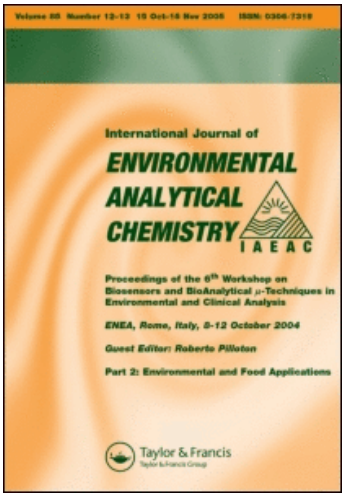

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Determination of Phthalates in Biological Samples†

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A cleanup of biological samples is described for the determination of phthalates. It can be incorporated into a cleanup—chromatography procedure used for the determination of chlorinated hydrocarbons. Phthalates, extracted from biological samples with hexane, are partially separated from lipids by chromatography on alumina to yield fractions in which the common phthalate plasticizers can be quantitated by gas chromatography. An additional cleanup is achieved by the extraction of phthalates from hexane into dimethyl formamide. Phthalates can then be confirmed by measurement of fluorescence in concentrated sulfuric acid. Analyses of spiked samples are reported. Dibutyl phthalate was detected in eggs of double-crested cormorants (*Phalacrocorax auritus*) and herring gulls (*Larus argentatus*) in levels from 11 to 19 mcg/g lipid. Di-2-ethylhexyl phthalate was detected in hatchery-reared juvenile Atlantic salmon (*Salmo salar*) at 13–16 mcg/g lipid, and in the blubber of a common seal pup (*Phoca vitulina*) at 11 mcg/g lipid.

INTRODUCTION

The contamination of food by phthalate plasticizers, migrating from plastic packaging materials, is well known.[1–3] Phthalates were recently identified in human plasma, stored in plastic transfusion packs.[4] The detection of di-2-ethylhexyl phthalate in the heavy mitochondrial fraction of bovine, rat, rabbit, and dog heart muscle[5] indicated that phthalate plasticizers may be environmental contaminants. This possibility was further corroborated by the

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detection of dibutyl and di-2-ethylhexyl phthalates in fish and in commercial fish feed.[6,7] It is obvious in view of these findings that the fate of phthalate plasticizers in the environment should be examined more closely.

Numerous methods for the determination of phthalates have been described. In samples such as water or cleansing solutions, containing few other organic compounds, the determination of phthalates does not present difficulties. Phthalates are extracted by a suitable organic solvent and quantitated by u.v. spectrophotometry,[8] thin-layer,[9] or gas[10] chromatography. The clean-up of lipid and tissue samples by thin-layer and column chromatography is more difficult and is often facilitated by the conversion of phthalate plasticizers into dimethyl phthalate,[5,11] or phthalic acid. The latter is subsequently determined spectrophotometrically, either as such,[12] or in the form of derivatives.[13–15] The alcohol liberated on hydrolysis of phthalate plasticizers may also be used for quantitation.[16,17]

This paper describes the chromatography of phthalates on alumina, activated as for the clean-up in the determination of chlorinated hydrocarbons,[18] the partition of phthalates between hexane and dimethyl formamide, and the confirmation of phthalates by fluorescence spectra in concentrated sulfuric acid. Analyses of samples spiked with phthalates are reported. Phthalates were also detected and measured in a few wildlife samples.

EXPERIMENTAL

A Barber–Coleman Model 5320 gas chromatograph with a flame-ionization detector, equipped with a stainless steel column (6 ft × $\frac{1}{4}$ in.), containing 3% SE–30 on Anakrom ABS, and operated isothermally at either 170 or 240°C, depending on the composition of the analysed mixture, was used to quantitate phthalates. The carrier gas was nitrogen at 80 ml/min^{-1}; the flow rates of hydrogen and air were adjusted to give the optimum response of the flame-ionization detector. The temperatures of the injection port and of the detector were 250 and 270°C, respectively. A Perkin–Elmer MPF–2A spectrofluorometer was used to record fluorescence spectra.

The phthalates originated from the Plasticizer Kit (Chemical Service, Media, Pa., U.S.A.). They gave single peaks on gas chromatography and were used without further purification. Pesticide-grade hexane (Fisher Scientific H–300), diethyl ether (Fisher Scientific E–134), dimethyl formamide (Fisher Scientific D–119), dimethyl sulfoxide (Fisher Scientific D–128), acetonitrile (Fisher Scientific A–999), chloroform (Fisher Scientific C–574), carbon disulfide (Fisher Scientific C–573), and sulfuric acid (B.D.H., microanalytical reagent) were used as received. Alumina (Fisher Scientific A–540) was activated at 800°C for 4 hr and deactivated by the addition of 5% water as described.[19]

A 45 × 0.7 cm glass column was charged with 2 g of alumina. The sample was applied to the column in 1.5 ml of pesticide-grade hexane and the column was percolated with the same solvent to collect 20 ml of effluent (fraction I), with 2% diethyl ether in hexane, collecting 20 ml of effluent (fraction II), and with 10% diethyl ether in hexane to yield 10 ml of effluent (fraction III). The fractions were evaporated to dryness in 25-ml round-bottomed flasks on a rotary evaporator, the residue was dissolved in a known volume of carbon disulfide (0.2–0.6 ml) and examined by gas chromatography.

For the determination of distribution coefficients, a calibrated and glass-stoppered 50-ml centrifuge tube was charged with 5 ml of dimethyl formamide and 5 ml of a solution of phthalate in pesticide-grade hexane. The mixture was shaken on a wrist-action shaker for 1 hr and the concentration of phthalate in the hexane phase was determined by gas chromatography. In a few experiments, dimethyl sulfoxide and acetonitrile were used instead of dimethyl formamide.

Fractions from alumina chromatography and hexane solutions of lipids were extracted with dimethyl formamide (2 × 5 ml, 2 × 1 hr) as described above. The dimethyl formamide extracts were combined, 8 ml of water was added and the aqueous dimethyl formamide was extracted with 5 ml of pesticide-grade hexane for 10 min on a wrist-action shaker. Hexane (4 ml) was withdrawn and evaporated to dryness. The residue was dissolved in carbon disulfide and examined by gas chromatography. Carbon disulfide was evaporated, and the residue was dissolved in dimethyl formamide for fluorescence analysis.

A solution of phthalate in dimethyl formamide (1 ml) was carefully added to 4 ml of concentrated sulfuric acid in a 15-ml glass-stoppered centrifuge tube for the determination of fluorescence spectra. The contents were mixed by inverting the tube a few times and the heat-generated pressure was released. After 10 min the tube was cooled to room temperature in a water bath and the fluorescence spectrum was recorded.

TLC was carried out on 0.25-mm layers of silica gel with u.v. indicator (Camag DF–5), using chloroform as the developing solvent. The spots of phthalates were either observed directly under the 254-nm u.v. light (Universal u.v. lamp Gelman–Camag), or visualized by spraying with resorcinol in acidified ethanol,[20] and the yellow fluorescence of phthalates on irradiation at 350 nm was observed.

Whole juvenile Atlantic salmon (*Salmo salar*), egg yolks of double-crested cormorants (*Phalacrocorax auritus*), herring gulls (*Larus argentatus*), and liver and blubber of a common seal (*Phoca vitulina*) pup were extracted as described.[18] Corn oil, herring oil, and fish oil were commercial samples.

All glassware was washed with a detergent solution and rinsed with distilled water, acetone, and pesticide-grade hexane.

RESULTS

Column Chromatography of Phthalates on Alumina

Phthalates, applied to the alumina column in pure hexane solutions or in lipid extracts, containing less than 20 mg of lipid per application, are eluted in fraction III (10% diethyl ether in hexane). When the lipid loading of the column is increased (50–100 mg of lipid per application), the activity of alumina is affected: di-2-ethylhexyl and diisodecyl phthalate are eluted in fraction I, dioctyl phthalate is distributed between fraction I and II, and dibutyl and diamyl phthalate are eluted in fraction II.

The results of analyses of samples spiked with di-2-ethylhexyl and dioctyl phthalate are presented in Table I. Interfering peaks were not encountered in

TABLE I
Recovery of phthalates from lipid extracts by chromatography on alumina

Sample	Phthalate, mcg/g lipid			
	Di-2-ethylhexyl		Dioctyl	
	Added	Found	Added	Found
Fish oil	44.4	51.6	56.5	55.3
Fish oil	44.4	46.4	56.5	41.8
Juvenile Atlantic salmon A	13.7	25.2	23.7	19.2
Juvenile Atlantic salmon B	37.6	53.6	65.4	52.5
Juvenile Atlantic salmon A	42.0	52.5	73.0	64.4
Juvenile Atlantic salmon B	83.9	103.0	146.0	127.0
Juvenile Atlantic salmon A	—	12.9	—	—
Juvenile Atlantic salmon B	—	16.4	—	—

the quantitation. The extracts of juvenile Atlantic salmon contained some di-2-ethylhexyl phthalate of their own. Dibutyl and diamyl phthalates could not be determined directly in these samples due to the presence of interfering peaks on gas chromatography. The interferences could be eliminated by operating the gas chromatograph at a lower temperature and thus achieving a better separation of the faster peaks, or after an additional clean-up by the dimethyl formamide partitioning. A sample of fish oil, spiked with 9.45 and 16.8 mcg/g lipid of dibutyl and diamyl phthalate, respectively, gave values of 8.30 and 11.7 mcg/g lipid after the dimethyl formamide clean-up. Dibutyl and diamyl phthalate were not detectable in the non-spiked extracts of juvenile Atlantic salmon.

Partition of Phthalates between Hexane and Dimethyl Formamide

High lipid loading of the alumina column, required for the detection of relatively low levels of phthalates, yields fractions containing considerable amounts of lipids. This makes the confirmation of phthalates by TLC difficult and the lipids may eventually contaminate the gas chromatographic system. An additional clean-up of the fractions from the alumina chromatography was therefore sought. The partition of phthalates between hexane and dimethyl formamide, hexane and dimethyl sulfoxide, and hexane and acetonitrile was investigated. As the data in Table II indicate, dimethyl formamide was a suitable partitioning solvent. Except for dimethyl and diethyl phthalate, phthalates could be recovered quantitatively from aqueous dimethyl formamide by extraction with hexane (Table III). Only about 10% of lipid present in the fractions from the alumina chromatography was extracted into dimethyl formamide (Table IV), and the recovery of dibutyl, diamyl, di-2-ethylhexyl, and dioctyl phthalate was 70–90%.

Direct extraction of phthalates by dimethyl formamide from solutions of lipids in hexane was also attempted. About 10% of the original lipids was extracted into dimethyl formamide. The material recovered from aqueous dimethyl formamide by extraction with hexane could not be examined directly by gas chromatography, due to the presence of very large interfering peaks, and had to be cleaned up by the described chromatography on alumina. The

TABLE II
Distribution coefficients of phthalates at 24°C

Phthalate	Initial concn. in hexane (mcg/ml)	C_{DMF}/C_{Hex}	C_{DMSO}/C_{Hex}	C_{MeCN}/C_{Hex}
Dimethyl	0.081	∞		
Diethyl	0.091	∞		
Dibutyl	0.126	∞		
Diamyl	0.112	6.0	1.6	3.9
	0.022	10		
Di-2-ethylhexyl	0.296	1.7	0.2	0.6
	0.059	1.6		
Dioctyl	0.378	1.8	0.2	0.5
	0.076	2.0		
Diisodecyl	3.53	1.2		

DMF = dimethyl formamide, DMSO = dimethyl sulfoxide, MeCN = acetonitrile, Hex = hexane

TABLE III

Distribution coefficients C_{DMF}/C_{Hex} at different concentrations of water in dimethyl formamide

Phthalate	Concentration of water in DMF (v/v) %				
	9	16.6	28.6	44.5	61.5
Dimethyl			14.3	13.2	6.4
Diethyl			3.2	1.3	0.7
Dibutyl			0.2	0	0
Diamyl	1	0.2	0		
Di-2-ethylhexyl	0.2	0.05	0		
Dioctyl	0.2	0.06	0		

DMF = dimethyl formamide, Hex = hexane

TABLE IV

Lipid removal by chromatography on alumina and subsequent dimethyl formamide partitioning

	Lipids (mg)	
	Fish oil	Herring gull, egg yolk
Applied to alumina	100	111
Fraction I (20 ml hexane)	43	41
after DMF partitioning	3.6	3.1
Fraction II		
(20 ml 2% ether in hexane)	29	16
after DMF partitioning	4.0	2.2
Fraction III		
(10 ml 10% ether in hexane)	20	3

DMF = dimethyl formamide

TABLE V

Recovery of phthalates from lipid extracts by dimethyl formamide partition[a]

Sample	Volume ratio Hexane/DMF	Phthalate, mcg/g lipid							
		Dibutyl		Diamyl		Di-2-ethylhexyl		Dioctyl	
		Added	Found	Added	Found	Added	Found	Added	Found
Corn oil	1	1.58	—	2.80	—	7.40	4.02	9.42	7.22
Corn oil	2	1.58	—	2.80	—	7.40	3.35	9.42	3.95
Corn oil	4	1.58	—	2.80	—	7.40	2.69	9.42	2.42
Herring oil	1	1.58	0.61	2.80	1.60	10.60	4.00	13.50	5.06
Spiked hexane[b]	1	3.15	3.32	5.60	5.02	14.80	13.03	18.85	23.40
Spiked hexane[b]	1	1.58	1.56	2.80	2.71	7.40	7.43	9.42	7.92
Spiked hexane[b]	1	0.63	0.53	1.12	0.83	2.96	2.66	3.77	2.78

[a] Starting with 1 g spiked lipid in 5 ml hexane.
[b] Amount of phthalates in mcg.

detectable levels of phthalates were lower than those in the "chromatography first" procedure, but the recoveries of phthalates were not quantitative (Table V). Lipids obviously increased the solubility of phthalates in hexane, since, under the same conditions, the recoveries of phthalates from pure hexane solutions were from 74 to 100%.

Fluorescence of Phthalates in Concentrated Sulfuric Acid

Phthalates (*o*- and *p*-) exhibit a strong fluorescence in concentrated sulfuric acid. No fluorescence was observed in the case of *m*-phthalates. The fluorescence excitation maxima of *o*- and *p*-phthalates are at 270 and 308 nm (Figure 1), the emission maximum of *o*-phthalates is at 360 nm, and that of *p*-phthalates is at 380 nm (Figure 2). The fluorescence of *o*-phthalates is due to phthalic anhydride and as the relative fluorescence data (Table VI) indicate, the alcohol moiety has no effect on the intensity of the fluorescence emission. Dimethyl formamide is a convenient solvent for the introduction of the sample into concentrated sulfuric acid. The measurement of fluorescence can be used to confirm phthalate plasticizers in the presence of a certain amount of lipids (up to 350 mcg/ml). Under such conditions, 0.2 mcg/ml of phthalates, expressed as di-2-ethylhexyl phthalate, is still detectable (Figure 3).

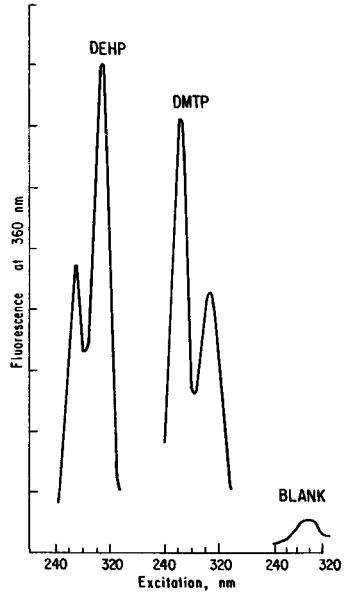

FIGURE 1 Fluorescence excitation spectra. DEHP = Di-2-ethylhexyl phthalate; DMTP = Dimethyl terephthalate.

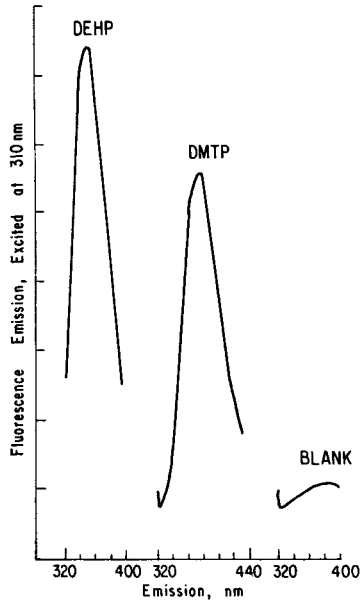

FIGURE 2 Fluorescence emission spectra. DEHP = Di-2-ethylhexyl phthalate; DMTP = Dimethyl terephthalate.

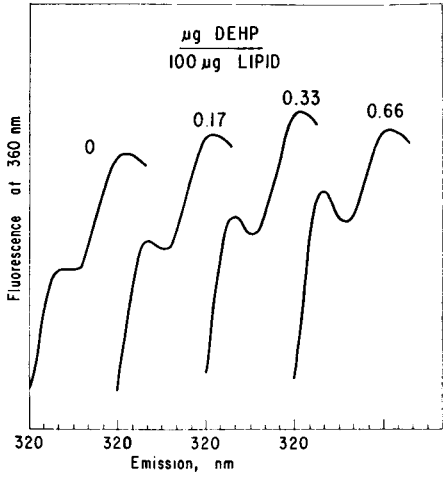

FIGURE 3 Confirmation of phthalates by fluorescence emission spectra in the presence of lipids. DEHP = Di-2-ethylhexyl phthalate.

TABLE VI
Fluorescence of phthalates in concentrated sulfuric acid on excitation at 310 nm

Phthalate	Relative fluorescence emission at 360 nm: Response per 1 mg	Response per 1 mmole
Dimethyl	24.4	4740
Dibutyl	17.6	4900
Di-2-ethylhexyl	11.1	4330
Diisodecyl	9.8	4350
Diphenyl	14.4	4580
Dimethyl tere-	8.1[a]	1570[a]
Dimethyl iso-	—	—
Phthalic acid	22.0	3670

[a] Emission maximum of dimethyl terephthalate is at 380 nm.

Phthalate Contamination in the Laboratory

Contamination of samples by phthalates in the laboratory was not encountered when the precautions routine in pesticide residue analysis[18] were observed. A batch of Whatman extraction thimbles contained dibutyl phthalate at a level of 4.25 mcg/g. It is common practice to pre-extract the thimbles with hexane to remove possibly present polychlorinated biphenyls (PCB) and this treatment simultaneously removes dibutyl phthalate.

Phthalates in Environmental Samples

Dibutyl phthalate was detected in eggs of double-crested cormorants and herring gulls (Table VII). Di-2-ethylhexyl phthalate was detected in hatchery-

TABLE VII
Dibutyl phthalate in egg yolks of double-crested cormorants and herring gulls

Sample	Dibutyl phthalate, mcg/g lipid: Added	Found
Double-crested cormorant	67.4	88.6
Double-crested cormorant	—	14.1
Herring gull A	—	17.1
Herring gull B	—	10.9
Herring gull D	—	19.1

reared juvenile Atlantic salmon (Table I), and at a level of 10.6 mcg/g on lipid basis in the blubber of common seal. Dioctyl phthalate was not detectable in any of the samples examined. The phthalates were confirmed by fluorescence measurement and by TLC.

DISCUSSION AND CONCLUSIONS

The determination of phthalates can be incorporated into the procedure used for the isolation of chlorinated hydrocarbons from biological samples. Depending on the lipid content of the samples, phthalates are either completely or partially separated from PCB and the common chlorinated hydrocarbon pesticides. Two additional fractions have to be collected from the alumina column in the former, and one fraction in the latter case. An additional clean-up, required for confirmation, can be achieved by the extraction of phthalates from hexane into dimethyl formamide, and the fluorescence of phthalates in concentrated sulfuric acid is a useful confirmatory test. Approximately 0.06 mcg of phthalates, expressed as di-2-ethylhexyl phthalate, can be detected in the presence of 100 mcg of lipids.

The detection limits of phthalates examined in this work are about 3–5 mcg/g lipid. This is much higher than the detection limits of chlorinated hydrocarbons which are generally in the ng/g lipid range. However, detection limits should be proportional to the biological effects of the measured compounds and it is possible that in the case of phthalates the detection limits are sufficiently low.

Literature data on the occurrence of phthalate plasticizers in biological samples, mentioned earlier, and the presented finding of di-2-ethylhexyl phthalate in hatchery-reared juvenile Atlantic salmon and in common seal, and of dibutyl phthalate in eggs of double-crested cormorants and herring gulls warrant a more detailed survey of the environment for these compounds. The performance of the methods for the determination of phthalates should, however, be tested in an interlaboratory exchange-sample program similar to that described for chlorinated hydrocarbons.[21]

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