Phthalate Ester Residues—Their Metabolism and Analysis in Fish

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During the course of our analysis of fish and other aquatic materials for pesticide residues, unknown components were often detected. Concern about the identity of late eluting gas–liquid chromatographic (GLC) peaks in fish and water extracts led to the identification of phthalic acid esters (PAEs) as environmental contaminants *(1)*. Further study of these contaminants with a GLC–mass spectrometer (GLC-MS) led to their identification as di-2-ethylhexyl phthalate (DEHP) and di-*n*-butyl phthalate (DBP). Preliminary data from acute toxicity tests indicate that these compounds are relatively low in toxicity. Chronic studies, however, indicate that they may have more subtle effects on reproduction of some aquatic species *(2)*.

Residue data obtained from a variety of areas, dietary materials, and samples related to fish rearing operations have established some information on the incidence of PAEs in relation to pesticides and polychlorinated biphenyl contaminants (Table 1). PAE residues in water appeared to be correlated with rivers draining industrialized or heavily populated areas (John Hesse, Michigan Department of Natural Resources, East Lansing, Michigan 1972 personal communication). However, the distribution of PAEs in the aquatic environment remains to be defined.

PAEs are commonly used as plasticizers, especially in poly(vinyl chloride) plastics. In addition, DBP and DEHP have been used as an insect repellent and pesticide, respectively *(3)*. Production of PAEs exceeded 800 million pounds in 1969 *(4)*. Despite their widespread use, only limited information exists on the fate of PAEs in biological systems or the aquatic environment. Water quality criteria have not been adopted for PAE compounds, but such criteria are currently under consideration.

Our study of PAEs was divided into four areas to better define the interaction of PAEs with aquatic organism. *In vivo* and *in vitro* metabolism studies were conducted to determine the uptake, degradation, and residue composition of PAEs in fish. Further studies were undertaken to develop analytical methods applicable to the GLC determination of residues which may be associated with the presence of PAEs in the aquatic environment. PAE and metabolite identities are also confirmed by GLC-MS and computer processing of spectral information.

In Vivo Metabolism of DEHP

PAEs are detected with existing GLC methods for pesticide residues. However, we did not know whether PAEs were degraded into other products as well. Therefore, we exposed channel catfish *(Ictalurus punctatus)* and fathead minnows *(Pimephales promelas)* to ^{14}C-carboxyl-labeled di-2-ethylhexyl phthalate to determine the identity and composition of PAE degradation products.

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Identification of the degradation products is a prerequisite for development of analytical methods and further toxicological studies of PAEs.

Experimental Procedures

Exposure—Six channel catfish (1—2 g) were exposed to 1 μg/l. DEHP at 23°C (C^{14}:nonradioactive, 1:9, w/w) for 24 hr in a 4-liter glass battery jar for each experiment. Fathead minnows (2 g) were exposed to 1.9 to 62.0 μg/l. DEHP for 56 days. (2).

Extraction—Several fish were combined and ground in an Osterizer blender with Na_2SO_4 (1:4, v/w). Each sample was then packed into a glass column (1–2 cm ID) and

Table 1. Phthalate ester, polychlorinated biphenyl, and insecticide residues found in selected samples from North America.

Source	Source	Sample	Residue, ng/g (ppb)			
			DBP [a]	DEHP [b]	PCB [c]	Insecticide
Mississippi and Arkansas (agriculture and industrial areas)		Channel catfish [d]	Trace	3200	400	DDT-300, dieldrin-40, endrin-30, toxaphene-2200
Fairport National Fish Hatchery, Iowa (water supply from industrial area of Mississippi River)		Channel catfish	200	400	700	–
		Dragonfly naiads	200	200	200	–
		Tadpoles	500	300	1000	Dieldrin-10
Black Bay, Lake Superior, Ontario (rural and industrial area)		Walleye	–	800	1300	DDT-120
		Water	–	300	–	–
		Sediment	100	200	40	–
Hammond Bay, Lake Huron, Michigan (forested area)		Water	0.040	–	0.003	–
Lake Huron, Michigan		Water	–	5.0	0.20	–
Spirit Lake, Iowa (agriculture area)		Yellow perch	–	–	–	DDT-160, dieldrin-120, chlordane-30
Clover Leaf Lake, California (10,300 ft elevation)		Brook trout	–	–	–	DDT-110
Missouri River McBaine, Missouri		Water (turbid)	0.09	4.9	0.20	–
Fish food and components		Total diets	–	2000	700	–
		Total diets	–	7000	60	DDT-30, dieldrin-60
		Casein	20	190	–	–
		Corn starch	20	170	80	–
		Gelatin	20	140	–	–
		Bone meal	30	400	–	–
		Wheat middlings	30	200	20	–
		Carboxymethyl cellulose	–	–	–	Chlordane-720

[a] Di-*n*-butyl phthalate.

[b] Di-2-ethylhexyl phthalate.

[c] Residues approximated by Aroclor 1254 (80%), 1248, and traces of 1232.

[d] Residues presented for this group are the means of 40 fish. Di-2-ethylhexyl phthalate residues ranged from 1000 to 7500 ng/g.

extracted with 1% H_3PO_4 in acetone *(5)*. The ratio of extraction solvent to sample size was 10:1 (v/w). Extraction efficiency for removal of radioactivity from the fish tissue was greater than 98% as determined by scintillation counting of tissue and the extracted activity.

After dilution of the extract to a known volume (10 ml/g tissue), an aliquot corresponding to 2 g of fish tissue was neutralized with concentrated NH_4OH. The neutralized solution was evaporated to 2–3 ml at 100°C using a hot plate. The sample was transferred to a centrifuge tube with the use of a 1:1 (v/v) mixture of methanol and acetone containing 0.1% acetic acid. The sample was centrifuged to remove insoluble material, i.e., $(NH_4)_3PO_4$, and the solution transferred into a conical centrifuge tube. The centrifuged material was resuspended in the methanol:acetone:acetic acid solution and recentrifuged. The tube was washed again to remove all of the radioactivity, and the supernatants combined and concentrated to 0.2 ml.

Chromatographic Characterization—The concentrated solution was applied in a 15 cm streak, 1.5 cm from the bottom of a Mallinckrodt Chrom AR—1000 chromatographic sheet (20 x 20 cm). Combined standard compounds were spotted at a point 1 cm from the vertical edge of the sheet. The sheet was suspended from a stainless steel rack with spring clips and placed in an equilibrated glass chromatographic tank and developed with petroleum ether:diethyl ether:acetic acid (80:20:5, v/v/v). The solvent front was allowed to migrate 19 cm (ca. 15 min). The sheet was then removed, dried, and examined under a shortwave ultraviolet lamp. The bands corresponding to phthalate esters and metabolites were located and marked (Fig. 1).

The radioactivity in each band, except for the conjugate band at the origin, was determined by shredding that section of the sheet in a liquid scintillation vial. Scintillation cocktail (20 ml of Beckman Fluoralloy in toluene) was added, each vial counted, and corrected for quench. Quench was minimal, and counting efficiency was 85–95%.

The band containing conjugates was shredded into a 25 ml centrifuge tube containing 15 ml of 0.5*N* acetate buffer and 1 ml of Ketodase and incubated overnight at 37°C. This suspension was acidified with 2 ml H_3PO_4, cooled, and extracted three times with 10-ml portions of diethyl ether (Caution! Cool the centrifuge tube after addition of H_3PO_4). The combined diethyl ether extracts were dried with Na_2SO_4 in an Erlenmeyer flask, transferred to a conical centrifuge tube, and evaporated to 0.2 ml. This solution was streaked in a 2.5 cm long band on a Chrom AR sheet. Authentic standards were spotted and the sheet developed as previously described. The origin and the bands corresponding to each standard were examined for radioactivity.

Derivatization—Material from the channel catfish extracts with an R_f corresponding to the phthalic acid standard was recovered from the chromatosheet fiber by extraction three times at pH 1.5 with 10 ml diethyl ether, dried with anhydrous Na_2SO_4, divided into equal aliquots, and evaporated to 1 ml. Then, one aliquot was reacted with ethereal diazomethane, and the esterified material and unreacted aliquot were evaporated to 0.1 ml. Esterified and unreacted aliquots, and a dimethyl phthalate (DMP) standard were spotted on an Eastman 6060

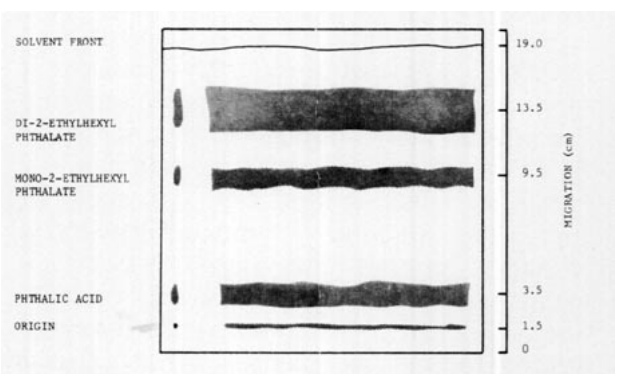


FIGURE 1. Channel catfish extract applied in 15 cm streak on 1 mm thick Chrom AR sheet. Developing solvent was petroleum ether, diethyl ether and acetic acid, (80:20:5). About 200 μg of each standard was applied in a spot 1 cm from the left side.

Chromatogram silicia gel sheet which was developed with petroleum ether:diethyl ether:acetic acid (80:20:2, v/v/v).

Chromatographic spots corresponding to DMP and phthalic acid were recovered and transferred to a scintillation vial. The radioactivity in the diazotized sample was recovered in the spot corresponding to DMP.

A similar derivatization study was made with the material corresponding to mono-2-ethylhexyl phthalate that had been separated from the channel catfish extract. Separation of the methylated 2-ethylhexyl phthalate and mono-2-ethylhexyl phthalate was not complete with the solvent system used previously. Thus, a GLC examination to determine the identity of the isolated methylated monoester was undertaken. The GLC elution of the methyl ester corresponded to that of methyl 2-ethylhexyl phthalate.

Results and Discussion

Exposure of fish to ^{14}C-labeled DEHP enabled us to separate biological degradation products by using TLC chromatosheets. The radioactivity was distributed among four products which were then identified by TLC or GLC, chromatography after enzymatic hydrolysis, and/or derivatization, to be mono-2-ethylhexyl phthalate, a monoester glucuronide, and phthalic acid and a phthalic acid glucuronide. The glucosides, probably glucuronides, remained near the origin during TLC. Hydrolysis of these materials with Ketodase, followed by TLC, showed that they were formed from phthalic acid and mono-2-ethylhexyl phthalate. The monoester aglycone represented 98% of the activity found as glucuronides. The presence of appreciable amounts of mono-2-ethylhexyl phthalate in the fish suggests that this metabolite is refractive to further degradation. The relative composition of separated metabolites in the channel catfish extracts were determined by scintillation counting (Table 2).

An additional study was made to characterize the composition of the material extracted with diethyl ether at pH 1.5 and to determine whether the glucuronides could be separated from unconjugated metabolites without prior chromatographic separation. The radioactive material remaining in the aqueous phase after extraction of an unhydrolyzed fish extract at pH 1.5 was determined. After hydrolysis of the ether extract, chromatographic examination revealed a much higher ratio of phthalic acid conjugate than in the initial sample extract. The monoester glucuronide was found to be partially extracted with diethyl ether at pH 1.5. Thus, chromatographic resolution of the glucuronide prior to hydrolysis was necessary to determine the ratio of the glucuronides of the monoester and phthalic acid.

Table 2. Composition of radioactive phthalate metabolites in channel catfish exposed to 1-μg/l of di-2-ethylhexyl phthalate for 24 hr.[a]

Material	Composition, %
Di-2-ethylhexyl phthalate	14.0
Mono-2-ethylhexyl phthalate	66.0
Phthalic acid	4.0
Conjugates	
Phthalic acid	0.3
Mono-2-ethylhexyl phthalate	13.7
Other	2.0

[a]Total residue equivalent to 2.6 μg/g di-2-ethylhexyl phthalate.

The composition of the residues differed from those determined in the channel catfish (Table 3). The ratio (Tables 2 and 3) of mono-2-ethylhexyl phthalate to DEHP was lower in the fathead minnows than channel catfish, as were the relative levels of conjugates. The significance of these differences cannot be assessed at this time because the concentration of DEHP in water, and the exposures were different. However, the monoester residues in fish represent an appreciable portion of the total DEHP residues that are accumulated. Therefore, the monoesters should be considered in analyses for phthalate ester residues and toxicological studies should be designed to assess the impact of phthalate esters on fish and other aquatic forms.

Table 3. Composition of residues in fathead minnows continuously exposed to C^{14}-di-2-ethylhexyl phthalate (DEHP).

Exposure, days [a]	Radioactivity as metabolite, % [b]					
	Phthalic acid	Phthalic acid conjugate	DEHP	MEHP [c]	MEHP conjugate	Other [d]
28	5.2	3.0	49.6	37.1	0.7	4.4
56	4.9	3.7	60.0	28.7	1.4	1.3

[a] Mean of residues determined from 4 fish at each of seven concentrations in the range of exposure 1.9 to 62 μg/l . DEHP.

[b] Radiolabeled components separated with Chrom AR sheets, recovered, and counted with liquid scintillation.

[c] Mono-2-ethylhexyl phthalate.

[d] Material remaining at origin after enzymatic hydrolysis.

In Vitro Metabolism of Phthalic Acid Esters

While PAE residues have been detected in fish from many locations *(1)*, metabolism of phthalates has not been reported. Metabolism of some xenobiotics by fish liver preparations has been studied *(6–9)*. In most reports to date, the compounds studied were organophosphate pesticides. Generally metabolism of xenobiotics by fish takes place in hepatic microsomes. Therefore, we examined the metabolism of ^{14}C-di-2-ethylhexyl phthalate and ^{14}C-di-*n*-butyl phthalate *in vitro* by microsomal preparations from channel catfish liver.

Experimental Procedures

Carboxyl-^{14}C-di-2-ethylhexyl phthalate (specific activity 1.64 mCi/mmole) and carboxyl-^{14}C-di-*n*-butyl phthalate (specific activity 1.53 mCi/mmole) were purchased from Mallinckrodt. An authentic standard of mono-2-ethylhexyl phthalate was prepared in this laboratory and characterized with TLC, GLC, and GLC-MS. Standards of DEHP and DBP were obtained from Monsanto Company and Chem. Services, Inc.

Male channel catfish (300–600 g) were obtained from the Fish Farming Experimental Station, Bureau of Sport Fisheries and Wildlife, Stuttgart, Arkansas. The spinal cord of each fish was severed, and the liver removed. Hepatic microsomal preparations were obtained by differential centrifugation *(10)*.

Unless exceptions are noted, the incubation conditions for these *in vitro* studies were as follows: 0.5 μmole of NADPH, 0.03–0.06 μmole of ^{14}C-carboxyl phthalic acid ester, and microsomal preparation equivalent to 200 mg of liver. Incubation was conducted in 2.0 ml of 0.05*M* Tris–HCl buffer (pH 7.4), with shaking in a water bath at 25°C. Incubation was usually for 2 hr. Following incubation the pH of the system was adjusted to <pH 2 with concentrated HCl. PAEs and metabolites were then extracted from the incubation medium by use of diethyl ether.

PAEs and certain metabolites were identified by using TLC, GLC, or GLC-MS. PAEs and their metabolites were separated by TLC on 0.2 mm silicia gel plates containing a fluorescent indicator. DEHP and DBP were cochromatographed with authentic standards. TLC plates were developed three times with petroleum ether:diethyl ether:acetic acid (77:20:3). ^{14}C-Phthalic acid esters and their metabolites were visualized using autoradiography. Kodak x-ray plates were exposed to thin-layer chromatograms. Radioactive areas on the TLC plates were scraped, placed in a disposable pipet, and extracted with diethyl ether or diethyl ether containing 5% acetic acid. Some radioactive areas were scraped directly into scintillation vials for quantitation. Derivatives of some PAE metabolites were prepared by reacting samples extracted from TLC scrapings with excess diazomethane to form methyl esters.

Radioactive PAEs and metabolites were quantitated by using liquid scintillation spectrometry.

Results and Discussion

At least four metabolites of DBP were detected by autoradiography (Fig. 2). Three of these metabolites (numbered 0, 1, and 2) have not been identified, and analogous metabolites were not found in the *in vivo* study with DEHP. The fourth metabolite has been identified in the *in vivo* and *in vitro* experiments as either mono-2-ethylhexyl or mono-*n*-butyl phthalate, depending on the parent compound.

The formation of the three as yet unidentified metabolites of either PAE was inhibited by carbon monoxide (Table 4). However, a requirement for oxygen could not be shown. Formation of the monoester metabolite of either di-2-ethylhexyl or di-*n*-butyl phthalate was unaffected by carbon monoxide or a lack of oxygen. Metabolism of DBP and DEHP was affected similarly by air, nitrogen, or carbon monoxide.

As with the effects of air, nitrogen or carbon monoxide, NADPH also had no effect on formation of the monoester metabolites of either phthalic acid diester (Table 5). It is obvious, however, that NADPH is required for the formation of metabolites, 0, 1, and 2.

Enzymatic degradation of DBP was more complete than that of DEHP (Table 6), 97% degradation of DBP and only 6% of DEHP occurring within 2 hrs. The total amount of metabolism, however, was found to vary considerably with hepatic microsomes prepared from different channel catfish.

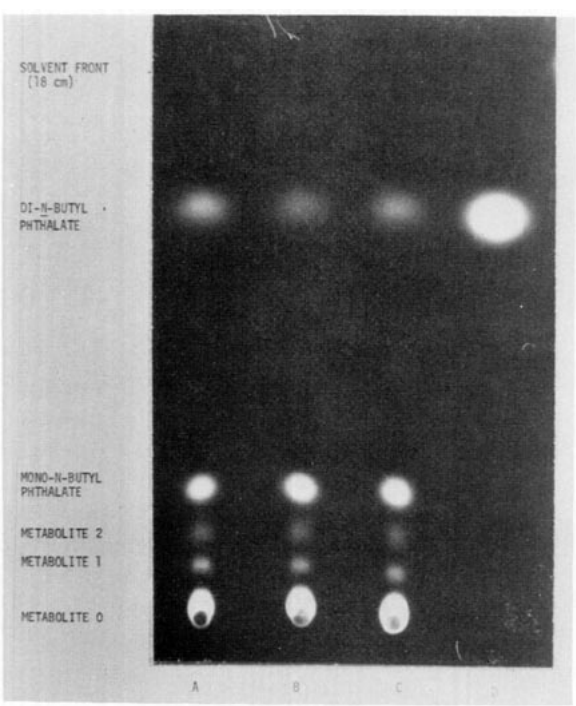


FIGURE 2. Thin-layer chromatogram of ^{14}C-di-*n*-butyl phthalate and metabolites. Samples A, B, and C are diethyl ether extracts from incubation of DBP-C^{14} with hepatic microsomal preparation from male channel catfish. Sample D is DBP-C^{14} incubated without microsomal preparation. The solvent system was petroleum ether:diethyl ether:acetic acid (77:20:3, v/v/v). TLC was developed three times.

Table 4. Effect of air, nitrogen, and carbon monoxide on metabolism of phthalic acid esters by hepatic microsomal enzymes from male channel catfish.

	Di-*n*-butyl phthalate, dpm			Di-2-ethylhexyl phthalate, dpm		
Metabolites	Air,	N_2	CO	Air	N_2	CO
Monoester	36,234	34,569	29,423	5,676	4,234	5,724
0, 1, and 2	21,125	26,980	6,436	6,130	2,768	639

Table 5. Effects of NADPH and O_2 on metabolism of phthalic acid esters by hepatic microsomal enzymes from male channel catfish.

Metabolites	Di-*n*-butyl phthalate, dpm	Di-2-ethylhexyl phthalate, dpm
Monoester		
+ NADPH, + O_2	36,234	5,676
- NADPH, + O_2	31,376	4,776
- NADPH, - O_2	27,662	5,498
+ NADPH, - O_2	34,569	4,259
0, 1, and 2		
+ NADPH, + O_2	21,125	6,130
- NADPH, + O_2	1,992	864
- NADPH, - O_2	2,955	478
+ NADPH, - O_2	26,980	2,768

Table 6. Metabolism of phthalic acid esters by a hepatic microsomal preparation from a male channel catfish.

Metabolite	Di-*n*-butyl phthalate, dpm (%) [a]	Di-2-ethylhexyl phthalate, dpm (%) [a]
Monoester	37,223 (55)	275 (1)
0, 1 and 2	27,979 (42)	3,183 (5)
Total	65,202	3,458
Parent compound recovered	1,908 (3)	58,902 (94)

[a] Expressed as per cent of total radioactivity recovered.

The length of incubation time has a definite effect on the metabolism of DBP (Fig. 3). Formation of the monoester metabolite (mono-*n*-butyl phthalate) appears to be relatively independent of time, at least for the initial 60 min of incubation. A slight increase in formation of mono-*n*-butyl phthalate was noted during the final 60 min of incubation but the maximum amount of metabolite 0 was formed in 120 min. Maximum amounts of metabolites 1 and 2 were present after 15 min incubation, and further incubation only decreased the amounts of these metabolites.

An additional study was undertaken to clarify whether the unidentified metabolites 0, 1, and 2 were derived from the monoester metabolite or parent PAE. Reincubation of monoester metabolites of DEHP and DBP with a microsomal preparation resulted in no further metabolism of the monoesters.

Results of the inhibition and cofactor experiments indicate that at least two separate and distinct microsomal enzyme systems degrade PAEs. One enzyme system, responsible for the formation of metabolites 0, 1, and 2, is carbon monoxide-sensitive and requires NADPH. The other enzyme, responsible for the formation of monoester metabolites, is not carbon monoxide sensitive and does not require NADPH. These observations indicate that the monoester is a terminal metabolite in this system and that all metabolites derive from the parent phthalic acid diester. While insufficient evidence is available to identify definitely the enzyme systems present, it does seem likely that metabolites 0, 1, and 2 are formed by a mixed function oxidase while the monoester metabolites may be formed by an esterase.

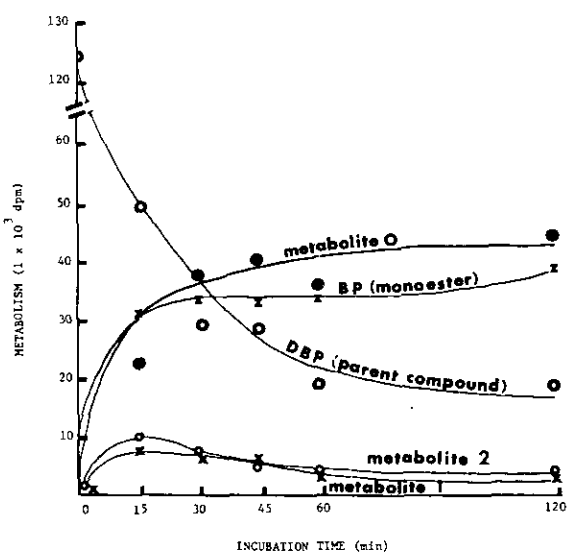


FIGURE 3. Effect of length of incubation time on metabolism of di-*n*-butyl phthalate by hepatic microsomal enzymes from male channel catfish.

Analysis of Phthalate Esters and Metabolic Products in Fish by GLC

The following discussion on GLC analytical techniques presents procedures we have developed in our study on PAEs and some of the problems which have been encountered. Analysis of PAEs and their degradation products in biological tissues has not, as yet, been reduced to routine analyses. Some progress toward this goal was achieved with the identification of the predominant metabolic products, the monoalkyl esters and corresponding glucuronides. These more polar products cannot be directly analyzed by GLC. GLC analyses are possible, however, if the glucuronides are chemically or enzymatically cleaved and the resulting acid group(s) derivatized to form methyl alkyl phthalates.

Extraction Procedures

The most satisfactory procedure for extraction of the phthalates and metabolic products was achieved with a variation of the column extraction procedure reported by Hesselberg and Johnson *(5)*.

Extraction procedure—The first step is to prepare a homogenous sample by blending tissue and anhydrous Na_2SO_4 in a 1:4 (w/w) ratio. Then an amount of sample–Na_2SO_4 mixture equivalent to 20 g of sample is transferred to the extraction column and prepared as described in the literature *(5)*. The sample is then extracted by percolation with 200 ml of 1% (v/v) H_3PO_4 in acetone and the eluate collected in a casserole dish. The column eluate, is evaporated to approxi-

mately 25 ml. The extract is then transferred to a 500 ml separatory funnel with two 5 ml acetone rinses and two 5 ml diethyl ether rinses. To this is added 200 ml of distilled water and the pH of the solution adjusted to 10.0 with 5*N* NaOH.

Cleanup of sample extract—Cleanup of the sample will depend upon the objectives of the analysis. The diesters are not appreciably soluble in water or base and will be removed from the basic solution along with the coextracted lipid by extraction with diethyl ether. The monoesters and phthalic acid can be separated by extraction at pH 1.5 along with some of the monoester conjugates.

After removal of the unconjugated material, the monoesters and phthalic acid conjugates are enzymatically cleaved by using Ketodase and then recovered by extraction. Cleanup and analysis of PAEs and metabolic products recovered in each isolation step are then possible.

Extraction procedure—(1) The basic solution in a separatory funnel is extracted three times with one 75 ml and two 50 ml portions of diethyl ether. (2) The diethyl ether extracts are combined and evaporated to ca. 2–4 ml. This is transferred to a calibrated 10 ml centrifuge tube and capped with a Teflon-lined cap. This extract contains the dialkyl phthalate esters and coextracted fish lipids.

To determine the unconjugated phthalate monoester and phthalic acid, proceed with the following step 3. To isolate total monoesters and phthalic acid proceed with step 5, omitting steps 3 and 4.

(3) The pH is adjusted to 1.5 with H_3PO_4 and extraction is performed twice with 50 ml of diethyl ether; (4) the extracts from step 3 are combined, evaporated to 2–4 ml, transferred to a 10 ml calibrated culture tube, and the monoesters and phthalic acid derivatized.

(5) The pH is adjusted from 10.0 to pH 4.5 with H_3PO_4 and 25 ml of 0.5*N*, pH 4.5 acetate buffer is added. (6) The aqueous solution is transferred to a 250-ml Erlenmeyer flask, warmed gently to remove the diethyl ether, and allowed to cool to room temperature. (7) A 5-ml portion of Ketodase is then added and incubated overnight at 37° C.

(8) The solution is transferred to a separatory funnel and the pH adjusted to 1.5 with H_3PO_4. (9) The extracts are combined and evaporated to 2–4 ml; the monoester and phthalic acid are transferred to a calibrated 10-ml culture tube with diethyl ether and derivatized.

Derivatization

Monoesters and phthalic acid are derivatized effectively by reaction with excess diazomethane (CH_2N_2) in diethyl ether. This reagent forms methyl esters rapidly and quantitatively. Diazomethane is readily prepared from Diazald *(11)*. Ethereal diazomethane is added to the solution obtained in the extraction procedure [step (4) or (10)] until the yellow color persists.

An aliquot is diluted or concentrated if required for cleanup or analysis.

Cleanup of sample extracts containing PAEs requires removal of coextracted fish lipids and two approaches may be utilized.

One approach is to employ conventional cleanup by using solvent partition as described in the *Official Methods of Analysis of the AOAC*, 11th edition, 1970, section 29.011, combined with chromatography on Florisil (section 20.014) *(12)*. We determined that DEHP has a *p* value in hexane–acetonitrile of 0.65 and, thus, its recovery suffers from the same difficulties as *p,p'*–DDE, while DBP has a *p* value of 0.15.

Chromatography of DBP and DEHP on Florisil is analogous to that of dieldrin. The PAEs are recovered in the 15% eluate from the Florisil column *(13)*. An examination of the 6% diethyl ether–petroleum ether eluate from a Florisil column should be made for DEHP, as this material may partially elute in the 6% fraction if the Florisil is too deactivated. Dieldrin may behave in an analagous manner.

An alternate and preferred approach to separation of lipids and PAEs is the substitution of gel permeation chromatography (GPC) for the partition step in the cleanup

procedure *(13)*. A modification of the collection volume is required as DEHP elutes somewhat earlier than the chlorinated insecticides. Collection of the GPC effluent is begun after 70 ml of cyclohexane is eluted instead of the recommended 100 ml. Recovery of DBP was quantitative (published procedure) when collection was initiated after 100 ml of elution, but only 55–60% of the DEHP was recovered.

Gas-Liquid Chromatographic Analyses

Di-*n*-butyl, di-2-ethylhexyl, methyl *n*-butyl, and methyl 2-ethylhexyl phthalate are readily detected by using the conventional electron capture cell and a lightly loaded GLC column (Fig. 4). Corning GLC-110 glass bead support coated with OV-7 or OV-11 is very satisfactory as GLC column packings for separation of PAEs *(13)*. Confirmation of the identity of the various esters is readily achieved from their GLC retention time combined with their mass spectrum.

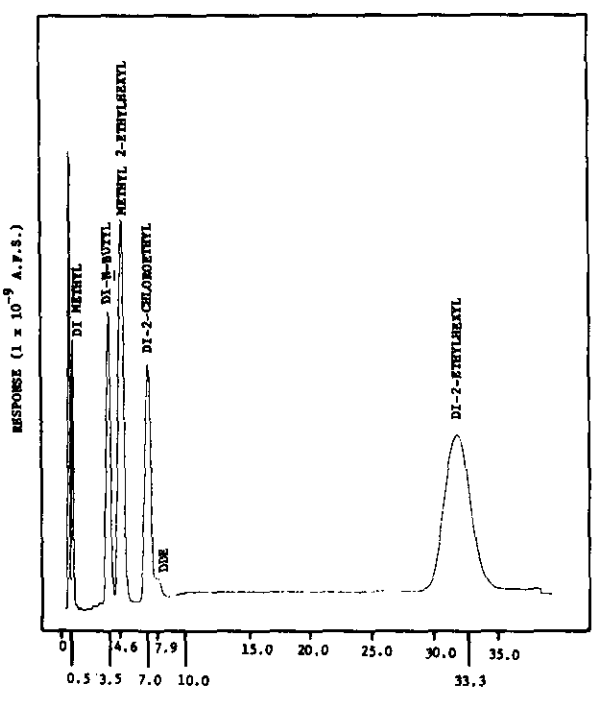


FIGURE 4. Gas—liquid chromatogram of phthalate esters. GLC column 183 cm long x 2 mm I D was packed with OV-11 (0.3%, w/w coated on Corning GLC-110 (80-100 mesh) glass beads: dimethyl phthalate, 3 ng; di-*n*-butyl phthalate, 6 ng; methyl 2-ethylhexyl phthalate, 15 ng; di-2-chloroethyl phthalate, 2 ng; di-2-ethylhexyl phthalate, 15 ng. Column temperature, 182°C; nitrogen carrier flow, 30 ml/min.

The GLC retention time of the PAEs does not pose a significant interference problem in pesticide analyses (Table 7). The GLC retention times relative to aldrin on an OV-7 column were 1.24 and 14.9 for DBP and DEHP, respectively. The retention time of methoxychlor relative to aldrin (OV-7) was 11.9. The OV-7 GLC column temperature was 155°C and the nitrogen carrier flow was 20 ml/min. The electron capture detector area response relative to an equal amount of DDT was 0.12 for DBP and 0.09 for DEHP.

Various components of laboratory equipment, such as blender seals, disposable plastic gloves and plastic tubing, were found to be sources of PAE contamination in residue samples. Reagent-grade anhydrous Na_2SO_4 also contained some DBP and prewashing of the Na_2SO_4 with petroleum ether was required before its use in tissue extractions.

A factor to be considered in the derivatization of phthalic acid is the volatily of (DMP). Quantitative analysis of DMP is difficult under GLC conditions which elute

Table 7. Retention time of phthalate esters and aldrin relative to DDE on an OV-11 column.[a]

Compound	Relative retention	Retention time, min.
Dimethyl phthalate	0.07	0.5
Di-*n*-butyl phthalate	0.45	3.5
Methyl 2-ethylhexyl phthalate	0.59	4.6
Di-2-chloroethyl phthalate	0.88	7.0
Di-2-ethylhexyl phthalate	4.26	33.3
p,p'—DDE	1.00	7.9
Aldrin	0.37	2.9

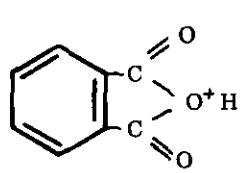
[a]Column conditions: 183 cm x 2 mm i.d. column packed with 0.3% (w/w) OV-11 on Corning GLC-110 (80–100 mesh) glass beads; nitrogen carrier gas; flow, 30 ml/min at 182°C.

DEHP in a reasonable time due to the short retention time of the dimethyl ester.

Derivatization of phthalic acid to a less volatile derivative can be achieved by esterification with 2-chloroethanol and BCl_3. The di-2-chloroethyl ester is well suited for chromatographic analysis under GLC conditions similar to those required for chlorinated insecticides (Fig. 4). Use of the 2-chloroethyl derivative requires that a separate aliquot of the recovered metabolic products be derivatized. If chemical hydrolysis was employed to recover phthalic acid from all of the esters and metabolic products in the sample, the 2-chloroethyl diester would be a good choice of derivative.

GLC-MS Identification of Phthalate Esters and Metabolites

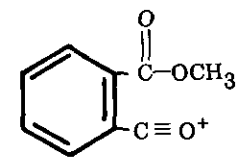
Characteristics of the electron impact mass spectra of phthalate esters permit ready identification of the esters as a group of compounds. The structure is indicated by the presence of a moderately intense ion corresponding to $(M\text{-}R+2H)^+$ in the spectra of symetrically substituted esters and an ion, R^+ or $(R\text{-}H)^+$ corresponding to the alcohol moiety *(14)*. For the higher esters, a very intense ion is observed at m/e^+ of 149 which corresponds to the protonated phthalic anhydride ion:



(1)

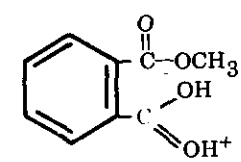
However, no parent ion is generally observed with the higher alkyl esters by using electron impact mass spectrometry *(15)*. Field ionization mass spectrometry was applied to the study of mixtures of phthalate esters and with this technique molecular ions are observed for mixed esters *(16)*.

The spectra of nonsymmetrical methyl alkyl esters we examined contained fragments of m/e^+ of 163 and 181 which correspond, respectively, to



(2)

and



(3)

in addition to the R^+ or $(R\text{-}H)^+$ ion. Dimethyl phthalate does not yield a significant 149 m/e^+ ion fragment, and a molecular ion peak is observed (m/e^+ 194). The base peak in the spectrum of dimethyl phthalate is 163 m/e^+ *(15)*. Combination of GLC with mass spectrometry (GLC-MS) simplifies interpretation of the spectrum as the occurrence of mixtures of compounds is greatly reduced. Computer reduction of the spectra from GLC-MS scans enables one to analyze environmental samples in more detail than is possible with manual data reduction.

Experimental Procedures

A PDP-12 LDP computer was interfaced to a Perkin-Elmer Model 270-B GLC-MS. The interface was expanded to contain an analogue squaring network with variable gain. The squaring network linearized the relation between the magnetic field intensity and m/e^+ ratio. Further, the network resulted in a uniform mass error throughout the mass range (14–550 amu) of approximately ± 0.15 amu. The low resolution software system MASH (as furnished by Digital Equipment Corporation) was used for data

acquisition and presentation *(17)*. Mass fragmentographs were constructed from sequentially acquired spectra using the program MCHROM.

A 3.6 m long x 2 mm ID glass column was filled with 0.3% (w/w) OV-7 coated on 80–100 mesh Corning GLC-110 glass bead support. The column temperature was programmed from 170°C at 5°C/min to 235°C with 18 psig helium as the carrier gas.

Spectra scans were initiated and acquired under computer program control every 8 sec with a scan duration of 4 sec. The scan rate was 3 sec/mass decade over a mass range 14–550, and 70 to 140 sequential scans were acquired during a GLC-MS run. Perfluorokerosine was used for calibration of the mass spectrometer and was introduced into the MS system through a heated variable leak.

After the parent compound or methylated metabolic products (dimethyl phthalate, methyl *n*-butyl phthalate, methyl 2-ethylhexyl phthalate, or di-2-ethylhexyl phthalate) were recovered from TLC and derivatized, the samples were concentrated to 1–5 μg/μl. and injected into the GLC-MS apparatus for analysis. Mass searches were then conducted on each set of spectra by using either mass 149 or 163 or both. The spectrum containing the specified base peak was then examined and compared to that of an authentic derivative.

Results and Discussion

A mass fragmentogram was constructed from 140 sequential scans of a mixture of dimethyl, methyl 2-ethylhexyl, and di-2-ethylhexyl phthalates by combining the intensities of ion fragments 149 and 163 in each spectrum (Fig. 5). The low apparent concentration of dimethyl phthalate is due to the lack of a significant 149 m/e^+ fragment in the spectrum of this compound (Table 8). The base peak in the spectrum of methyl 2-ethylhexyl phthalate is 163 (Fig. 6). Note the ion fragment of 112 m/e^+ which corresponds to $C_8H_{16}^+$, the long chain alkyl portion of the ester. As previously stated, the base peak of the spectrum of

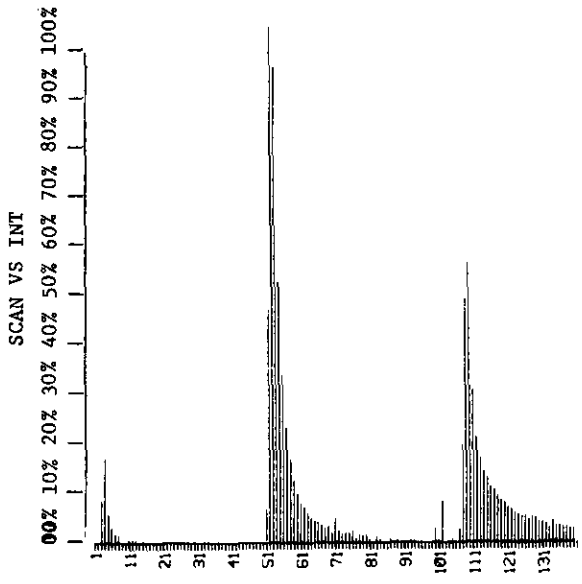


FIGURE 5. Mass fragmentogram of phthalate esters. Computer search for masses 149 and 163 in 140 sequential scans. A 5 μg portion of each ester was injected into the GLC column, and scans were initiated at 8-sec intervals.

di-2-ethylhexyl phthalate contains an intense 149 m/e^+ ion (base peak) and an ion fragment corresponding to $C_8H_{16}^+$ (112 m/e^+) (Table 9).

Confirmation of the identity of several of the *in vitro* degradation products of DBP and DEHP was achieved from the spectrum of the methylated monoesters of phthalic acid. The metabolic products were separated by TLC, recovered, and reacted with diazomethane. The reaction products were examined by GLC-MS, and, by using the computer the spectra were examined for masses 149 and 163. Upon location of a GLC peak containing the specified mass(es), the corresponding spectrum of the compound was compared to that of a standard spectrum.

Identification of methyl *n*-butyl phthalate demonstrated that one ester group was hydrolyzed during *in vitro* metabolism of DBP (Table 10). A similar product, methyl

Table 8. Mass spectrum of dimethyl phthalate. Fragment intensity not corrected for changes in sample concentration during scan.

Peak	Mass	Intensity
9	32.10	18.26
10	44.09	2.11
11	50.10	10.05
12	51.09	1.52
13	52.10	2.11
14	62.80	1.12
15	63.90	2.44
16	65.89	2.18
17	74.00	1.58
18	75.00	3.63
19	75.89	10.78
20	76.89	20.38
21	78.00	1.52
22	79.10	2.05
23	92.00	9.92
24	104.20	1.12
25	105.00	4.10
26	119.90	2.58
27	128.70	1.12
28	133.00	5.62
29	135.00	7.80
30	163.00	100.00
31	163.90	13.76
32	165.00	2.38
33	194.00	9.26
34	195.00	2.18

2-ethylhexyl phthalate (Fig. 7), was identified from the *in vitro* studies with DEHP. The mass spectra of the methylated metabolic compounds from the *in vivo* exposures were consistent with those of the *in vitro* studies.

TLC of the *in vivo* extracts revealed that one spot of lower R_f than phthalic acid remained near or at the origin. This compound has not been identified but could be a hydroxylated derivative of either phthalic acid or the monoester. Acylation of the material remaining at the origin with trifluroacetic anhydride and subsequent reaction with diazomethane may yield a suitable derivative for GLC-MS identification.

The monoesters of phthalic acid are formed by fish upon exposure to the diester and should be considered in the analysis of environmental samples. Their toxicity at present, however, remains undetermined.

Table 9. Mass spectrum of di-2-ethylhexyl phthalate. Fragment intensity not corrected for changes in sample concentration during scan.

Peak	Mass	Intensity
10	41.19	21.79
11	42.20	4.47
12	43.20	27.43
13	44.20	6.76
14	50.10	.85
15	55.10	15.87
16	56.09	7.77
17	57.10	44.21
18	62.69	.85
19	64.89	2.02
20	67.40	.85
21	69.00	5.70
22	70.00	22.80
23	71.10	27.81
24	72.00	3.51
25	83.19	9.27
26	84.09	4.20
27	93.10	2.34
28	104.20	6.12
29	105.20	.85
30	112.19	9.27
31	113.30	13.53
32	121.20	2.02
33	112.20	1.86
34	132.10	2.77
35	135.10	2.45
36	144.20	.85
37	149.20	100.00
38	150.10	13.10
39	151.00	1.49
40	163.00	3.30
41	167.00	37.34
42	168.00	3.72
43	207.10	1.97
44	269.2	.85
45	279.0	9.90
46	280.0	2.50
47	281.0	1.65

Summary

Fish from various locations in the United States have been analyzed for DEHP by GLC. Residue levels ranged from 0.2 to 10.0 μg/g on a whole fish basis. Higher phthalate ester residues appear to be associated with industrialized areas. Significant residues also occurred in commercially reared channel catfish and dietary contamination was suspected as the source of the phthalate ester. Laboratory contamination from plastics was

Table 10. Spectrum of methyl *n*-butyl phthalate from *in vitro* degradation of di-*n*-butyl phthalate by male channel catfish.

Peak	Mass	Intensity
1	17.20	18.83
2	18.10	7.62
3	20.10	13.45
4	27.10	9.86
5	28.10	13.45
6	29.10	28.25
7	40.10	8.96
8	41.20	22.42
9	45.10	20.62
10	57.00	8.96
11	76.10	15.69
12	77.19	13.45
13	92.00	13.45
14	104.10	9.41
15	135.00	12.10
16	149.00	67.71
17	163.00	100.00
18	165.00	10.76
19	181.20	8.96

a critical problem and was minimized by replacement of blender seals and tubing with Teflon or polyethylene.

Metabolism of the esters occurred in *in vivo* and *in vitro* studies with fish. Static exposures of catfish (2 g) to 1 μg/l. of ^{14}C-labeled DEHP for 24 hr resulted in tissue residues of 2.6 μg/g. The composition of the radioactive residues was examined by TLC. Four metabolic products were separated and identified as: mono-2-ethylhexyl phthalate, the corresponding monoester glucuronide (unknown aglycone), phthalic acid, and a phthalic acid glucuronide. The monoester was the predominant metabolite identified by TLC.

DBP was metabolized *in vitro* sixteen times more rapidly than DEHP by hepatic microsomes from male channel catfish. Formation of monoester metabolites of di-*n*-butyl and di-2-ethylhexyl phthalates was not inhibited by carbon monoxide and did not require NADPH. Formation of three other unidentified metabolites, however, was inhibited by carbon monoxide and did require NADPH. The phthalic acid monoesters were not further metabolized and appeared to be a terminal metabolite for the *in vitro* system under study. At least two separate and distinct enzyme systems appeared to metabolize phthalic acid diesters *in vitro*.

Metabolic products were separated by TLC by using Brinkman 0.25 mm silica gel plates and a petroleum ether:diethyl ether: acetic acid (77:20:3, v/v/v) developer. Individual components were recovered from

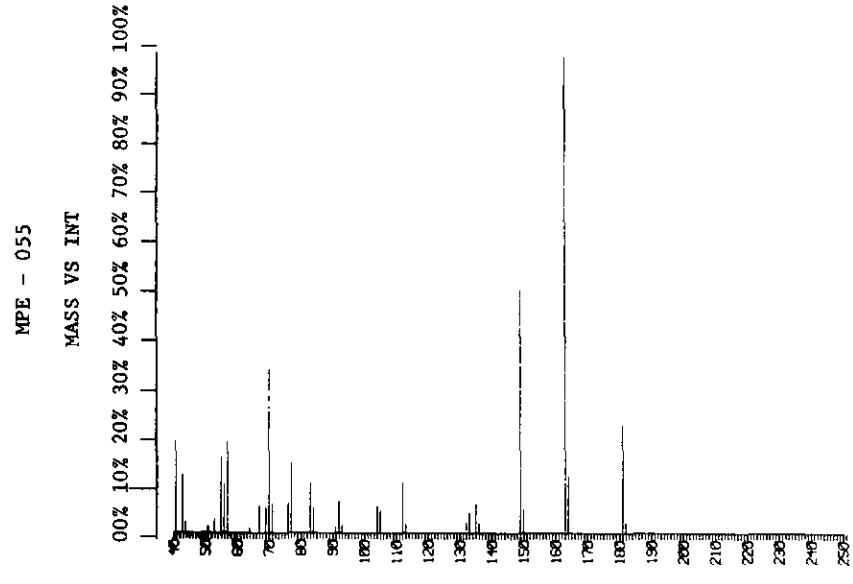


FIGURE 6. Spectrum of authentic methyl 2-ethylhexyl phthalate.

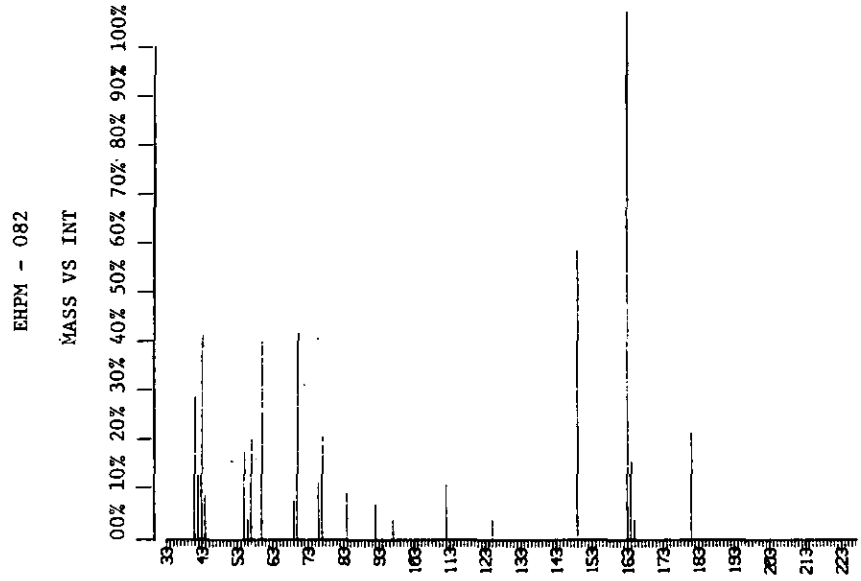


FIGURE 7. Spectrum of methyl 2-ethylhexyl phthalate derived from *in vitro* microsomal degradation of di-2-ethylhexyl phthalate.

the TLC plates, derivatized with diazomethane, and identified by GLC-MS. The spectra of resultant dimethyl phthalate, methyl *n*-butyl phthalate, and methyl 2-ethylhexyl phthalate were identical to those of the corresponding synthetic esters. Graphical presentation of mass fragments in sequential GLC-MS scans using the PDP-12 LDP computer was extremely useful in examining the metabolites and parent compounds.

Routine analysis of phthalate esters and metabolic products required multiple cleanup techniques prior to GLC analysis. Diesters were recovered by conventional residue extraction techniques. Chromatography of these esters of Florisil was analagous to chromatography of dieldrin. Glucuronide conjugates of the phthalic acid monoesters must be enzymatically hydrolyzed and recovered prior to derivatization and GLC analysis.

Recovery of the glucuronides from tissue (20 g) was achieved by column extraction using 200 ml of 1% H_3PO_4 in acetone. The extract concentrated to 20 ml, was dissolved in 200 ml of water and the pH was adjusted to pH 4.5 and buffered with acetate buffer. The glucuronide was hydrolyzed by incubation with Ketodase (5 ml) for 24–48 hr at 37°C. The pH was lowered to 1.5 with H_3PO_4, and the monoester and phthalic acid extracted with diethyl ether. After evaporation, the extract was dried with Na_2SO_4 (anhydrous) and derivatized with diazomethane prior to GLC analysis.

Derivatization of the PAE monoesters with diazomethane was satisfactory. Dimethyl phthalate, however, has an extremely short GLC retention time, and di-2-chloroethyl phthalate was a more suitable derivative for phthalic acid. The electron capture detector was sensitive to the phthalate esters and detector response was approximately 20% of the response for DDE.

Additional investigations have been undertaken to establish which derivatives of the phthalate ester metabolites are suitable for gas chromatographic analysis. The methyl and *n*-butyl 3-*O*-trifluoroacetyl phthalates and trimethylsilyl derivative of 3-hydroxyphthalatic acid were examined by GC-MS. Only the trimethylsilyl derivative gave satisfactory chromatographic peaks. The mass spectrum of the TMS derivative

(MW=398) was as follows *(m/e⁺:I)*: 73:100; 221:60; 147:58; 383:52 (P^+-15); 193:21; 295:14; 282:6; 398:2. The TMS derivative eluted at 220°C by use of a 10% SE-30 column programmed from 120 to 240°C at 6.5°C/min with ca. 20 ml/min of a He carrier flow.

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