b]	Metabolites/Total Compounds[c] X 100 (%)
Shrimp muscle				
No hydrolysis	SMB 2-3	0.031 ± 0.014	<0.01	0
Hydrolysis			10 ± 7	99
No hydrolysis	PV 7-3	0.85 ± 0.38	<0.01	0
Hydrolysis			4.8 ± 1.7	85
Scorpionfish liver				
No hydrolysis	SMB 2-3	14 ± 11	<0.01	0
Hydrolysis			13 ± 7	48
No hydrolysis	PV 7-3	28 ± 10	<0.01	0
Hydrolysis			15 ± 9	35
Sediment				
No hydrolysis	SMB 2-3	0.067 ± 0.011	<0.01	0
Hydrolysis			0.48 ± 0.92	88
No hydrolysis	PV 7-3	6.3 ± 2.7	<0.01	0
Hydrolysis			26 ± 14	80

[a] o,p'-DDE + p,p'-DDE + o,p'-DDT + p,p'-DDT + o,p'-DDD + p,p'-DDD.
[b] p,p'-DDA + p,p'-DDOH.
[c] Parent compounds + oxygenated metabolites.

RESULTS

The oxygenated metabolites of DDTs and PCBs were not detectable in any samples without inclusion of heat-catalyzed base-hydrolysis (Tables 1 and 2). With the inclusion of this step, the oxy-metabolites of DDT (DDA + DDOH) represented from 35 to 98% of the total chlorinated hydrocarbons (parent compounds plus oxy-metabolites) present in

Table 2. Mean (± 1 standard deviation; n = 5) concentrations of Aroclor 1254 and its oxygenated metabolites comparing samples extracted with and without hydrolysis.

Sample Description	Station	Mean Concentration (mg/wet kg) Parent PCB Compounds[a]	Oxygenated PCB Metabolites[b]	Metabolites/Total Compounds[c] X 100 (%)
Shrimp muscle				
No hydrolysis	SMB 2-3	0.016 ± 0.009	<0.1	0
Hydrolysis			1.3 ± 0.6	99
No hydrolysis	PV 7-3	0.039 ± 0.027	<0.1	0
Hydrolysis			0.38 ± 0.86	91
Scorpionfish liver				
No hydrolysis	SMB 2-3	2.0 ± 1.3	<0.1	0
Hydrolysis			18 ± 20	90
No hydrolysis	PV 7-3	1.9 ± 0.5	<0.1	0
Hydrolysis			16 ± 10	89
Sediment				
No hydrolysis	SMB 2-3	0.011 ± 0.003	<0.1	0
Hydrolysis			4.0 ± 1.8	99
No hydrolysis	PV 7-3	0.22 ± 0.09	<0.1	0
Hydrolysis			12 ± 11	98

[a] Aroclor 1254.
[b] 3,3',5,5'-tetrachloro-4,4'-biphenylol + 2',3',4',5'-tetrachloro-4-biphenylol + 2',3,3',4',5'-pentachloro-2-biphenylol + 2',3',4',5,5'-pentachloro-2-biphenylol.
[c] Parent compounds + oxygenated metabolites.

sediments, shrimp muscle, and scorpionfish liver from either station (Table 1). The oxy-metabolites of PCBs represented from 89 to 99% of this total (Table 2).

Figures 2-4 contain the total ion chromatograms of the six oxy-metabolite standards analyzed by this lab. These chlorinated oxy-metabolites are ideal for GC/MS analysis because their fragmentation patterns are mainly the molecular ions which have a natural isotopic

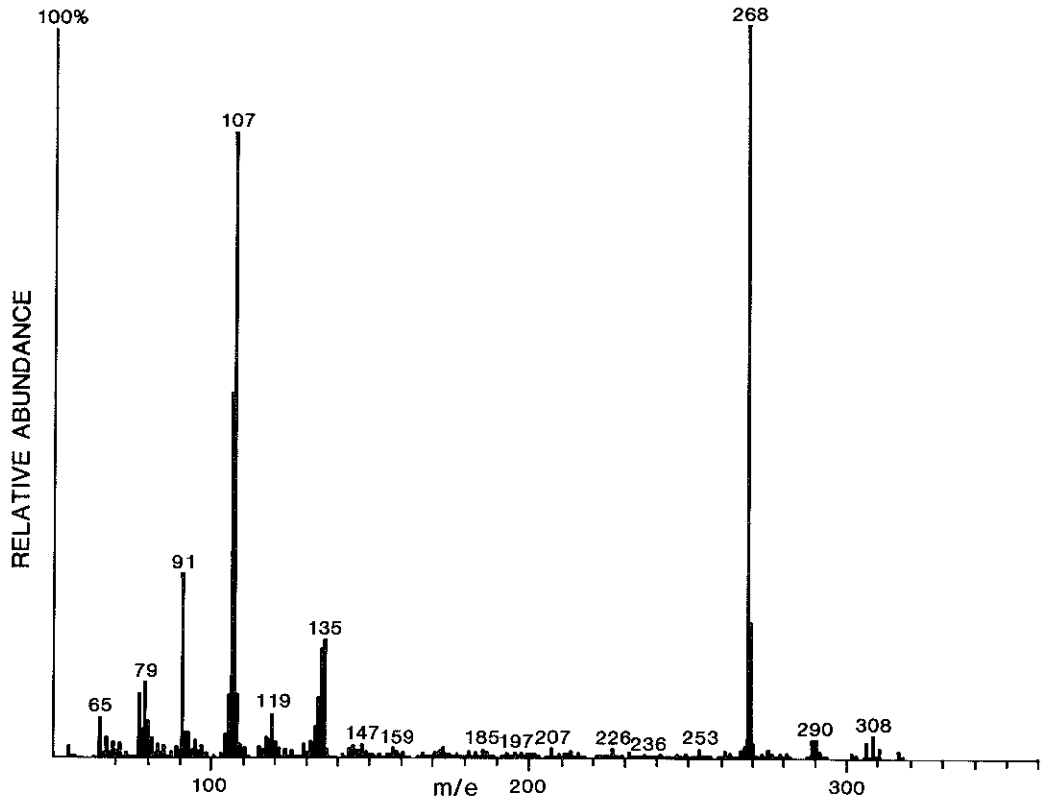

Figure 1. Total ion chromatogram of a scorpionfish liver extracted for oxy-metabolites at pH = <2, demonstrating the inteference caused by the presence of biogenic compounds in the extract. The retention time of this spectrum corresponds to that of 2',3',4',5'-tetrachloro-4-biphenylol (Figure 3b).

abundance ratio that is easy to identify. Figures 5-8 are the total ion chromatograms of a scorpionfish liver extract. These spectra are very similar to the standard spectra, indicating qualitatively that the compounds identified in this sample are the same as those in the standards.

To test the extraction efficiency of the technique described above (see METHODS), one water sample and three scorpionfish livers were split and spiked with the compounds of interest. The results of this extraction test, presented in Table 3, indicate reasonable extraction efficiencies for the oxy-metabolites using this technique. However, these recoveries can probably be improved by adjusting the solvent volumes or type.

To ensure that the heat-catalyzed base-hydrolysis step used did not create oxygenated metabolites from any parent compounds that might be remaining in the samples, we spiked water with standards of p,p'-DDE,

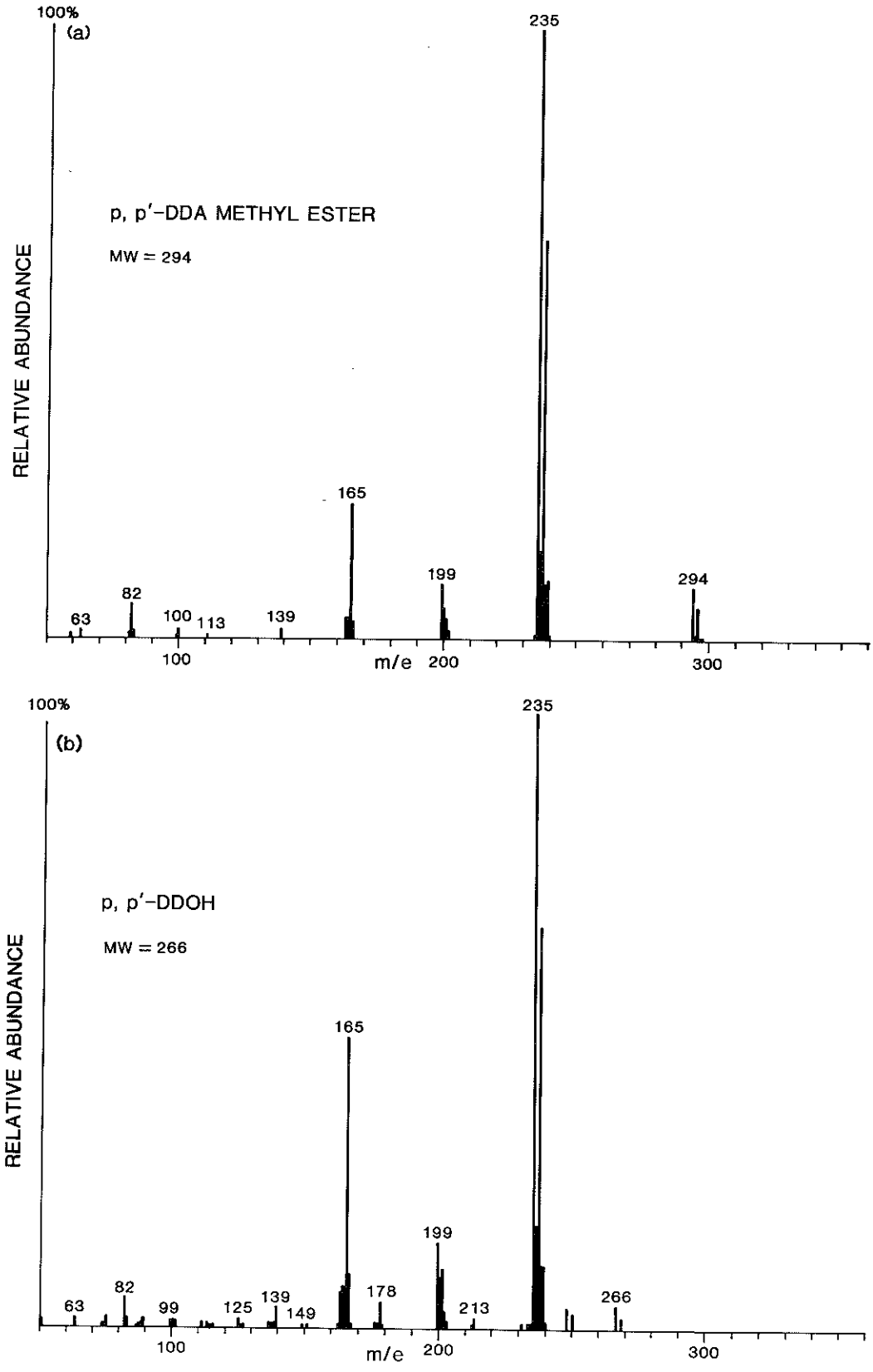

Figure 2. Total ion chromatograms of a) p,p'-DDA and b) p,p'-DDOH standards.

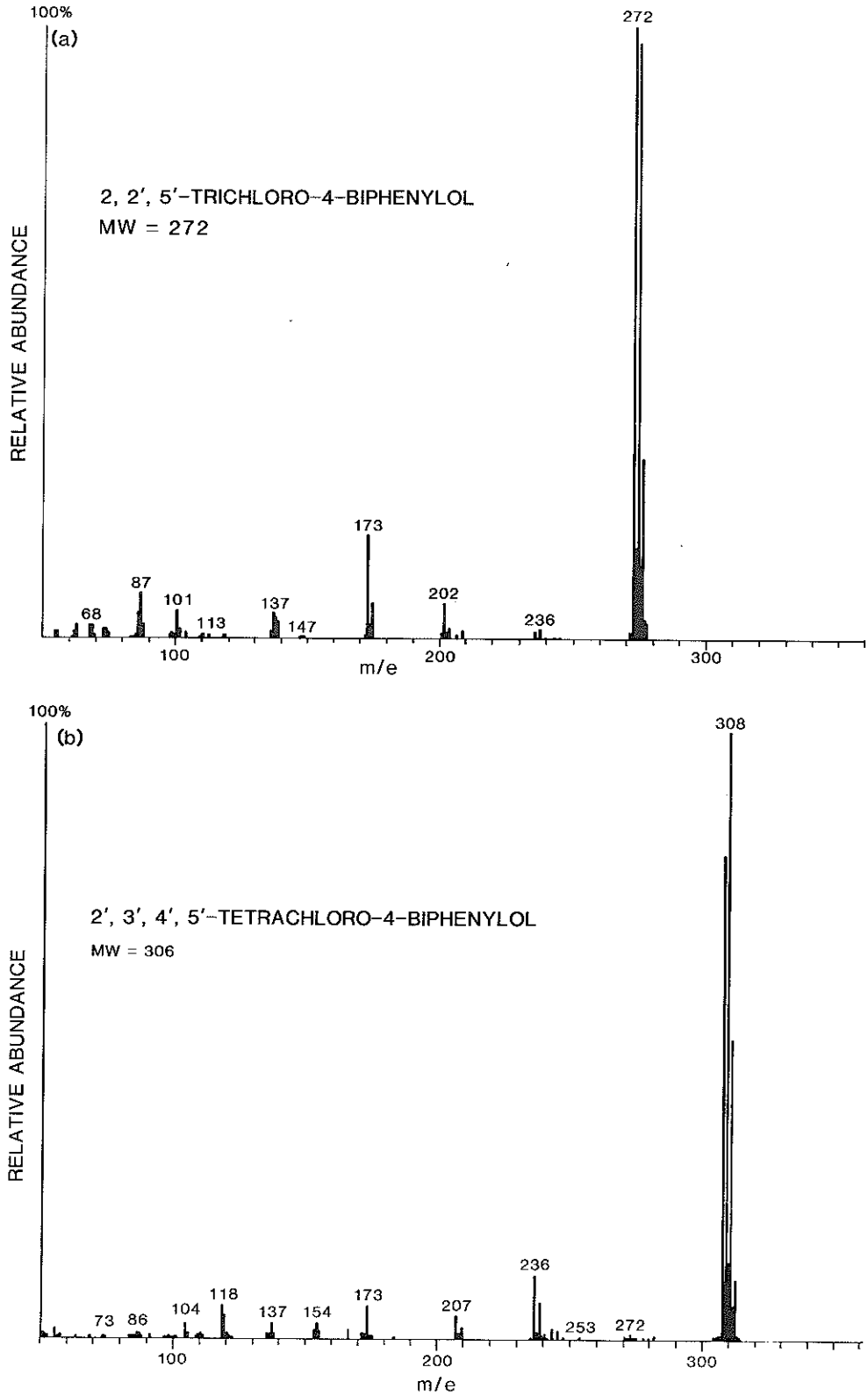

Figure 3. Total ion chromatograms of a) 2,2',5'-trichloro-4-biphenylol and b) 2',3',4',5'-tetrachloro-4-biphenylol standards.

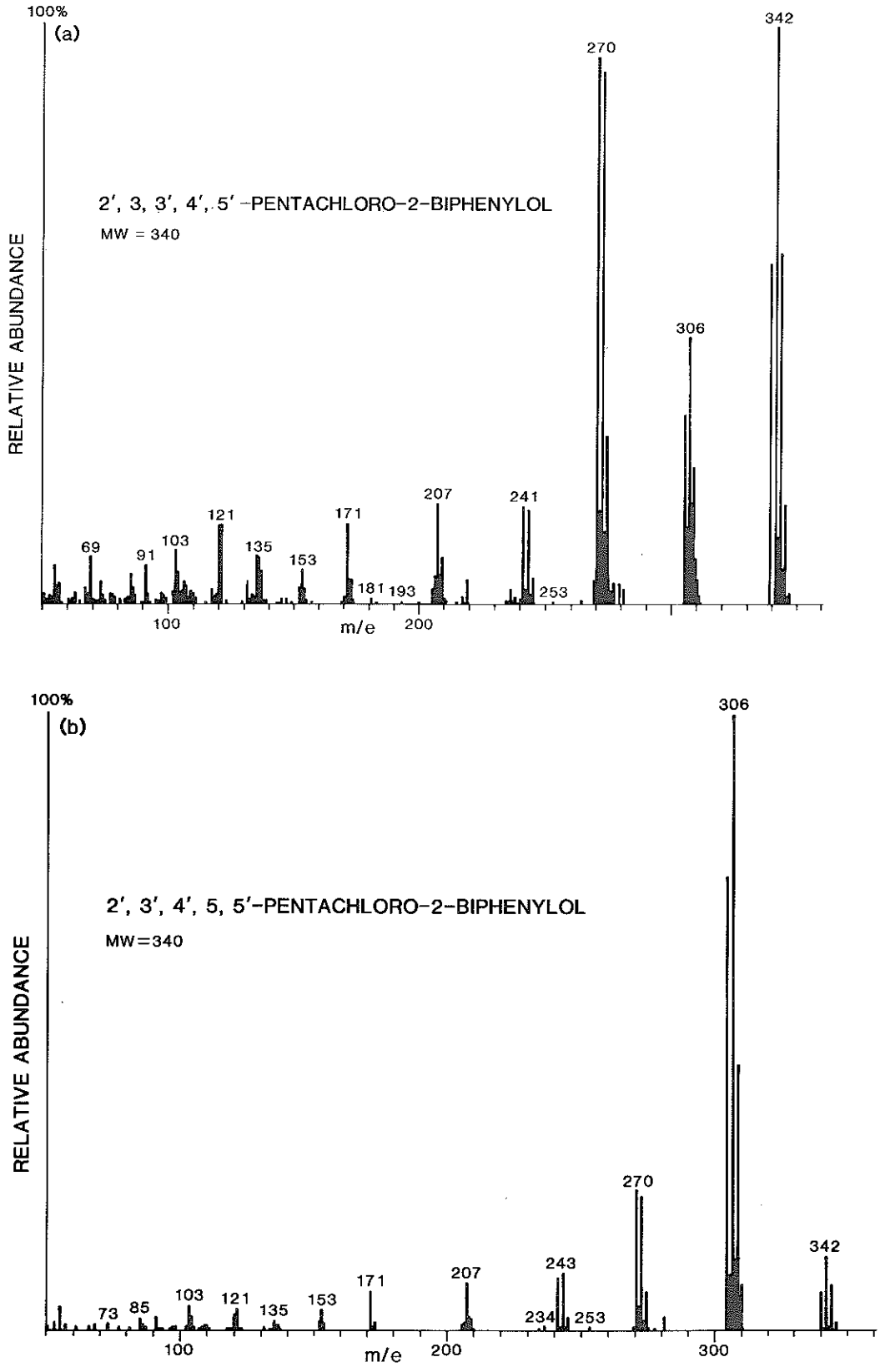

Figure 4. Total ion chromatograms of a) 2',3,3',4',5'-pentachloro-2-biphenylol and b) 2',3',4',5,5'-pentachloro-2-biphenylol standards.

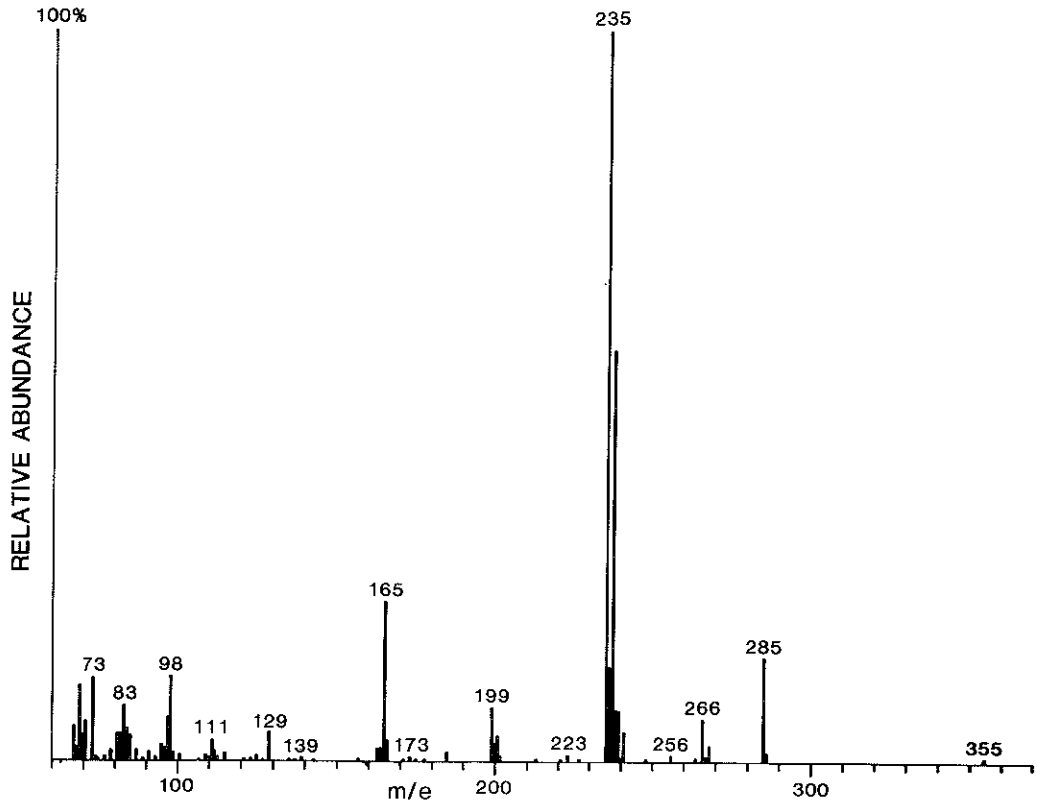

Figure 5. Mass spectrograph of a PV scorpionfish liver extract corresponding to the p,p'-DDOH standard (Figure 2b).

o,p'-DDE, p,p'-DDT and o,p'-DDT, heated them at 90°C for 30 min in a 2% NaOH:acetone (2:1) solution and followed with solvent extraction at a pH <2. These results indicated that none of the parent compounds tested were converted to either p,p'-DDA or p,p'-DDOH by this hydrolysis.

DISCUSSION

The presence of oxy-metabolites in our samples indicates that DDTs and PCBs are metabolized in the marine environment. Because oxygenated metabolites represent the largest portion of the xenobiotic compounds, the rate of this metabolism must be rapid or the rate of depletion of the oxy-metabolites must be slower than that of parent compounds.

The source of the oxy-metabolites in animal tissues is uncertain. The MFO system is known to be inducible in marine fish in response to exposure to PCBs, but not DDTs (Franklin et al. 1980). Gossett et al. (this volume) reported that the levels of oxy-metabolites of DDT were

Table 3. Extraction recovery efficiencies from spiked water (n = 1) and spiked scorpionfish liver (n = 3) and percent total mass extracted by individual fractions.

	Compounds Spiked[a]				
	I	II	III	IV	V
Amount spiked (µg)	72.0	20.0	16.0	20.0	26.0
Water (% recovery of total mass extracted)					
Fraction 1	46.9	44.5	44.0	32.2	32.5
Fraction 2	46.2	47.1	47.2	54.9	51.4
Fraction 3	6.9	8.4	8.8	12.9	16.1
% spike recovered	71.0	22.4	83.8	85.1	81.5
Scorpionfish liver (% recovery of total mass extracted)					
Fraction 1	72.1	71.1	67.3	61.6	63.8
SD	2.2	2.8	5.2	7.7	8.3
Fraction 2	23.7	23.9	25.5	27.7	26.8
SD	0.6	3.4	2.9	2.5	6.7
Fraction 3	4.1	5.0	7.2	10.7	9.3
SD	1.6	1.8	3.3	8.8	6.5
% spike recovered	53.9	64.8	74.6	52.7	57.4
SD	30.0	21.0	19.8	19.7	9.6

[a] I = p,p'-DDOH; II = 2,2',5'-trichloro-4-biphenylol; III = 2',3',4',5'-tetrachloro-4-biphenylol; IV = 2',3,3',4',5'-pentachloro-2-biphenylol; V = 2',3',4',5,5'-pentachloro-2-biphenylol.

highly correlated with liver size in scorpionfish, which suggests that metabolism does occur in these fish. Alternatively, DDE, the dominant form of total DDT in southern California coastal waters, can be metabolized by microorganisms in marine sediments (Lee and Ryan 1978). In addition, the oxy-metabolites of DDT have octanol/water partition coefficients, which suggests that these could be bioaccumulated from sediments (Brown et al. 1982).

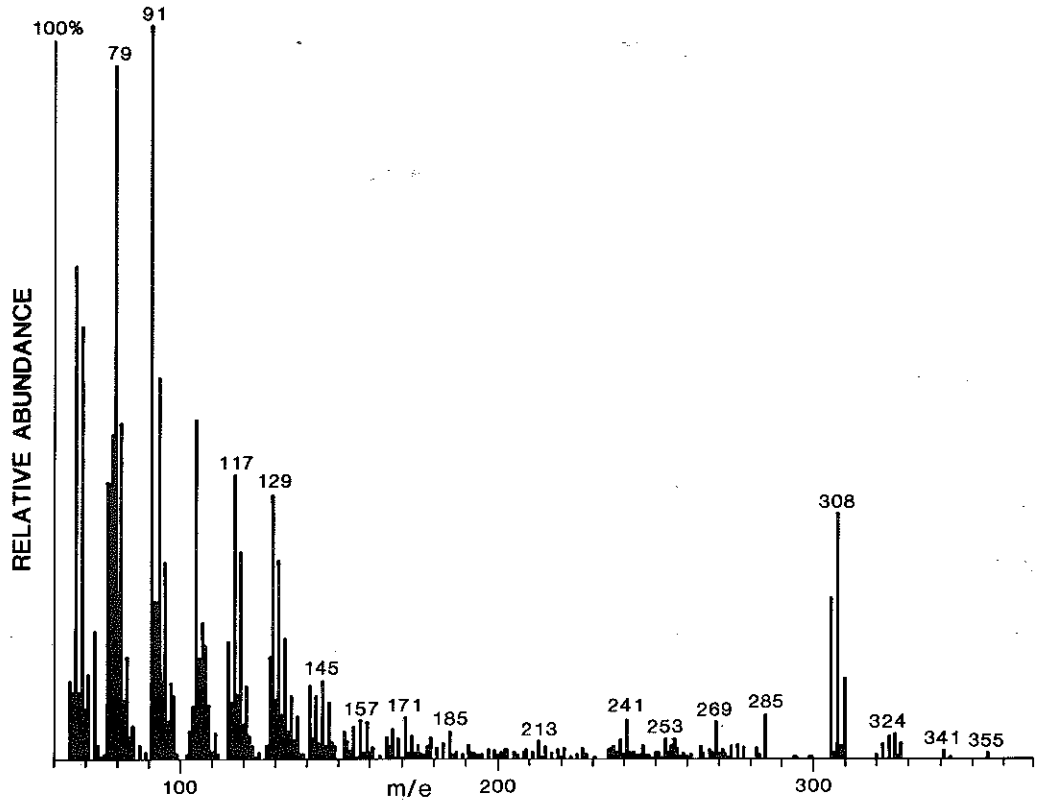

Figure 6. Mass spectrograph of a PV scorpionfish liver extract corresponding to the 2',3',4',5'-tetrachloro-4-biphenylol standard (Figure 3b).

Although the concentrations (n = 10) of parent compounds and oxy-metabolites were correlated in sediments (r_{DDT} = 0.857, r_{PCB} = 0.808, $p<0.01$), these were not significantly correlated in shrimp muscle (r_{DDT} =-0.496, r_{PCB} = -0.126, $p>0.10$) or scorpionfish liver (r_{DDT} = 0.126, r_{PCB} = 0.080, $p>0.2$). That is, the sediment concentration of parent compounds and metabolites increases the closer an area lies to the source of these compounds, which is the Palos Verdes outfall zone. But a corresponding increase does not occur in shrimp muscle or scorpionfish liver. Two possible explanations of this noncorrelation are 1) the organisms move around, affecting the rate of uptake from the sediments by varying the exposure rate, or 2) the rate of metabolism and excretion of metabolites is independently regulated from that of parent compounds.

CONCLUSIONS

The presence of chlorinated oxy-metabolites measured with GC/EC has been confirmed by GC/MS comparison of sample extract spectra with

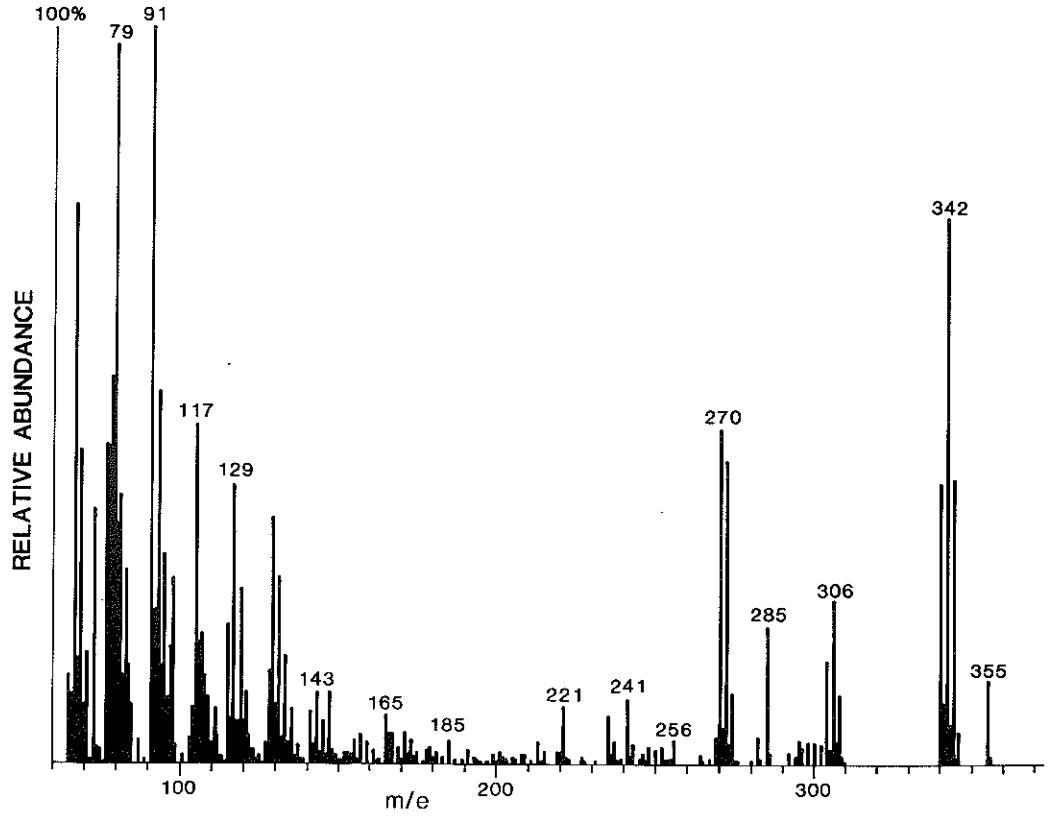

Figure 7. Mass spectrograph of a PV scorpionfish liver extract corresponding to the 2',3,3',4',5'-pentachloro-2-biphenylol standard (Figure 4a).

those of standards. These results indicate that the oxygenated metabolites of DDTs and PCBs were present in tissues of fish collected from the southern California Bight. Further research is needed regarding detoxification/toxification of these compounds to determine their source to organisms and what effects they may produce in the marine ecosystem.

ACKNOWLEDGEMENTS

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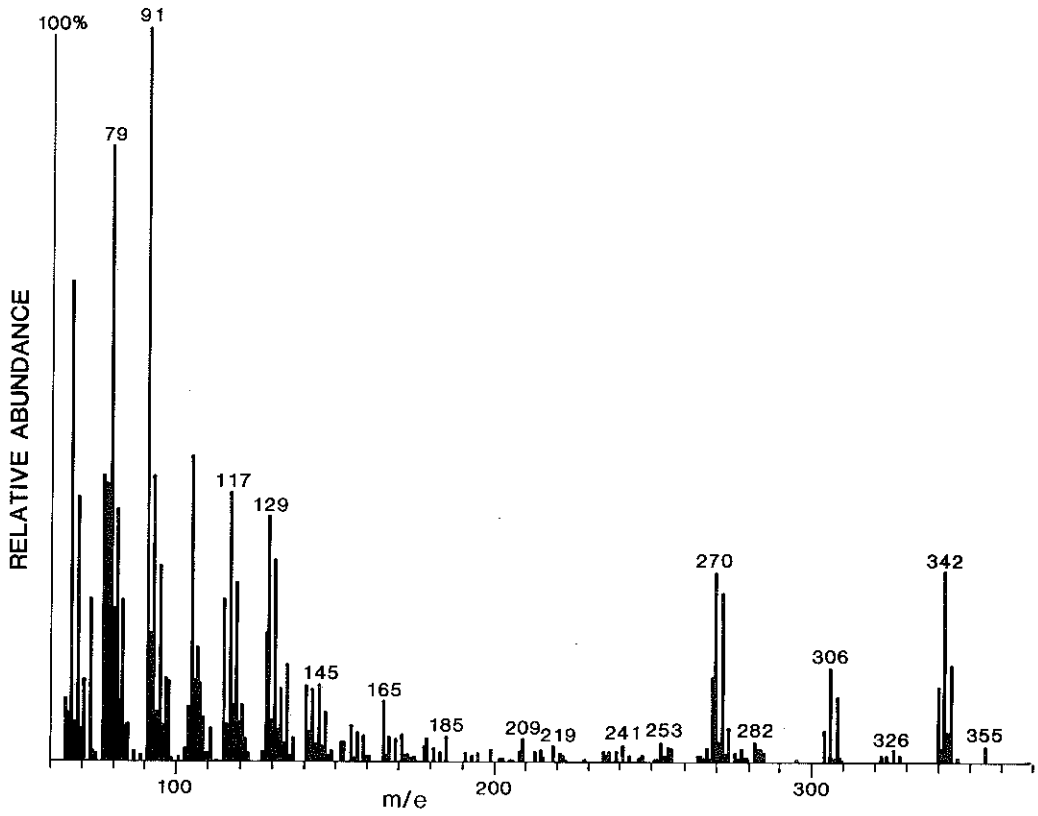

Figure 8. Mass spectrograph of a PV scorpionfish liver extract corresponding to the 2',3',4',5,5'-pentachloro-2-biphenylol standard (Figure 4b).

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