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Abstract - The production, properties, uses, toxicity, and determination of phthalates are reviewed. A cleanup of biological samples is described for the determination of phthalates. The cleanup can be incorporated into a 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 be then confirmed by measurement of fluorescence in concentrated sulfuric acid. Dibutyl phthalate was detected in eggs of double-crested cormorants (Phalacrocorax auritus) and herring gulls (Larus argentatus) in levels from 11 to 19 μg/g lipid. Di-2-ethylhexyl phthalate was detected in hatchery-reared juvenile Atlantic salmon (Salmo salar) at 13-16 μg/g lipid, in the blubber of a common seal pup (Phoca vitulina) at 11 μg/g lipid, and in commercial fish food at 8-9 μg/g lipid. The food also contained 5-8 μg/g lipid of dibutyl phthalate.

Literature Review

Several reports on the occurrence of phthalate plasticizers in the environment generated renewed interest in the determination of phthalates in environmental samples. This report reviews the recent literature on the determination of phthalates, their occurrence in the environment, metabolism and toxicity.

The production of phthalate plasticizers in the U.S.A. has grown exponentially from 1950 until 1966 and then more or less levelled off (Anon. 1971a). The production figures for the most important phthalate esters for 1968 (Anon. 1970) and 1970 (Anon. 1971a) are presented in Table 1.

Table 1. U.S.A. annual production of phthalate plasticizers, millions of pounds.

	1968	1970
Di-2-ethylhexyl phthalate	321	350
Diisodecyl phthalate	123	123
Diisooctyl phthalate	84	85
n-octyl-n-decyl phthalate	38	59
Dibutyl phthalate	27	23
Diethyl phthalate	18	21

About 75% of plasticizers of all types (phosphates, epoxidised esters, and adipates) are used in polyvinyl chloride and copolymers, which were produced at a rate of 325 million pounds per year in 1950, and 3.44 billion pounds per year in 1971 (Anon. 1972a). For comparison, the production of high and low density polyethylene was 6.4 billion pounds in 1971.

Some properties of phthalate plasticizers are summarized in Table 2. The preparation of odourless phthalate plasticizers by catalytic esterification (catalyst butyl titanate) and steam stripping was patented (Klein 1970).

Phthalates may be found in a wide variety of other products. Phthalate plasticizers are used in vehicle solids of paints, lacquers, and in adhesives. Dicyclohexyl, diphenyl, and dioctyl phthalate are used in lacquers suitable for water and light resistance, respectively. Paper lacquers may contain dibutyl and dioctyl phthalate. The latter is also used in

Table 2. Properties of phthalate plasticizers (Darby et al. 1969).

	Boiling point		Loss, %*	
	Mol.wt.	°C, (mm Hg)	Volatility	Water extn.
Di-2-ethylhexyl	390	231 (5 mm)	4.5	0.01
Diisodecyl	446	255 (5 mm)	1.8	0.02
Diisooctyl	391	220-248 (4 mm)	4.6	0.04-0.14
n-octyl-n-decyl	418	250 (5 mm)	3.5	0.03
Dibutyl	278	340 (760 mm)	44.0	0.25
Diethyl	222	296 (760 mm)		

*Volatility data for 24 h at 87°C, water extraction for 24 h at 50°C, both for 40% concentration in Opalon 650 (Monsanto).

wood lacquers. Polyvinyl acetate-based adhesives contain usually dibutyl phthalate, which may sometimes be replaced by the less volatile butylcyclohexyl phthalate (Focsaneanu 1971). Dibutyl phthalate is used as plasticizer in inks for printing on cellophane, polyethylene, and aluminum foil (Levchenko et al. 1972). Postage stamp adhesives may also contain phthalates (Cleverley et al. 1972). Dioctyl phthalate was detected in some polyvinyl chloride toys (Tsankov et al. 1971). Dimethyl and diethyl phthalate were used as plasticizers for controlled-release pesticides strips (Behrenz et al. 1969).

The preparation of alkyl tetrachloro phthalates was described (Guseinov et al. 1968). Tetrachloro-o-phthalic acid was used in fire-retardant polyester coatings (Abbott, 1972), and tetrabromophthalic anhydride was used for the same purpose in polypropylene (Dalzell 1972).

Phthalate esters were isolated from a variety of natural products such as cranberries, lingon berries, oxidised lipids, and lipids of microorganisms. Di-2-ethylhexyl, dibutyl, diisobutyl, and small amounts of dimethyl, diisoamyl, diamyl, and dihexyl phthalate were isolated from Cryptotaemia canadensis (Hayashi et al. 1967). Monomethyl phthalate was isolated from fungi (Batta et al. 1969). A large amount of phthalic acid (0.01% of whole animals body weight) was reported in the deep sea jellyfish Atolla (Morris 1970). It is possible that at least some of these findings are artifacts caused by phthalate contamination of solvents used in the extractions, or by contact of the samples with plastic materials. Dibutyl

phthalate and di-2-ethylhexyl phthalate were detected in commercial first-grade benzene. Chloroform, hexane, and petroleum ether contained dibutyl phthalate. Small amounts of diisobutyl phthalate and iso-n-butyl phthalate were also present in some of the solvents. Vinyl tubing yielded diisobutyl, dibutyl, and di-2-ethylhexyl phthalate, filter paper and plastic tape contained dibutyl phthalate, whereas plastic (polyethylene) bucket contained tentatively identified diethyl phthalate (Asakawa et al. 1970).

Lower phthalates such as dimethyl and diethyl phthalate have insect repellent properties, however, phthalates as a group were considered practically nontoxic. In the U.S.A. diethyl phthalate, di-2-ethylhexyl phthalate, diisoctyl phthalate, butylbenzyl phthalate, dicyclohexyl phthalate, dihexyl, and diphenyl phthalate are permitted as plasticizers in food packaging materials. Diisodecyl phthalate is allowed for food-packaging materials at levels below 0.2 mg/in^2 of food-contact surface (Anon. 1969). In Great Britain phthalates are classified as compounds with "doubtful toxicity", in Germany, dibutyl, di-2-ethylhexyl, dicyclohexyl, and benzylbutyl phthalate are allowed for food-packaging materials, but the maximum amounts of leachable material per surface area are specified (Weinmann 1968).

It was reported that free phthalic anhydride, possibly present as impurity in phthalate plasticizers, is a skin irritant and sensitiser in combination with maleic anhydride and naphthoquinone compounds (Weinmann 1968). Commercial di-2-ethylhexyl phthalate contains traces of ethylhexanol, ethylhexyl butyrate, ethylhexyl oxide, and ethylhexyl benzoate (Courtier et al. 1969).

An excellent paper dealing with general toxicity problems of plastics was published (Estevez, 1969). The problem of the migration of additives used in plastics into food is discussed in detail and it is emphasized that changes in physical parameters of plastics such as the thickness of the plastic film, or slight changes of composition of the formulation may have pronounced effects on the migration of the ingredients. The extractability of phthalate plasticizers from polyvinyl chloride increases with increasing molecular weight (Efremova et al. 1972). It was noted that heptane does not adequately simulate the migration of dioctyl phthalate into fatty foods (Sampaolo et al. 1972).

The teratogenicity of phthalates in rats was studied (Singh et al. 1972). The paper also contains references to older toxicity studies. Phthalates were administered by intraperitoneal injections at 1/10, 1/5, and 1/3 of LD50 on the 5th, 10th, and 15th day of gestation. Both dioctyl- and di-2-ethylhexyl phthalate, which have very high LD50 (Table 3) were given at 5 ml/kg and 10 ml/kg levels. All phthalate

esters examined had deleterious effects and a significant number of malformed fetuses was observed in most treated animals. Particularly dimethoxyethyl and dimethyl phthalate were highly active.

Dibutoxyethyl phthalate was teratogenic in chick embryos, and together with di-2-methoxyethyl and octyl-isodecyl phthalate was damaging the central nervous system of the developing chick embryo (Bower et al. 1970).

Table 3. Acute toxicity of phthalates in rats (Singh et al. 1972).

Phthalate	LD50 ml/kg	Specific Gravity at 25°C (Darby et al. 1969)
Dimethyl	3.3751	1.190
Dimethoxyethyl	3.7355	1.220
Diethyl	5.0579	1.121
Dibutyl	3.0496	1.046
Diisobutyl	3.7498	
Butylcarbobutoxymethyl	6.8892	1.097
Dioctyl	>50	0.978
Di-2-ethylhexyl	>50	0.986

Low molecular weight phthalates were more toxic in rats and mice than those containing the C_6-C_9 alcohol moieties. The latter were practically non-toxic in mammalian cell cultures (Nematollahi et al. 1967), and an LD50 of more than 26 g/kg was found for di-2-ethylhexyl, dioctyl, and dinonyl phthalate in rats and mice (Brown et al. 1970). On the other hand, it was reported that mice are more sensitive to phthalates than rats. Dioctyl phthalate was more toxic than diethyl phthalate in mice, and both diisooctyl and dioctyl phthalate were more toxic than dibutyl phthalate in rats (Antonyuk 1969).

The toxicity of a variety of plastics to 6 phytoplankton species, to the copepod Enterpina acutifrons and to larvae of the sea urchin Arbacia lixula was studied (Bernhard et al. 1970). Materials such as natural rubbers and PVC were found highly toxic. Teflon, polyolefins (polyethylene, polypropylene), polycarbonates and a polyester were non-toxic.

Di-2-ethylhexyl phthalate accumulates in the lung, liver, and spleen after intravenous injection in the rat.

Under the same conditions, butylglycolyl butyl phthalate is hydrolyzed to glycolyl phthalate (Jaeger 1971). Human plasma extracted efficiently di-2-ethylhexyl phthalate from a plastic transfusion pack. No phthalates were however detected in freshly collected blood (Marcel et al. 1970). Di-2-ethylhexyl phthalate was detected in tissues of humans, exposed to blood contaminated with this ester. Phthalic acid was isolated after hydrolysis of human urine, which may indicate exposure to some environment-contaminating phthalates (Jaeger 1971). This conclusion should be viewed with caution, however, since phthalic acid occurs in many natural materials.

Jaeger's dissertation also contains a detailed review of toxicity studies on di-2-ethylhexyl phthalate. Jaeger himself detected an enhancement of hexobarbital sleeping times after fairly high (500 mg/kg) doses of intraperitoneally administered di-2-ethylhexyl phthalate, whereas intravenous injections caused a loss in body weight in rats and mice. A recent meeting sponsored by the U.S. National Institute of Environmental Health Sciences concluded that the effect of phthalates on human health is not clear. Residues of phthalates in humans should be avoided, however, "the U.S.A. cannot afford to be stampeded into substitutes" (Anon. 1972b).

Di-2-ethylhexyl phthalate was detected in the heavy mitochondrial fraction of bovine, rat, rabbit, and dog heart muscle at a level of 13,500, 129, 118, and 36 μg/100 g heart muscle (Nazir et al. 1971). All possible precautions were taken to avoid contamination during the work-up procedure and it is therefore likely that di-2-ethylhexyl phthalate in heart muscle mitochondria is not an artifact. There is no evidence whether its origin is natural, or whether it is due to environmental contamination. The alcohol moiety (2-ethyl-1-hexanol) would rather strongly indicate that the latter is the case.

Di-2-ethylhexyl phthalate is not attacked by non-specific esterases (Jaeger et al. 1970).

Di-2-ethylhexyl phthalate and small amounts of dicyclohexyl and dibutyl phthalate were detected in fulvic acid (Ogner et al. 1970) and a subsequent study showed that fulvic acid solubilizes very efficiently these compounds. Thus 125 mg of fulvic acid solubilized 203 mg of di-2-ethylhexyl phthalate, 65 mg of dicyclohexyl phthalate, and 35 mg of dibutyl phthalate at pH = 2.45, and approximately 25% less at pH = 7 (Matsuda et al. 1971).

The growth and reproduction of Daphnia magna showed a 60% inhibition in water containing 3 μg/l of di-2-ethylhexyl phthalate. Food containing phthalates caused abortions in guppies and large mortalities in zebrafish (Anon. 1971b).

No detailed results have been provided as yet. The same report states that levels of dibutyl phthalate in fish ranged from not detectable to 0.5 μg/g, those of di-2-ethylhexyl phthalate were up to 3.2 μg/g.

The acute toxicity (TL50) of dibutyl phthalate to 1 g bluegills at 17°C was 1,230 and 731 μg/l at 24 and 96 h, respectively. The toxicity to Gammarus pseudolimnaeus and to crayfish (Orconectes nais) was low, with an TL50 of >10,000 μg/l (Anon. 1970a).

Channel catfish contained 0.26-0.58 μg/g (on wet weight basis) of dibutyl phthalate and from 1.0 to 7.5, mean 3.2 μg/g, of di-2-ethylhexyl phthalate. One batch of commercial fish feed was reported to contain 0.39 μg/g of dibutyl phthalate, another batch contained 2.0 μg/g of di-2-ethylhexyl phthalate (Anon. 1970a).

Dibutyl phthalate imparts a bitter salty taste to water and a tolerance level of 0.2 mg/l was suggested (Maslenko 1967). Dibutyl and di-2-ethylhexyl phthalate, the latter in concentrations of about 1 μg/l, were detected in water from the Charles River, Boston, Massachusetts (Hites et al. 1972). The concentration of phthalates was quite variable and was not correlated with the runoff.

Dibutyl phthalate is appreciably volatile (Table 2). Air in contact with polyvinyl chloride (1 $m^3/1\ m^2$) contained dibutyl phthalate in a concentration of 6.4 mg/m^3 after 30 h at 20°C, 31.2 mg/m^3 after 1 h at 100°C (Popov et al. 1967), and vapors of dioctyl phthalate are emitted in the effluent air of PVC processing plants (Staschik 1969). A new laboratory building was thoroughly contaminated by phthalates originating from phthalate-impregnated air filters of the central air distribution system (Blumer 1965). It was suggested that the concentration of dibutyl phthalate in the air of ship's cabins should be less than 0.1 mg/m^3 (Men'shikova 1971), and a permissible concentration of 1 mg/m^3 was also proposed (D'yakova 1970). Workers were reported to show signs of discomfort when the concentration of phthalates in air reached 50 mg/m^3 (Armeli et al. 1968).

Monoethyl and monobutyl phthalate were isolated from rat urine after oral administration of diethyl and dibutyl phthalate, respectively (Chambon et al. 1971). The monophthalates had higher intraperitoneal toxicity than the diphthalates (0.7 and 1 g/kg as compared to 2.83 and 4 g/kg, respectively).

The determination of phthalates in biological material consists of extraction, separation of interferring substances (in pesticide residue analysis commonly referred

to as cleanup), and quantitation. The type of substrates most frequently investigated includes food items - milk, cheese, and vegetable oil.

From aqueous solutions containing few other organic compounds phthalates were extracted with ether and identified by TLC on silica with xylene-ethyl acetate-hexane (9:1:1) as the developing solvent. Phthalates were detected by spraying with 4N sulfuric acid - 20% ethanolic resorcinol (1:1) (Schettino et al. 1969a). Phthalates from cleansing solution were extracted with light petroleum, transferred into ethanol and quantified by UV spectrophotometry at 274 nm (Wildbrett et al. 1969). In a similar procedure phthalates were extracted with hexane and quantified by gas chromatography on a column containing 1% of polyvinylpyrrolidine and 1% of Carbowax 20M on DMCS Chromosorb G 80-100 mesh (Bunting et al. 1967).

Dioctyl phthalate was extracted from plastics with ether and determined after hydrolysis by phenolphthalein formation (Chubarova, 1967; Rapaport 1970).

TLC of phthalates, extracted from polystyrene was carried out on silica gel using methylene chloride as solvent, resorcinol and methyl red as developer (Damyanova-Gudeva 1971). Phthalates were chromatographed on silica gel with ethyl acetate-cyclohexane (1:9) as the developing solvent (Swiatecka et al. 1969), and were visualized by Draggendorff reagents or by spraying with anisaldehyde (Nelson 1969).

Phthalates were determined by gas chromatography in small-arms propellants (Norwitz et al. 1971; Alley et al. 1972).

Dibutyl phthalate from air was absorbed in cooled ethanol and determined spectrophotometrically (Bartenev et al. 1969).

The isolation of dioctyl phthalate from milk by dialysis, freeze-drying, and extraction with light petroleum was described (Cerbulis et al. 1967). The light-petroleum extract was further purified by column chromatography on alumina and TLC on silica. The phthalate was finally identified by IR spectrophotometry. Extraction of di-2-ethylhexyl phthalate from milk and its quantification by UV spectrophotometry of the liberated phthalic acid was described (Wildbrett et al. 1968).

Infrared spectrophotometry is a fast technique for the identification of plasticizers. A catalog of spectra has been published (Kendall et al. 1955).

A method for the determination of phthalates by formation of hydroxamates was described (Lehmann et al. 1968). Hydroxamates formed from other esters were separated by TLC.

Phthalates and other dibasic esters were determined by treating a 10-mg sample of the esters or a 10-g sample of olive oil with 2-ml concentrated sulfuric acid and 1 g hydroquinone for 1 h at 145°C. The mixture was taken up in water and extracted with chloroform. The chloroform-extracted products were separated by TLC on silica using either chloroform or a mixture of benzene-chloroform-methanol (2:7:1) as the developing solvent. Samples of natural olive oil contained 1-5 μg/g of phthalates (Covello et al. 1967). Interferences from other compounds were negligible (Ranaudo et al. 1969). The reaction of o-phthalic acid with hydroquinone was described previously (Swann 1957).

A determination of phthalates in lipids by direct injection into a gas chromatograph was described. Lipids were removed on a pre-column and the detection limit of phthalates was 30 μg/g lipid (Rohleder et al. 1972).

Di-2-ethylhexyl phthalate was detected in synthetic glycerides and in solid fats stored in polyvinyl chloride containers (Rost 1970).

For the determination of di-2-ethylhexyl phthalate in tissues, the tissues were freeze-dried and extracted with 20 volumes of chloroform-methanol (2:1 v/v). The extract was diluted with an equal volume of saline. The chloroform phase was treated with silicic acid, filtered, and evaporated to dryness. The residue was dissolved in methanol, centrifuged, and the supernatant was evaporated to dryness with a stream of nitrogen. The residue was taken up in hexane and injected into a gas chromatograph (6' x 1/4' column, 3% SE-30 on gas-chrom Q 80-100 mesh, 225°C). The recoveries ranged from 60 to 98% (Jaeger 1970).

Di-2-ethylhexyl phthalate from heart muscle was eluted in the 4% ether in hexane fraction of silicic acid chromatography. Saponification with 30% aqueous potassium hydroxide for 2 h yielded 2-ethyl-1-hexanol and, after esterification with methanol, dimethyl phthalate. Elemental analysis, infrared, and mass spectrum (molecular ion 390.2765; 149, M-241; 167, M-223; 279, M-111) were consistent with those of di-2-ethylhexyl phthalate. GLC on 5% SE-30 on Chromosorb W (6 ft x 6 mm) at 225°C resulted in good separation of several phthalates including di-2-ethylhexyl and dioctyl phthalate (Nazir et al. 1971).

Several methods for the determination of phthalate plasticizers are also based on the measurement of the alcohol moiety. Dicyclohexyl phthalate and dibutyl phthalate in

cheese and lard were determined by extracting fat with dichloromethane, followed by saponification. Butanol and cyclohexanol were separated by steam distillation and quantified by gas chromatography (Pfab 1967). A similar procedure was used to determine di-2-ethylhexyl phthalate in milk (Reichle et al. 1968).

Phthalates after conversion into dimethyl phthalate were determined in vegetable oils. The phthalic acid content of virgin olive oil was 0.5-1 μg/g and that of sansa oil 1-5 μg/g (Giannesi 1968).

Phthalate plasticizers were saponified by refluxing with 1N ethanolic potassium hydroxide for 1 h. The generated acids were treated with phosphorus pentoxide at 200 C for 10 min, and phthalic anhydride was isolated by sublimation from the reaction mixture (Schettino et al. 1969b). Phthalic anhydride was determined spectrophotometrically after the reaction with 1,3-dihydroxyamino acetone. The methanolysis of phthalates is a first order reaction in the methoxide concentration with the hydrolysis rate about 2-3 times higher for the first than for the second ester group (Ciola et al. 1971).

Polarography was used to determine phthalic acid after extraction from resins and plasticizers and saponification (Traxton 1966).

Liquid chromatography of phthalates was described (Majors 1970).

Dibutyl phthalate in air was determined turbidimetrically after the conversion into lead phthalate (Stepanenko et al. 1969), or spectrophotometrically after the absorption in an organic solvent (Dregval et al. 1969). The latter method was also adopted for the determination of dibutyl phthalate in water.

Fuchsine dyes were used to detect phthalates on thin-layer chromatograms (Sliwiok 1968).

Phthalates were extracted from oils by boiling methanol and detected by IR spectrophotometry (Cianetti et al. 1969).

Condensation of phthalic anhydride with resorcinol to fluorescein was suggested for the identification of phthalates. Quantitative determination was based on the formation of sodium n-hydroxyphthalimide and spectrophotometry at 410 nm. Thin-layer chromatography on silica gel, with chloroform or methylene chloride as the developing solvent, was used for the separation of phthalates (Fickentscher 1970).

A review of the determination of phthalates and their biological properties was recently published (Fishbein et al. 1972).

Experimental

A Barber-Coleman Model 5320 gas chromatograph with a flame-ionization detector, equipped with a stainless steel column (6 ft x 1/4 inch), 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 $mlmin^{-1}$, 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 h and deactivated by the addition of 5% water as described (Holden et al. 1969).

A 45 x 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-bottom flasks on a rotatory 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 h 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 x 5 ml, 2 x 1 h) 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.

Thin-layer chromatography was carried out on 0.25 mm layers of silica gel with UV indicator (Camag DF-5), using chloroform as the developing solvent. The spots of phthalates were either observed directly under the 254 nm UV light (Universal UV lamp Gelman-Camag), or visualized by spraying with resorcinol in acidified ethanol (Kirchner 1967), 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), and herring gulls (Larus argentatus), liver and blubber of a common seal (Phoca vitulina) pup, and commercial fish food were extracted as described (Zitko 1972). Corn oil, herring oil, and fish oil were commercial samples.

Polyvinyl chloride tubing, i.d. 0.9 cm, and 0.3 cm, widely used in the fish-holding facilities, and polyvinyl chloride swimming pool lining used for holding Atlantic salmon smolts, were extracted with chloroform in a single-batch extraction for 6 h. Chloroform was evaporated in a rotatory evaporator and the residue (40, 43, and 34% on weight basis, respectively) was examined by gas chromatography and IR spectrophotometry (Perkin-Elmer 700).

Toxicity of dimethyl, dibutyl, and di-2-ethylhexyl phthalate to juvenile Atlantic salmon (size range 8-11.5 cm, weight range 5.6-14.0 g) at 14-15°C was tested under static conditions using 5 fish per 60 l of solution. The phthalates were solubilized with Corexit 7664 (weight ratio phthalate: Corexit 1:19).

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 4. Interfering peaks were not encountered in 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. GLC separation of some common phthalates is illustrated on Figures 1-5. 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 cleanup by the dimethyl formamide partitioning. A sample of fish oil, spiked with 9.45 and 16.8 μg/g lipid of dibutyl and diamyl phthalate, respectively, gave values of 8.30 and 11.7 μg/g lipid after the dimethyl formamide cleanup. Dibutyl and diamyl phthalate were not detectable in the non-spiked extracts of juvenile Atlantic salmon.

Table 4. The recovery of phthalates from lipid extracts by chromatography on alumina.

Sample		Phthalate, μg/g lipid			
		Di-2-ethylhexyl		Dioctyl	
		Added	Found	Added	Found
Fish oil		44.4	51.6	56.5	55.3
" "		44.4	46.4	56.5	41.8
Juvenile Atlantic salmon	A	13.7	25.2	23.7	19.2
" " "	B	37.6	53.6	65.4	52.5
" " "	A	42.0	52.5	73.0	64.4
" " "	B	83.9	103	146	127
" " "	A	-	12.9	-	-
" " "	B	-	16.4	-	-

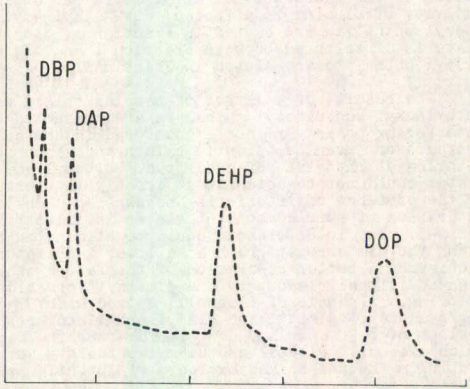

Fig. 1. GLC separation of dibutyl (DBP), diamyl (DAP), di-2-ethylhexyl (DEHP), and dioctyl (DOP) phthalates. Column temperature 229°C.

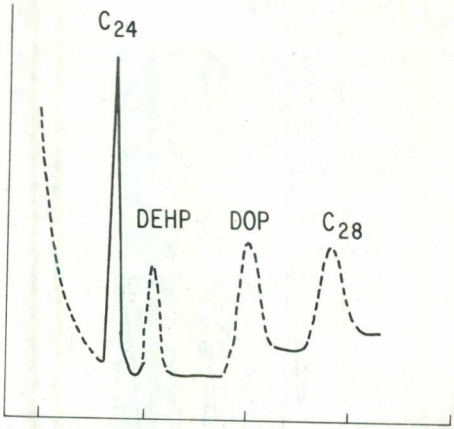

Fig. 2. GLC separation of di-2-ethylhexyl (DEHP) and dioctyl (DOP) phthalate. Tetracosane (C_{24}) and octacosane (C_{28}) used as internal standards. Column temperature 248°C.

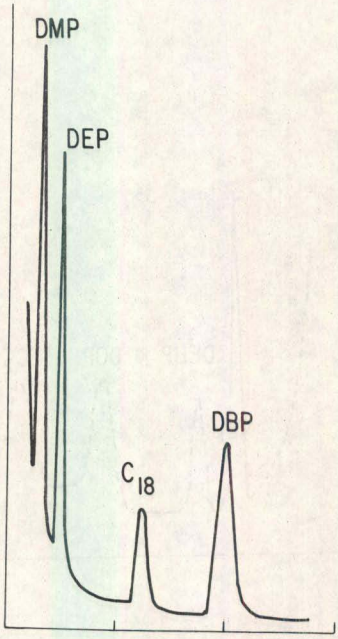

Fig. 3. GLC separation of dimethyl (DMP), diethyl (DEP), and dibutyl (DBP) phthalate. Octadecane (C_{18}) used as internal standard. Colume temperature 170°C.

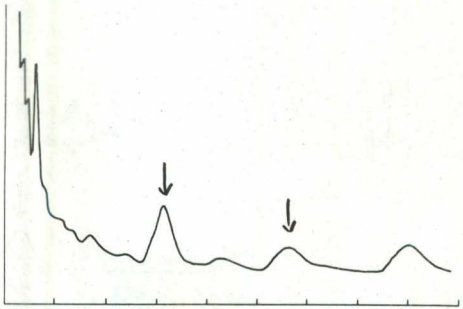

Fig. 4. GLC of fish oil spiked with di-2-ethylhexyl and dioctyl phthalate to 44 and 56 μg/g, respectively. Fraction I after DMF cleanup.

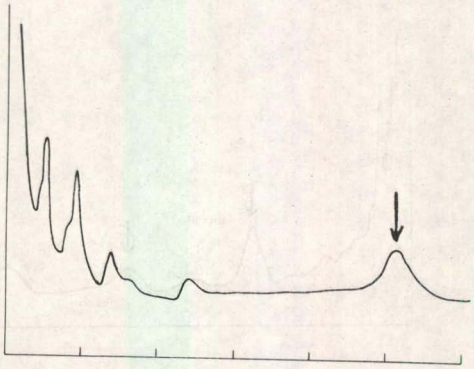

Fig. 5. GLC of fish oil spiked with di-2-ethylhexyl and dioctyl phthalate to 44 and 56 μg/g, respectively. Fraction II after DMF cleanup. The peak of dioctyl phthalate is marked.

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 thin-layer chromatography difficult and the lipids may eventually contaminate the gas chromatographic system. An additional cleanup 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 5 indicate, dimethyl formamide was a suitable partitioning solvent. Except for dimethyl and diethyl phthalate, phthalates

Table 5. Distribution coefficients of phthalates at 24°C.

Phthalate	Initial concn. in hexane, μg/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

could be quantitatively recovered from aqueous dimethyl formamide by extraction with hexane (Table 6). Only about 10%

Table 6. Distribution coefficients C_{DMF}/C_{Hex} at different concentrations of water in dimethyl formamide

Concentrations of water in DMF (v/v) %	9	16.6	28.6	44.5	61.5
Phthalate					
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		

of lipid present in the fractions from the alumina chromatography was extracted into dimethyl formamide (Table 7), and the recovery of dibutyl, diamyl, di-2-ethylhexyl, and dioctyl phthalate was 70-90%.

Table 7. 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

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 detectable levels of phthalates were lower than those in the "chromatography first" procedure, but the recoveries of phthalates were not quantitative (Table 8). 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 (Fig. 6), the emission maximum of o-phthalates is at 360 nm, and that of p-phthalates is at 380 nm (Fig. 7). The fluorescence of o-phthalates is due to phthalic anhydride and as relative

Table 8. Recovery of phthalates from lipid extracts by dimethyl formamide partition*.

Sample	Volume ratio hexane/DMF	Phthalate, μg/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
" "	2	1.58	-	2.80	-	7.40	3.35	9.42	3.95
" "	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**	1	3.15	3.32	5.60	5.02	14.80	13.03	18.85	23.40
" "	1	1.58	1.56	2.80	2.71	7.40	7.43	9.42	7.92
" "	1	0.63	0.53	1.12	0.83	2.96	2.66	3.77	2.78

*Starting with 1 g spiked lipid in 5 ml hexane.

**Amount of phthalates in μg.

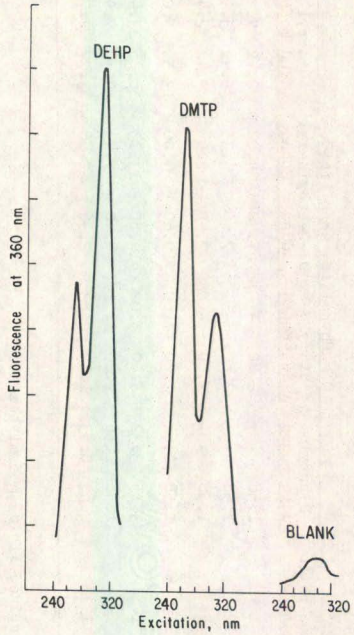

Fig. 6. Fluorescence excitation spectra.
DEHP = Di-2-ethylhexyl phthalate,
DMTP = Dimethyl terephthalate.

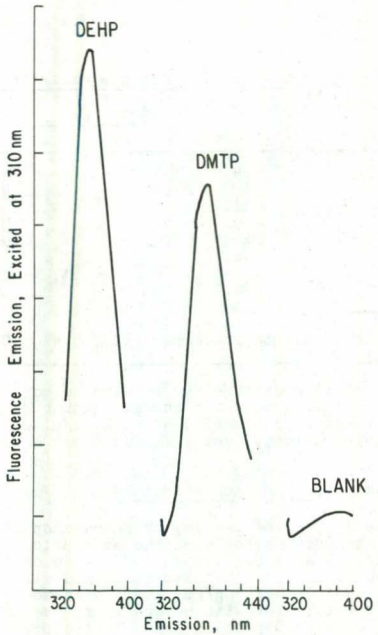

Fig. 7. Fluorescence emission spectra.
DEHP = Di-2-ethylhexyl phthalate,
DMTP = Dimethyl terephthalate.

fluorescence data (Table 9) 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.

Table 9. Fluorescence of phthalates in concentrated sulfuric acid on excitation at 310 nm.

	Relative fluorescence emission at 360 nm	
	Response per	
Phthalate	1 mg	1 millimole
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*	1570*
Dimethyl iso	-	-
Phthalic acid	22.0	3670

*Emission maximum of dimethylterephthalate is at 380 nm

The measurement of fluorescence can be used to confirm phthalate plasticizers in the presence of a certain amount of lipids (up to 350 μg/ml). Under such conditions, 0.2 μg/ml of phthalates, expressed as di-2-ethylhexyl phthalate is still detectable (Fig. 8).

Phthalate contamination in the laboratory

A contamination of samples by phthalates in the laboratory was not encountered when the precautions routine in pesticide residue analysis (Zitko 1972) were observed. A batch of Whatman extraction thimbles contained dibutyl phthalate at a level of 4.25 μg/g. It is a common practice to pre-extract the thimbles with hexane to remove possibly present polychlorinated biphenyls and this treatment simultaneously removes dibutyl phthalate. Dibutyl and di-2-ethylhexyl phthalate were also detected in some batches of anhydrous sodium sulfate. Again, the phthalates together with PCB are removed by pre-extraction with hexane.

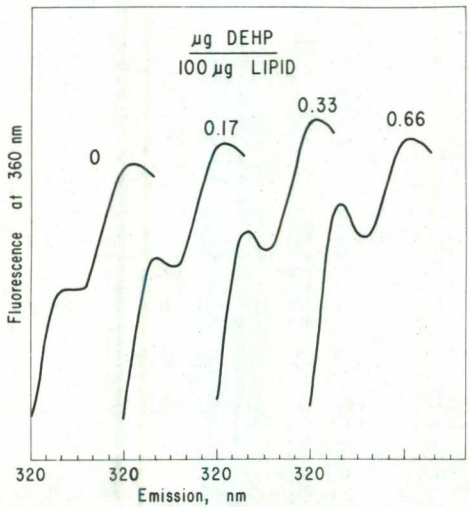

Fig. 8. Confirmation of phthalates by fluorescence emission spectra in the presence of lipids.
DEHP = Di-2-ethylhexyl phthalate

Phthalates in environmental samples

Dibutyl phthalate was detected in eggs of double-crested cormorants and herring gulls (Table 10). Di-2-ethylhexyl phthalate was detected in hatchery-reared juvenile Atlantic salmon (Table 4), and at a level of 10.6 μg/g on lipid basis in the blubber of common seal.

Table 10. Dibutyl phthalate in egg yolks of double-crested cormorants and herring gulls.

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

Dioctyl phthalate was not detectable in any of the samples examined. The phthalates were confirmed by fluorescence measurement and by thin-layer chromatography. Two batches of commercial fish food contained dibutyl phthalate, 5.3 and 8.5 μg/g lipid, respectively, and di-2-ethylhexyl phthalate at 8.8 and 8.5 μg/g lipid, respectively.

Phthalates in plastics from the fish-holding facilities

Polyvinyl-chloride tubing, i.d. 0.3 cm contained a mixture of di-2-ethylhexyl and diisoctyl phthalate. The swimming pool lining contained di-2-ethylhexyl phthalate. IR spectrum of the material extracted from the polyvinyl-chloride tubing i.d. 0.9 cm indicated the presence of a phthalate, but contained two additional absorption maxima at 1210 and 1420 cm^{-1}, respectively. On gas chromatography the residue yielded two peaks with retention times relative to di-2-ethylhexyl phthalate of 0.18 and 0.5, respectively.

Toxicity of phthalates to fish

In the preliminary bioassay experiments with juvenile Atlantic salmon no mortality in 96 h was observed at 10 mg/l of dimethyl and di-2-ethylhexyl phthalate. At this concentration dibutyl phthalate caused 100% mortality in 3 h (LT50 = 1.25 h), but no mortality in 96 h occurred at 1 mg/l.

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 cleanup, 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 µg of phthalates, expressed as di-2-ethylhexyl phthalate can be detected in the presence of 100 µg of lipids.

The detection limits of phthalates examined in this work are about 3-5 µg/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 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 (Holden 1970).

It is obvious from the literature review and the new data presented in this report that phthalates are widely distributed in the environment. According to the available toxicological evidence the environmental concentration of phthalates are very likely at the "no effect" levels. On the other hand, phthalates as any other synthetic chemicals, persisting in the environment, are a cause for concern and their uncontrolled discharge into the environment should be limited as much as possible.

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APPENDIX

The following additional information became available when this report was in typing:

The behaviour of several phthalates in the AOAC Multiresidue Method for pesticides in fatty foods was determined (Kamps 1972). Phenethyl or p-chlorophenethyl phthalimide were described as useful derivatives for the determination of phthalates in fish extracts (Breder et al. 1972). Di-2-chloroethyl phthalate was suggested for the same purpose (Stalling et al. 1972).

Fish metabolise di-2-ethylhexyl phthalate to 2-ethylhexyl phthalate (main metabolite), its glucuronide, phthalic acid, and phthalic acid glucuronide (Stalling et al. 1972).

The toxicity of a number of phthalates to larvae of Leptimotarsa decemlimeata and Tribolium destructor was determined (Vasechko et al. 1970). Dioctyl and dibutyl phthalate administered to Wistar rats at 1, and 1 and 10% of LD50 respectively, caused enlargement of the liver. In addition, dioctyl phthalate increased the activity of glutamic-pyruvic transaminase. Feeding a diet containing dioctyl phthalate at a level of 3.5 g/kg caused a fall in body weight in males, an increase of liver and kidney weight in females and a rise in the activity glutamic-pyruvic and glutamic-oxalacetic transaminases (Piekacz 1971).

The acceptable daily intake (ADI) of di-2-ethylhexyl phthalate is 1 mg/kg body weight (Woggon et al. 1971).

Dipropyl and dibutyl or diisobutyl phthalate were detected in lipids of bacteria (Thiele et al. 1972). Phthalates were also detected in filter paper (Remberg et al. 1971).

A spectrophotometric method for simultaneous determination of dibutyl phthalate and tricresyl phosphate in air was described (Druyan 1972).

The use of ethylene tetrabromophthalate and tetrachlorophthalate for flame-proofing of polyethylene terephthalate fiber was patented (Yanagi et al. 1971).

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