Toxicity of Phthalate Esters in Fish and Shellfish from Virginia Beach Using Matrix Solid Phase Dispersion (MSPD) and GC-MS

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Summary: This study presents the outcome of an investigation on the occurrence of phthalates, in eight edible marine fish species from lower James River, Chesapeake Bay along the Virginia Coast of Atlantic ocean. These include shell fish like Crab(Callinectes sapidus), Clam(Merccnaria), Oyster(C.ariakensis), and white shrimp (Litopenaeus vannamei) from aquaculture center of Virginia Tech. These were analyzed for their content of Dimethyl Phthalate, Diethyl Phthalate, Di-n-butyl Phthalate , butyl benzyl Phthalate , bis(2-ethylhexyl) Phthalate ,and di-n-octyl Phthalate Mean concentrations (range) of Di(2-ethylhexyl) phthalate (DEHP), Butyl benzyl phthalate (BBP) and di-n-butyl phthalate (DBP) in fish samples were 1.1 (<0.01-1.5), 0.22 (<0.01-1.1) and 0.14 (<0.01-1.3) μg g−1; those in shell fishery were 1.2 (<0.02-1.3), 0.13 (<0.01-0.27) and 0.09 (<0.02-0.22) μg g−1, respectively. The highest concentration of bis (2-ethylhexyl) Phthalate in fish samples were found in Atlantic mackerel (Centropristis striate) (1.98±0.92) μg/g (wet weight) and Oyster (C.ariakensis) (2.30 μg/g (wet weight), were higher than those in other fish species.

Keywords: GC-MS; phthalates; Endocrine disruptor; Seafood; and Virginia Beach.

Introduction

During the last two decades, there has been a growing concern for the study of the impact of man-made chemicals on wildlife and humans. Phthalates and their metabolites are excreted from human urine and wastewater (such as water that washes off cosmetics, facial cream, lotion, shampoo). Phthalate-containing wastewater reaches the environment via untreated sewage discharged into streams, rivers, lakes, oceans, and other bodies of water [1] and worldwide commonly used phthalates in consumer and industrial products has been listed as below

Table-1: List of phthalates commonly used in consumer and industrial products worldwide

Dimethyl phthalate	DMP	$C_6H_4(COOCH_3)_2$
Diethyl phthalate	DEP	$C_6H_4(COOC_2H_5)_2$
Diallyl phthalate	DAP	$C_6H_4(COOCH_2CH{=}CH_2)_2$
Di-n-propyl phthalate	DPP	$C_6H_4[COO(CH_2)_2CH_3]_2$
Di-n-butyl phthalate	DBP	$C_6H_4[COO(CH_2)_3CH_3]_2$
Diisobutyl phthalate	DIBP	$C_6H_4[COOCH_2CH(CH_3)_2]_2$
Butyl cyclohexyl phthalate	BCP	$CH_3(CH_2)_3OOCC_6H_4COOC_6H_{11}$
Di-n-pentyl phthalate	DNPP	$C_6H_4[COO(CH_2)_4CH_3]_2$
Dicyclohexyl phthalate	DCP	$C_6H_4[COOC_6H_{11}]_2$
Butyl benzyl phthalate	BBP	$CH_3(CH_2)_3OOCC_6H_4COOCH_2C_6H_5$
Di-n-hexyl phthalate	DNHP	$C_6H_4[COO(CH_2)_5CH_3]_2$
Diisohexyl phthalate	DIHxP	$C_6H_4[COO(CH_2)_3CH(CH_3)_2]_2$
Diisoheptyl phthalate	DIHpP	$C_6H_4[COO(CH_2)_4CH(CH_3)_2]_2$
Butyl decyl phthalate	BDP	$CH_3(CH_2)_3OOCC_6H_4COO(CH_2)_9CH_3$
Di(2-ethylhexyl) phthalate	DEHP DOP	$C_6H_4[COOCH_2CH(C_2H_5)(CH_2)_3CH_3]_2$
Di(n-octyl) phthalate	DNOP	$C_6H_4[COO(CH_2)_7CH_3]_2$
Diisooctyl phthalate	DIOP	$C_6H_4[COO(CH_2)_5CH(CH_3)_2]_2$
n-Octyl n-decyl phthalate	ODP	$CH_3(CH_2)_7OOCC_6H_4COO(CH_2)_9CH_3$
Diisononyl phthalate	DINP	$C_6H_4[COO(CH_2)_6CH(CH_3)_2]_2$
Diisodecyl phthalate	DIDP	$C_6H_4[COO(CH_2)_7CH(CH_3)_2]_2$
Diundecyl phthalate	DUP	$C_6H_4[COO(CH_2)_{10}CH_3]_2$
Diisoundecyl phthalate	DIUP	$C_6H_4[COO(CH_2)_8CH(CH_3)_2]_2$
Ditridecyl phthalate	DTDP	$C_6H_4[COO(CH_2)_{12}CH_3]_2$
Diisotridecyl phthalate	DIUP	$C_6H_4[COO(CH_2)_{10}CH(CH_3)_2]_2$

Phthalates also reach the natural environment via pesticides, industrial lubricants, and phthalate-containing garbage that humans throw away. Because everything of humans use eventually get disposed of into the environment, it is inevitable that phthalates are found in the environment. As team of British scientists from the University of Exeter, University of Plymouth, and Brunel University found that when freshwater fish (belonging to species *Rutilus rutilus*) were exposed for 300 days to treated sewage effluent (containing phthalates and other endocrine disruptors). The reproductive, endocrine, immune, genotoxic, and nephrotoxic effects were observed in fish on exposure [2]. These studies have suggested that synthetic and naturally occurring substance in the environment may affect the normal function of the endocrine systems. These substances are also referred to as endocrine disrupting chemicals (EDCs) [3]. Today, phthalates and BPA are found in many mass-produced products including medical devices, food packaging, perfumes, cosmetics, children's toys, flooring materials, computers, CDs, etc [4] . In wildlife, alterations in sexual reproductive behavior have been reported in areas of contamination with EDCs. For example, malformations in the sexual organs of alligators have been reported in Lake Apopka, Florida, where high concentrations of DDT and its degradation products

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have been detected [5], and feminization of trout in the Great Lakes has been associated with high levels of polychlorinated biphenyls (PCBs) in water samples. Other studies have indicated that many chemicals including phthalate esters may affect development and reproduction, including germ cells, sperm mobility, chryptorchidism and hypospadias, in laboratory animals [6].

Phthalate ester plasticizers are widely used in synthetic polymers, especially polyvinyl-chloride commonly used for packaging, storing and preserving food [7]. in insect repellant preparations, cosmetics, decorative inks, muni-tions, and industrial and lubricating oil. Scientists from Colorado State University have found that African clawed frogs (*Xenopus laevis*) exposed to very low concentrations of di-*n*-butyl phthalate (DBP) suffered numerous problems [8].

The ubiquity of phthalate esters have been widely reported in various environmental samples in the developed countries of Europe and America. Their occurrence have been reported in the Greater Manchester River [9] Phthalates were found in tap water [10] . They were found in the water, fish, and other aquatic organisms of the Gulf of Mexico [11]. They are suspected to be carcinogenic [12]. (They are lipophilic and tend to concentrate along the ecological food chains, a process known as bioamplification [13]. Since humans are usually at the top of the food chain, high concentrations of such toxic substances may occur in the human diet with undesirable results. The reports of their toxicity should make it important to have knowledge of the presence of these compounds in our environment. One of the main routes of exposure is via water as these chemicals find their ways into rivers through effluent discharges, leaching from waste dumps and through diffuse sources.

Several attempts have been made to determine phthalate esters in the aquatic environment by gas liquid chromatography (GLC) with electron capture detector [14] . and FID [15] Other methods include the use of GCMS [16] and differential pulse polarography [17] .A major problem in the analyses of environmental samples is the reduction of background contamination to levels less than the very low (parts per billion or ppb) levels generally present in the samples. This problem of background contamination has been more serious in the trace analysis of phthalates than in the studies of many other pollutants (including the chlorinated hydrocarbons) because phthalates are present in almost all equipment and reagents used in the laboratory. The Matrix solid phase dispersion (MSPD) was used to extract residual phthalates in fish tissue due to high average recoveries and relative standard deviations (RSDs) below 20% as compared to all other conventional solid phase extraction techniques such as soxhlet extraction and liquid-liquid extraction (LLE). In MSPD analytical method uncertainties were found lower than 23.5% and 30%, with and without recovery correction, respectively and sample size is very small only two grams tissue is required for single analysis. The rapid and practical MSPD technique has a particular application in determining phthalates of different physicochemical properties in fish with satisfactory validation parameters. The study estimated that MSPD has significant advantages over all conventional extraction techniques and liquid-liquid extraction (LLE) LLE because, coupled with simultaneous stage of purification, it allowed for a radical reduction time of analysis and its cost. MSPD fulfilled the requirements of multi-residue techniques. The method is reliable and can be useful for routine monitoring of phthalates in fish.

Non-plastic materials like cork, glass wool, Teflon sheets and aluminum foil have been found to contain the most prevalent of the phthalates, di-2-ethylhexyl phthalate (DEHP) that often results in high background levels [16].

A list of phthalates commonly used in consumer and industrial products worldwide has been given in a Table-2. The data relating to the carcinogenicity of phthalates in general and DEHP in particular have been reviewed by both national and international expert scientific groups and they have reached the following conclusions.

European Union (1990). The EU Commission Decision 90/420/EEC of 25 July, 1990 states that DEHP shall not be classified or labeled as a carcinogenic substance [18].

EU Scientific Committee for Toxicity, Ecotoxicity and the Environment (CSTEE) (1998) [19] is assumed that the carcinogenic effect is related to peroxisome proliferation in rats. A carcinogenic effect solely related to the peroxisome proliferation in rodents may have little relevance for humans."

World Health Organization (1992) The IPCS Environmental Health Criteria No 131 on DEHP (1) concludes that "Currently, there is not sufficient evidence to suggest that DEHP is a potential human carcinogen [20].

Canada (2009) A Priority Substance Assessment on DEHP published by the Canadian government classified DEHP in Group IV - "unlikely to be Carcinogenic to Humans.

USA. The American Conference of Governmental Industrial Hygienists (ACGIH) has classified DEHP as an animal carcinogen [21].

The aim of this study was to develop a solid phase extraction method for the determination of phthalates in fish tissue samples along with geographical representation of background contamination.

Results and Discussion

The GC/GC-MS results of triplicate extraction showed respective recovery efficiencies of 95.5% ± 0.6, 89% ± 2.5, 91% ± 1.9 and 59% ± 8.5 for DMP, DEP, DBP, and DEHP (Table-2). Good laboratory practice (GLP) was applied throughout and procedural blank were analyzed. All blanks had values below the detection limit of the method.

The limits of detection were 0.020 µg/g for DMP, 0.025 µg g^{-1} for DEP, 0.030 µg/g for DBP and 0.05 µg g^{-1} for DEHP and recoveries obtained from spiking experiments and elution with 50/50 ratio (v/v) of CH_3OH in CH_2Cl_2 were DMP, 94.0±0.07%, DEP, 96±04%, DBP89±0.05% and DEHP 83±0.09%, . Phthalates identification and quantification parameters of the studied compounds and abundance of target ions are given in Table-2.

Total ion chromatogram of rock fish sample and oyster and their combined samples were determined by spiked concentration of internal standard (Fig. 1-3). The use of solid phase extraction and GC-Mass provides the basis for the selective determination of phthalate esters in tissue samples .Of the several solvent ratios (methanol in dichloromethane) used for selective elution of phthalate esters from prepacked florisil column through C 18 solid phase cartridge, the 50/50 ratio, CH_3OH in CH_3Cl_2 gave the best result.

The highest concentration of Dimethyl Phthalate (DMP) and Diethyl Phthalate (DEP) in fish samples was found in Rock (1.35 µg g^{-1}) and (1.60 µg g^{-1}) respectively and *white* shrimp and eggs of flounder were contaminated at maximum level of (1.96 µg $g^{-1}$and 0.98 µg g^{-1}) among shell fishery respectively. These compounds however were either not detected or below limit of detection in Croaker and Atlantic Mackerel as well as in eggs of flounder and Clam. Highest level of Di-n-butyl phthalate (DBP) and Butylbenzyl phthalate (BBP)) in fish samples was found in Tilapia and Bluefish as (1.09µg g^{-1}) and (1.81 µg g^{-1}) while in shellfishery *Clam* and Oyster were contaminated at maximum level of (2.00 µg g^{-1}) and (1.56 µg g^{-1}) respectively. These compounds however were either not detected or below limit of detection in Trout, Rock and Perch as well as in oyster, Clam, *white* shrimp and eggs of flounder.

Other studies phthalates were observed in such a range among fishes as DEHP was (0-2.01 µg g^{-1}) , DNoP (0-2.10 µg g^{-1}) and DNoP-d_4 (0-0.21 µg g^{-1}) and in shellfishery DEHP was found in a range of 0-2.01 µg g^{-1} ,DNoP (0-2.10 µg g^{-1}) and DNoP-d_4 as 0-0.21 µg $g^{-1,}$ DEHP was 0-2.50 µg g^{-1}, DNoP as 0-2.30 µg g^{-1}) and DNoP-d_4 was 0-0.19 µg $g^{-1.}$

Our data suggested that phthalates level in collected samples were affected by fish habitat and physiochemical properties of polluted contaminants and not found alarming Table-3 and 4. DEHP in aquatic environments presents a significant challenge due to established biological effects as it has low water solubility and its tendency to form colloidal dispersions above the 3 mg amount in water [22] . Given the widespread occurrence of phthalates in the aquatic environment, fish are also likely to be exposed to phthalates via the water column, food and/or via fish feed packing materials. Occurrence of phthalates in fishery was studied by any scientists and found that the highest concentration of DEHP in fish samples were found in Liza subviridis (253.9 mg/kg dw) and *Oreochromis miloticus* niloticus (129.5mg/kg dw). BSAF of DEHP in *L. subviridis* (13.8-40.9) and O. miloticus niloticus (2.4-28.5) were higher than those in other fish species, indicating that the living habits of fish and physical-chemical properties of phthalates, like log Kow, may influence the bioavailability of phthalates in fish. [23] .

Table-2: Mean recoveries (%) of phthalate esters from spiked fish sample.

	Methanol–Dichloromethane	Percentage recovery (means±S.D.a, n = 3)		
mixture (v/v) %	**DMP %**	**DEP %**	**DBP %**	**DEPH %**
10:90	**62.0±0.1**	**64.0±0.1**	**57.0±0.3**	**42.0±0.4**
30:70	**69.0±0.1**	**70.0±0.1**	**66.0±0.1**	**49.0±0.4**
50:50	**94.0±0.07**	**96.0±0.04**	**89.0±0.06**	**83.0±0.1**
70:30	**74.0±0.08**	**68.0±0.04**	**71.0±0.07**	**65.0±0.09**
90:10	**66.0±0.1**	**59.0±0.1**	**60.0±0.08**	**48.0±0.2**

S.D. = Standard deviation.
DMP, dimethyl phthalate; DEP, diethyl phthalate; DBP, dibutyl phthalate; DEHP, diethylhexyl phthalate.

Table-3: Identification and quantification parameters of the studied compounds.
[a] Internal standard.

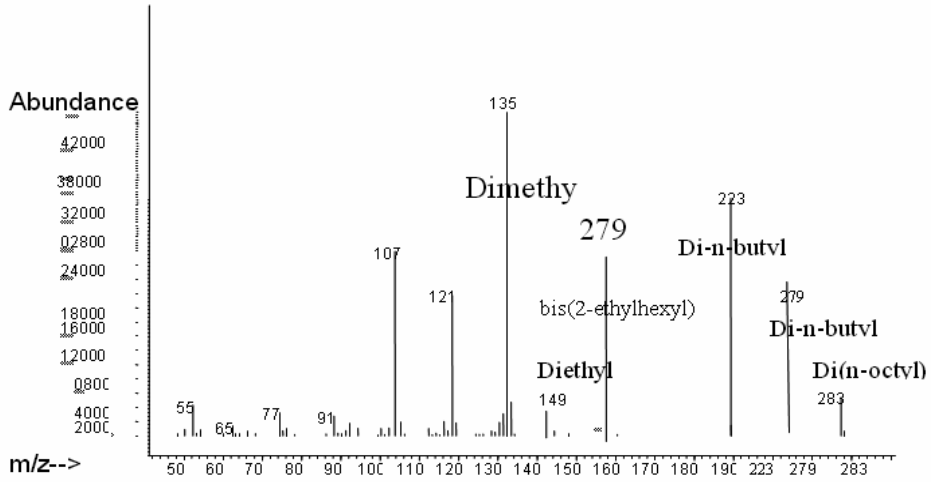
Compounds	Abbreviation	Chemical type	Target ions (abundance) (*m*/*z*)	Quantitation ions (*m*/*z*)
Dimethyl phthalate	DMP	$C_{10}H_{10}O_4$	135 (15.0), 163 (100), 194 (15.0)	163
Diethyl phthalate	DEP	$C_{12}H_{14}O_4$	149 (100), 177 (28.0)	149
Di-n-butyl phthalate	DBP	$C_{16}H_{22}O_4$	149 (100), 205 (6.0), 223 (6.2)	149
Butylbenzyl phthalate	BBP	$C_{19}H_{20}O_4$	91 (71.5), 149 (100), 205 (21.5)	149
bis(2-ethylhexyl) Phthalate	DEHP	$C_{24}H_{38}O_4$	149 (100), 167 (50.0), 279 (35.5)	149
di(n-octyl)phthalate	DNOP	$C_{24}H_{38}O_4$	149 (100), 279 (18.0)	149
Di(n-octyl)phthalate	DNOP-d_4		153 (100), 283 (20.5)	153

Table-4: Results for Phthalates compounds determined in edible fishes from Virginia Coast µg/g (wet weight).

Species / No of samples / Types of Phthalates	Bluefish *(Pomatomus saltatrix)* (8)	Trout (Cynoscion nebulosus) (2)	Rock (*Morone saxatilis*) (2)	Flounder (Paralichthys dentatus) (4)	Croaker (Micropogon undulatus) (5)	Perch (Bairdiella chrysoura) (3)	Tilapia Tilapia sp (6)	Atlantic mackerel *Centropristis striate* (2)
Dimethyl Phthalate	0.20±1.03	1.20±0.20	1.35±0.31	0.98±0.50	<LOD	1.08±0.53	1.00±2.33	0.36±2.00
Diethyl Phthalate	1.60± 0.63	1.36±0.00	0.81±0.15	0.28±0.05	0.62±0.32	1.00±0.27	1.23±2.03	nd
Di-n-butyl Phthalate	<LOD	nd	nd	<LOD	1.03±0.05	<LOD	1.09±0.30	1.08±0.84
butyl benzyl Phthalate	1.81±0.15	1.23±0.70	0.23±0.99	1.00±0.99	1.08±0.20	0.45±0.15	1.02±0.92	<LOD
bis(2-ethylhexyl) Phthalate	nd	1.69±0.35	nd	1.34±0.35	1.50±0.98	2.01±2.03	0.04±2.03	1.98±0.92
di-n-octyl Phthalate	0.42±0.35	<LOD	0.55±0.31	0.85±0.31	1.36±0.20	2.10±2.03	1.36±2.03	nd
Di(n-octyl)phthalate	0.21±0.13	nd	<LOD	<LOD	0.20±0.05	<LOD	0.19±0.10	nd

Table-5: Results for Phthalates compounds determined in shell fishes from Virginia Coast µg/g (wet weight)

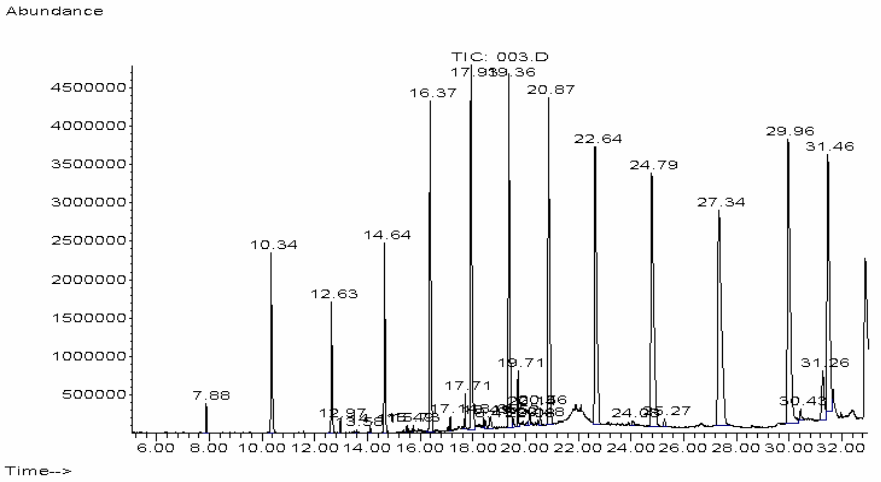
No of samples Phthalates	Oyster (*C.ariakensis*) (15)	Clam *(Merccnaria*) (12)	Crab *(Callinectes sapidus)* (12)	White shrimp *Litopenaeus vannamei)* (30)	Eggs of flounder (04 pairs)
Dimethyl Phthalate	1.21±1.05	1.12±0.60	1.00±1.74	1.96±2.41	nd
Diethyl Phthalate	0.21±1.05	nd	<LOD	<LOD	0.98±1.74
Di-n-butyl Phthalate	<LOD	2.00±1.74	1.89±1.74	<LOD	1.21±1.74
Butyl benzyl Phthalate	1.56±1.04	<LOD	1.30±2.13	0.30±0.50	<LOD
bis(2-ethylhexyl) Phthalate	2.50±3.13	0.50±0.32	1.43±0.68	1.53±2.02	2.21±0.34
di-n-octyl Phthalate	1.00±3.12	1.80±0.14	0.61±4.55	nd	2.30±0.27
Di(n-octyl)phthalate	0.19±1.16	nd	<LOD	nd	0.17±1.16



Target Compounds

Dimethyl phthalate	0.54 mg/l	135 (15.0), 163 (100), 194 (15.0)
Diethyl phthalate	0.36 mg/l	149 (100), 177 (28.0)
Di-n-butyl phthalate	0.25 mg/l	149 (100), 205 (6.0), 223 (6.2)
Butylbenzyl phthalate	0.65 mg/l	91 (71.5), 149 (100), 205 (21.5)
bis(2-ethylhexyl) Phthalate	0.35 mg/l	149 (100), 167 (50.0), 279 (35.5)
di(n-octyl)phthalate	0.41 mg/l	149 (100), 279 (18.0)
Di(n-octyl)phthalate	1.53 mg/l	153 (100), 283 (20.5)

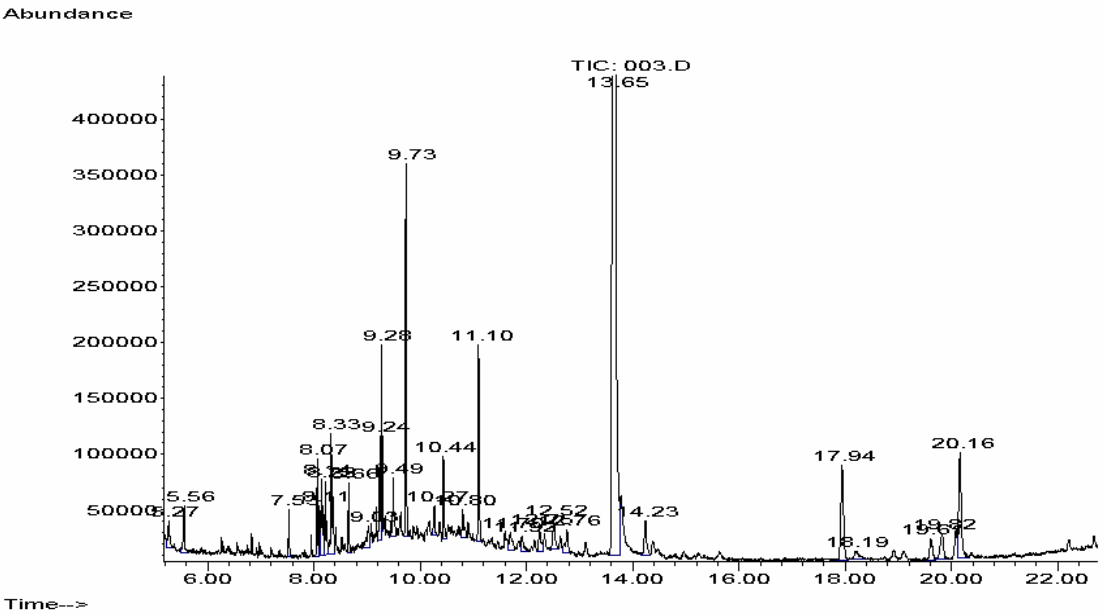
Fig. 1: Typical total ion chromatogram of rock fish sample, spiked with internal standard. Quant Method: C:\MSDCHEM\1\METHODS\PTHALATES.M (RTE Integrator).



Target Compounds

1	Dimethyl phthalate	135 (15.0), 163 (100), 194 (15.0)
2	Diethyl phthalate	149 (100), 177 (28.0)
3	Di-n-butyl phthalate	149 (100), 205 (6.0), 223 (6.2)
4	Butylbenzyl phthalate	91 (71.5), 149 (100), 205 (21.5)
5	bis(2-ethylhexyl) Phthalate	149 (100), 167 (50.0), 279 (35.5)
6	di(n-octyl)phthalate	149 (100), 279 (18.0)
7	Di(n-octyl)phthalate	153 (100), 283 (20.5)

Fig. 2: Typical total ion chromatogram of oyster sample, spiked with internal standard. Quant Method: C:\MSDCHEM\1\METHODS\PHENOLS.M (RTE Integrator).



Target Compounds

1	Dimethyl phthalate	135 (15.0), 163 (100), 194 (15.0)
2	Diethyl phthalate	149 (100), 177 (28.0)
3	Di-n-butyl phthalate	149 (100), 205 (6.0), 223 (6.2)
4	Butylbenzyl phthalate	91 (71.5), 149 (100), 205 (21.5)
5	bis(2-ethylhexyl) Phthalate	149 (100), 167 (50.0), 279 (35.5)
6	di(n-octyl)phthalate	149 (100), 279 (18.0)
7	Di(n-octyl)phthalate	153 (100), 283 (20.5)

Fig. 3: Collective total ion chromatogram of fish and shell fish sample, spiked with internal standard. Quant Method: C:\MSDCHEM\1\METHODS\PHENOLS.M (RTE Integrator).

This method has been developed and validated for animal tissue after determination of recovery of each phthalates, instrument LOD by following the steps of internal spiking, linearity and repeativity etc. It was difficult to give all details in single manuscript.

In early 70th a very low level of DBP (0-78ppb) and DEHP (0-160ppb) was found in 21 samples of fish available to the Canadian consumers. The bulk of the human intake of phthalates is likely to occur or have occurred through direct contamination of foodstuffs, from food packaging such as cellophane, rayophane, aluminium paper-foil laminate packaging (such as on some butters) or foil lids. This has lead to butylbenzylphthalate (BBP) concentrations of 13 – 15 mg/kg in sandwiches and up to 12 mg/kg in meat pies packed in nitrocellulose-coated regenerated cellulose film, as well as up to 47.8mg/kg in butter and margarine packed in aluminum paper-foil laminate [24]. Similarly, many studies reported the presence of plasticizer residues in fishery that can be \attributed to (a) fishery contamination in the seafood processing plant, (b) contamination of plasticizers from the packing material of boxes to the fishery since quality may vary depending on the raw material as well as the technology used in plastic boxes production, and (c) cross-contamination during analytical procedure due to the wide use of plasticizers their occurrence in the environment has been reported widely, possibly arguing against a rapid biodegradation in some environments [25] .

It may be concluded that the selected phthalates were found at low level in fish depending upon the variety of fish, location, season and environmental condition of the sea.

Experimental

Samples study Area and Sampling Sites

The department of Civil and Environmental Engr. of Virginia Tech has undertaken a study (2004–2005) to measure the ecotoxicological effect of phthalates as endocrine disrupters in commonly edible shellfishes and fishes. A number of fishes and shell fishes were procured along the Virginia Beach. Virginia is the third largest seafood producer and the largest on America's Atlantic coast. The waters of the Chesapeake Bay are the largest and most biologically diverse estuary of America, yielding more fishery than any of the 840 other estuaries in America. Virginia's shellfish, including scallops, clams, blue crab, soft-shell crab, oysters and fish such as flounder, mackerel, croaker, and striped bass, White shrimp (*Litopenaeus vannamei*) and Tilapia were collected from Aqua Cultured farm in Southern Virginia (Table-5).

Instrumentation

The instrument used was an Agilent 6890 series GC with an Agilent 5973 Mass Selective Detector (EI 70 eV) coupled with the computer data system Agilent Chem station.

Reagents

All chemicals used were of analytical reagent grade. All solvents used were further purified by distillation. All the standards of Phthalates such as Dimethyl Phthalate, Diethyl Phthalate, Di-n-butyl Phthalate , butyl benzyl Phthalate bis(2-ethylhexyl) Phthalate ,and di-n-octyl Phthalate were purchased from Chem Services (West Chester,PA,USA) and Aldrich-Sigma (Milwaukee,WI,USA). (With percentage assay greater than 99%) were used.

The internal standard (n-butyl benzoate) was chased from Fisher and was at 99% grade purity**.** For derivatization the Trimethylchlorosilane (TMCS) or N-Methyl-N-trimethylsilyltri-fluoroacetamide+ 1% Trimethylchlorosilane and N,o-Bis(trimethylsilyl) trifluoroacetamide (BSTFA) PI-48915, Pierce Chemical Company, No.:48915, was purchased.

Preparation of Stock Standard Solution and Determination of Response Factors

A stock solution (1.0 mg/L) of the mixture of esters dimethyl (DMP), diethyl (DEP), dibutyl (DBP) and diethylhexyl (DEHP) inmethanol was prepared. The required volume of ester was calculated from the density of each of the esters. A 1.0 mg/ L n-butyl benzoate (a non-aqueous pollutant) in methanol was used as internal standard. The stock solution containing the internal standard was run on the GC-MS.

The response factor was calculated from: Area of the peak of phthalate ester / Area of the peak of internal standard.

Determination of the Limit of Detection (LOD) of GC System

The LOD was calculated considering yb + 3Sb for each calibration curve (Miller and Miller, 1998) for each phthalate esters (DMP-, DEP-, DBP- and DEHP-), with the range 2.5–50 mg L−1 (Sb =

standard error of the regression line and Y*b* is the blank value).

Quality Assurance Studies

A twelve- (12) port glass Manifold extractor tank was used. The column (syringe) barrels were the Florisil (1 g packing) designed for environmental samples and purchased from Supelco, U.S.A. The vacuum in the tank was created using a pump. Columns were first conditioned by passing about 2 mL CH_3OH.

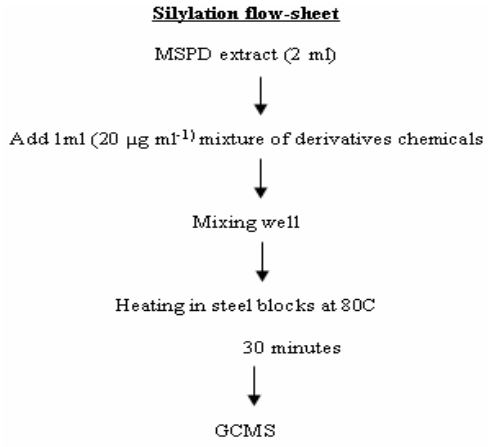
Sample Preparation, Extraction, Recoveries and Reproducibility

Samples storage, sample preparation for extraction, purification and identification are carrying on in Pesticides Residues Laboratory, FST and Civil and environmental Eng Department .After dissection, samples of some tissues and organs were removed, wrapped in aluminum foil and stored in a deep freezer (−20 °C) until analysis. All samples were thawed and cut into small pieces that were thoroughly mixed and analyzed by Matrix solid phase dispersion (MSPD). The Matrix solid phase dispersion (MSPD) was validated used to extract and average recoveries for these compounds and standard deviations (RSDs) was determined. Finely chop frozen tissue was homogenized in Glass mortar and pestle and Weighed 0.5 g of tissue in a small plastic weigh boat. Samples were extracted in a single step with surface modified bonded silica sorbent (C18) using a mortar and pestle and was spiked with 1 µL of 5 mg 100ml−1 of standard mixtures of dimethyl (DMP), diethyl (DEP), dibutyl (DBP) and diethylhexyl (DEHP) containing 1.00µl each standard solution (DMP, DEP, DBP, DEHP) were added to each.

The preconditioned Florisil columns have been used for the cleanup of phthalate esters. However a number of methods have been described for the cleanup of phthalate esters prior to their analysis by gas chromatography [15]. The most commonly used solid phases for separation/clean up of environmental samples are deactivated florisil (3% water v/v) Giam, 1975), alumina and silica gel (5% water) [15]. The spiked samples/C18 mixture is transferred to a syringe barrel column containing pre packed Florisil, a frit is tapped on the surface of column. Thus samples were passed through the preconditioned Florisil columns at a flow rate of 1 mL min−1.15 mL mixture each, of variable portions of CH_3OH in CH_2Cl_2 (10:90, 30:70, 50:50, 70:30 and 90:10, v/v) was used separately to elute the columns. The eluent from each column was evaporated and dried under nitrogen -vacuum in sample vials. Once dry, 1 µL of 1.0 mg L−1 n-butyl benzoate in methanol was added as internal standard to each of the residues.

Silylation

Silylation was done to derivatized all samples for GC/MS analysis by which , an active hydrogen is replaced by an alkylsilyl group such as Trimethylchlorosilane (TMCS) or N-Methyl-N-trimethylsilyltri-fluoroacetamide+ 1% Trimethyl-chlorosilane and N,o- Bis(trimethylsilyl) trifluoroacetamide (BSTFA) .As compared to their parent compounds, silyl derivatives are more volatile, less polar, and more thermally stable. As a result, GC separation is improved and detection is enhanced then re-dissolved in methanol containing Triton-101 as an internal standard.



Flow chart of *Silylation*

The instrument used was an Agilent 6890 series GC with an Agilent 5973 Mass Selective Detector (EI 70 eV) coupled with the computer data system Agilent Chem station. The GC column, a 30 m DB-5MS (0.25 mm i.d.; 5% biphenyl, 95% dimethylpolysiloxane), was connected directly to the MS detector. The transfer line was kept at 280 °C. The sample (1 ul) was injected in a split less mode at 260 °C. The temperature program was: 70 °C (2 min)10 °C per min up to 240 °C2 per min up to 260 °C- 20 °C per min up to 300 °C (2 min).

Calibration standard solutions were used to generate response factors in relation to the internal standard. The concentration of each phthalates was calculated by rationing the analyte integrated peak area with that of the internal standard, by the use of

relative response factor. For quantification using the internal standard (n-butyl benzoate), the quantifiable limits of detection were of 0.05µg/g 0.01µg/g and 0.02µg/g for DMP, DEP, DBP and DEPH respectively. Owing to the very low response of DEPH in GC/MS analysis we excluded this compound from the study. Identification of compounds in the extract was based on comparison of the relative retentions of the phthalate ester standards with those in the sample. Quantitation was done by internal standardization, using n-butyl benzoate.

Triplicate analyses were performed for each ratio of solvent mixture using the GC conditions as described below.

Determination of Blank Levels

0.5 g fish tissue was extracted and eluent was passed through a pre-conditioned column (same as above) at a flow of 1 mL min−1 without the standard phthalate esters. 15 mL of CH_3OH in CH_2Cl_2 (50:50, v/v) were used to elute the column. The eluent was dried under nitrogen-vacuum and 1 μL of internal standard in methanol was added to dissolve the residue. 0.1 μL of resulting solution from a 1 μL syringe was run on the GC using similar GC conditions as described below.

Conclusions

The concentrations of phthalates were generally very low, if not below the limits of detection, in all the fish considered in this study, but the concentration of each phthalates in fish tissue was found to be at very low level and in many samples values were below the limit of detection during this study.

The highest concentration of Dimethyl Phthalate (DMP) and Diethyl Phthalate (DEP) in all fish samples was ranged as < LOD -1.81 µg g^{-1} and shellfishery having level of DEHP, DNoP and DNoP-d4 in a range of < LOD -2.50 µg g^{-1}. Thus overall trace level of Σ phthalates was found <LOD (Mean minimum value was detected 0.001µg/g) in both (fishery and shellfishery) that is except in few samples at very low level indicating that the living habits of fish and physico-chemical effects of phthalates may influence the bioavailability of phthalates in fish therefore it is concluded that DEHP in fish was affected by fish habitat and physiochemical properties of phthalates .The daily intake of DEHP, BPA and NP via consumption of seafood was low, contributing <0.05% to respective tolerable intakes. The daily intake calculated from the DEHP, DEP and BPA concentrations in fishery is below the maximum safe doses for chronically food exposure.

Scientific studies on phthalates as EDCs have triggered public concern about their possible occurrence in seafood. This can be controlled by taking all possible remedial measures and to stop dumping of plastic and polymer waste which contains high level of phthalates. As issue of phthalates pollution in marine environment is still under discussion and currently there is no international legislation specific to phthalates in fishery. Further research in this field is necessary.

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