Scientific Research Publishing

American Journal of Analytical Chemistry, 2019, 10, 445-458
https://www.scirp.org/journal/ajac
ISSN Online: 2156-8278
ISSN Print: 2156-8251

Validated Method for the Detection of Three Phthalates Derived from Marine Invertebrates

D. Avisar[1*], A. Kaplan[1], G. Ronen-Eliraz[1], G. Vered[2], N. Shenkar[2], I. Gozlan[1]

[1]The Water Research Center, School of Environment and Earth Sciences, Faculty of Exact Sciences, Tel Aviv University, Tel Aviv, Israel
[2]School of Zoology, Faculty of life Sciences, Tel Aviv University, Tel Aviv, Israel
Email: *droravi@tauex.tau.ac.il

How to cite this paper: Avisar, D., Kaplan, A., Ronen-Eliraz, G., Vered, G., Shenkar, N. and Gozlan, I. (2019) Validated Method for the Detection of Three Phthalates Derived from Marine Invertebrates. *American Journal of Analytical Chemistry*, **10**, 445-458.
https://doi.org/10.4236/ajac.2019.1010032

Received: August 7, 2019
Accepted: October 9, 2019
Published: October 12, 2019

Abstract

This study represents, detailly, the validated method for the extraction and quantification of widespread phthalic acid esters (PAEs) bis(2-ethylhexyl) phthalate (DEHP), di-n-butyl phthalate (DBP) and di-n-octyl phthalate (DnOP) from solitary ascidians collected from a marine environment. The extraction was based on a pressurized liquid extraction method, using n-hexane as the solvent to extract the target PAEs from dry biological tissues, and was performed in an accelerated solvent extraction instrument. The average recovery of 89.2% was obtained from samples subjected to a pressure of ~1500 psi and 120˚C in two 10-min cycles. GC-MS was used for quantification, conducted in single-ion monitoring mode. Following careful and rigorous cleanup procedures to prevent cross-contamination from laboratory glassware, PAE standards showed signals with good specificity. The obtained limits of detection were 130, 122 and 89 ng/g for DEHP, DBP and DnOP, respectively. Accordingly, the calculated limits of quantification were 394, 370 and 270 ng/g for DEHP, DBP and DnOP, respectively. The obtained linearity ranged from 5.4 to 269 ng/ml (equivalent to 135 - 6725 ng/g dry weight), with $R^2 \geq 0.998$. Concentrations in the range of 200 to 9000 and 400 to 5000 ng/g sample dry weight, for DEHP and DBP, respectively, were obtained from the ascidians. No DnOP was detected in any of the samples. These results indicate that the method presented in this study is applicable for detection of low and trace concentrations of the target PAEs in samples collected from a marine organism, which can serve as a bioindicator of plastic contamination.

Keywords

Phthalate, Plastic Contamination, Bioindicator, GC-MS, ASE, Marine Invertebrate

1. Introduction

Phthalic acid esters (PAEs) are the main group of plasticizers used to increase flexibility, pliability, and elasticity of plastics. Among the most common PAEs are di-n-butyl phthalate (DBP), dimethyl and diethyl phthalates, bis(2-ethylhexyl) phthalate (DEHP) and di-n-octyl phthalate (DnOP). PAEs have been widely used as industrial chemicals for more than 40 years and are also very common in cosmetics, personal care products, and many other products that are used on a daily basis [1] [2].

Today, PAEs are detected in most environments, including various water bodies, air, soil [3] [4], natural vegetation, agricultural products and food [2], as well as in human and animal tissues, plasma, urine, breast milk and more [5] [6]. As PAEs are additives and not chemically bound to other materials, they can simply leach and migrate into the environment [1]. In the marine environment, the extent of plastic pollution is estimated at millions of metric tons per year [7]. Therefore, it is crucial to understand the significant effect of pollution by plastics and their additives on the marine environment [3].

Various studies have demonstrated a toxicological effect of PAEs on human and other organisms in the natural environment [2] [8] [9]. Of particular concern is the primary effect associated with PAEs, *i.e.*, altered functioning in the endocrine systems of different biota, both female and male. PAEs act as endocrine-disrupting chemicals, even at very low concentrations [2] [10] [11]. Benzyl butyl phthalate, DBP and DEHP have been shown to have a proliferative effect on normal breast cells [10]. Radke *et al.* [11] demonstrated a negative impact of DEHP and DBP on human male reproductive function. That study indicated a dramatic decrease in semen quality associated with increasing exposure to DBP. Consequently, PAEs have been proven to have a variety of adverse effects on living organisms and the human body, emphasizing the importance of monitoring these chemicals in the environment.

The detection of PAEs in environmental samples is challenging. On the one hand, they are ubiquitous environmental contaminants, making contamination during sampling and handling common, but on the other, in many cases, they are only present in trace concentrations [12] [13]. Furthermore, because PAEs are so common in the environment, it is important to be able to detect these molecules in various types of environmental samples, such as water, soil, sludge, and biological matrices.

To cope with these challenges, this study detailly presents the development and validation of an analytical method for the DEHP, DBP and DnOP quantification, which further was demonstrated and implemented by the authors for an examination of solitary ascidians sampled at different sites along the Israeli coastline [3]. As sessile filter feeders, ascidians have the potential to act as excellent bioaccumulators and bioindicators for PAEs in the marine environment. Moreover, being an invasive species, their wide distribution worldwide may offer the unique possibility of applying the same method to study the same organism in

different locations [3].

2. Materials and Methods

2.1. Chemicals and Standards

The three targeted PAEs, DEHP, DBP and DnOP were purchased from Sigma-Aldrich and their chemical structures are shown in **Figure 1**. HPLC-grade acetone, pesti-grade n-hexan (C_6H_{14}) and dichloromethane were purchased from Bio-Lab (Israel). The internal standard (IS) bis-(3,3,5-trimethylcyclohexyl) phthalate (TMCHP) was from Sigma-Aldrich. Phthalic acid and bis-2-ethylhexyl ester-3,4,5,6-d_4 isotope-labeled DEHP (DEHP-d_4) were purchased from Analytical Standard Solutions. Diatomaceous earth was from Thermo Fisher Scientific.

PAE stock solutions were prepared with n-hexane to a final concentration of ~22.4 μg/mL. TMCHP was selected as the IS since it is not a common PAE; it is rarely used in the industry and is unlikely to be found in environmental samples or to interfere with the studied samples. TMCHP was prepared with n-hexane to a final concentration of ~5.2 μg/mL. A surrogate solution of DEHP-d_4, used for spiking to test method accuracy, was prepared with n-hexane to a final concentration of ~6.0 μg/mL.

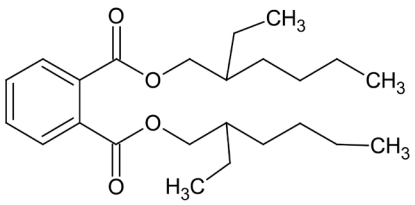
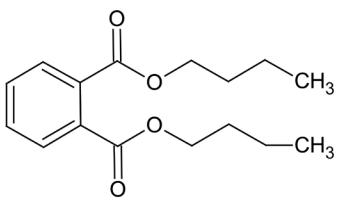
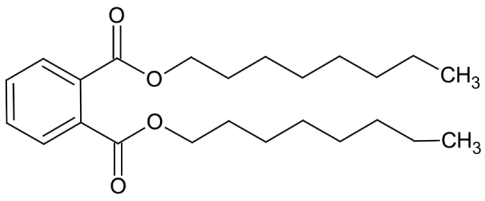
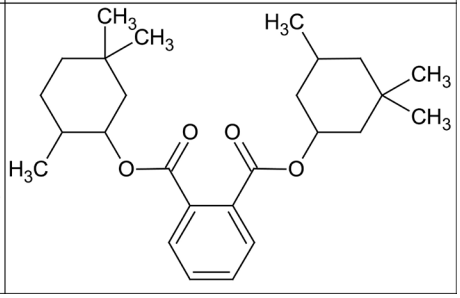
Bis(2-ethylhexyl) phthalate	Di-n-butyl phthalate
(DEHP)	(DBP)
$C_{24}H_{38}O_4$	$C_{16}H_{22}O_4$
390.6 g/mol	278.3 g/mol
CAS: 117-81-7	CAS: 84-74-2
	
Di-n-octyl phthalate	Bis-(3,3,5-trimethylcyclohexyl) phthalate
(DnOP)	(TMCHP)
$C_{24}H_{38}O_4$	$C_{26}H_{38}O_4$
390.6 g/mol	414.6 g/mol
CAS: 117-84-0	CAS: 37832-65-8
	

Figure 1. Properties of the targeted compounds and the internal standard.

2.2. Instrumentation

An accelerated solvent extraction (ASE) instrument (Thermo Fisher Scientific, model 350) was used for the extraction of PAEs from biological samples consisting of dried ascidian tissues. It was equipped with 10-mL stainless-steel ASE cells and suitable glass-fiber filters. A gas chromatograph (Agilent, model 6890) equipped with a Restek Rxi-1MS column (15 m × 0.25 mm × 0.25 mm) and mass spectrometer (Agilent, model 5973) was used for the PAE analyses.

2.3. Sample Preparation

2.3.1. Sample Collection

Two common species of solitary ascidians, *Herdmania momus* and *Microcosmus exasperatus*, were periodically collected and used as the bioindicators in this study. Mature individuals were detached from substrates at a depth of 2 to 20 m below sea level, at different sites along the Israeli Mediterranean and Red Sea coasts. The inner body was immediately separated from the outer tunic, and the tissues were wrapped in aluminum foil and frozen at −20˚C. The samples were freeze-dried and ground to a fine powder, and their dry weight recorded. As this procedure was part of the sample preparation before the extraction stage, plastic instruments were completely avoided to prevent PAE cross-contamination.

2.3.2. Extraction Method

The extraction method was based on a pressurized liquid extraction method that was developed and optimized using ASE. Sequences of extraction cycles were subjected to a combination of pressure (constant on ~1500, ASE default), temperature (up to 140), static time (residence time in the sample cell), solvent type, rinse volume (60% of cell volume) and purge time (60 seconds of nitrogen flow through the cell). In this procedure, a 10-mL stainless-steel extraction cell filled with a mixture of sample and inert filler (diatomaceous earth) was subjected to high temperature in a high-pressure solvent system for several minutes. To avoid PAE cross-contamination, a glass-fiber filter (Thermo Fisher Scientific) was placed at the bottom of the extraction cell. The studied PAEs (DBP, DEHP, DnOP) are considered to be hydrophobic compounds, which are not water-soluble, and a lipophilic solvent was required for efficient extraction. PAEs are easily soluble in n-hexane and it is GC-MS compatible, and it was therefore selected as the extraction solvent. Only high-grade n-hexane was used, to avoid PAE cross-contamination. Following the extraction procedure, extracts were poured into 25-mL volumetric flasks, mixed with 1 mL of TMCHP (IS) stock solution, and n-hexane was added to a final volume of 25 mL. The extraction method was also applied to inert filler (blank) samples, to confirm the absence of PAE cross-contamination.

2.4. Chromatographic Method

The developed chromatographic method was based on GC-MS. Quantitative analysis was conducted in single-ion monitoring mode after recording full-scan

spectra from m/z 50 to m/z 500 for qualitative analysis. The target ion was m/z 149 for the PAE standards, and 153 for DEHP-d_4. The qualifier peaks included m/z 205, 223, 167, 279, 109, 283 and 171 (Table 1). The following parameters were examined for method optimization: injection volume (1 µL, 2 µL, 3 µL), pulse time (no pulse, 15 sec, 30 sec, 45 sec, 60 sec) and MS dwell time (100 ms, 80 ms, 60 ms, 40 ms) to achieve maximum sensitivity and injection repeatability. Optimal parameters were: injection volume 2 µL, pulse splitless inlet mode set to 20 psi for 30 s, and MS dwell time set to 40 ms. Column flow was set to a constant 1.9 mL/min, the injector temperature was set to 250˚C, and the transfer line to 300˚C. The GC oven was programmed as follows: 70˚C held for 1 min, then raised to 320˚C at a rate of 15˚C/min, and held for 9 min.

2.5. PAE Calculation

A 12-point calibration curve ranging from 3.2 to 269 ng/mL was obtained. The target PAEs were quantified by normalizing their peak area (S) to the corresponding IS peak area. PAE concentration (ng/g) in the dried samples (PAE_{conc}) was calculated as:

$$\mathrm{PAE}_{\mathrm{conc}} = 25 \times \left[\left(NP - b \right) / \left(a \times dw \right) \right] \quad (1)$$

where NP is the normalized peak area (S/IS), a and b are derived from the linear equation $y = ax + b$, and dw is the sample dry weight. The dilution factor was 25.

2.6. Method Validation

The method was validated by examining the following parameters: specificity, linearity, limit of detection (LOD), limit of quantification (LOQ), accuracy, repeatability, and stability. Specificity was demonstrated by running blank samples, which indicated negligible interference below the specified LOD. Ion abundance (relative to the maximum ion intensity) was tested for all qualifiers in the analyzed samples and confirmed according to the criteria of ±20% for >50% ion abundance, ±25% for >20% - 50%, ±30% for >10% - 20% and ±25% for ≤10% [14] [15]. Linearity was tested by calculating the linear regression and the correlation coefficient (R^2) for each PAE. Each set of analyses was accompanied by a calibration curve. For the method proposed in this study, LOD was set to an analyte peak signal-to-noise ratio (S/N) of >3 and the LOQ was set to S/N > 9, where N represents the interference value. LOD and LOQ were also expressed by

Table 1. Chromatographic parameters for the selected compounds.

Compound	m/z (for quantitation)	m/z (qualifier)	RT (min)
DBP	149.1	205.1, 223.1	8.701
DEHP	149.1	167.1, 279.1	12.060
DnOP	149.1	279.1	12.942
TMCHP	149.1	109.0	12.602
DEHP-d4	153.1	171.0, 283.2	12.054

equations (2.1) and (2.2), respectively:

$$\mathrm{LOD} = 3.3 \times \mathrm{SD_b} / \mathrm{Avg_a} \times 25 \quad (2.1)$$

$$\mathrm{LOQ} = 10 \times \mathrm{SD_b} / \mathrm{Avg_a} \times 25 \quad (2.2)$$

where SD_b is the standard deviation of the y-intercepts and Avg_a is the slope average derived from the linearity curves [16]. The dilution factor was 25.

Accuracy was evaluated by the calculated recovery of the isotopic DEHP-d_4 (surrogate), which was added to each sample as a spike and underwent the same process (ASE conditions, dilution) as the sample. The recovery calculation based on value obtained from spiked sample versus matched surrogate concentration. Repeatability was evaluated by the relative standard deviation (RSD) of six replicates taken from one homogenized pooled sample. On machine stability was tested by leaving samples in GC vials in the autosampler for 22 h. Storage stability was tested by placing sample solutions in volumetric flasks at 4˚C for 72 h. The relative difference (RD) was calculated as:

$$\mathrm{RD} = \left(C_{\text{time=o}} - C_{\text{time of storage}}\right) \Big/ \left[\left(C_{\text{time=o}} + C_{\text{time of storage}}\right) \Big/ 2\right] \times 100 \quad (3)$$

3. Results and Discussion

An efficient extraction procedure for PAEs is required for their analysis in biological samples, such as marine organism tissues and organs. The use of an organic solvent is a must, and extraction has been traditionally performed using the Soxhlet method, although it is time- and solvent-consuming, labor-intensive and may create background PAE residues [17]. Other common extraction methods for PAEs are ultrasonic bath [18], vortex [19], shaker [20] and ion pairing [21].

This study adopted a different method, the ASE instrument, as the main technique to extract PAEs from biological organisms. The ASE technique has become popular for extraction from solid and biological samples due to its high extraction efficiency, full automation, high repeatability and lower solvent use [17]. Use of ASE has been successfully demonstrated for the extraction of polyaromatic hydrocarbons and polychlorinated biphenyls from mussel tissue [22], for endocrine-disrupting chemical (bisphenol A, 4-nonylphenol) extraction from liver and muscles of the Greenland shark [23], and for detection of pharmaceutical residues in fish tissue [24].

To the best of our knowledge, the use of ASE technique for the extraction of PAEs trace concentrations from marine biological matrix had never been published.

3.1. Optimization of Sample Extraction

The efficiency of the extraction procedure was assessed by the recovery values obtained with the ASE instrument. A comparison of four methods using different ASE parameters was applied by spiking a mixture of standards (DBP, DEHP, DnOP at 500 μg) into extraction cells filled only with diatomaceous earth filler

(Table 2). In parallel, non-spiked blank cells (filler only) were extracted by the same methods. A high PAE signal was found in the non-spiked samples, indicating contamination in the laboratory.

PAE ubiquity poses a severe challenge for process development, due to potential sample contamination during collection, preparation and measurement. Although precautions were taken for the strategies and work procedures, including the elimination of plastic labware throughout the procedures and the use of high-grade solvent, contamination of blanks and samples still occurred.

Extraction and analysis of 10 procedural non-spiked blank samples (conducted with diatomaceous earth filler) showed high and inconsistent results for DBP and DEHP, ranging from 41 to 170 and 38 to 582 ng/g, respectively (Figure 2(a)). Those values make up a significant amount of the environmental samples, and therefore contamination had to be reduced to improve method reliability. However, since no DnOP contamination was found in the non-spiked blank samples and method C demonstrated 97% recovery for DnOP (up to 20% more than other methods), it (method C) was selected for further method development.

A comprehensive study was performed during method development to reveal the sources of contamination. It was found that disposable and reusable glassware and the ASE cell components contribute small quantities of contaminating PAEs. Moreover, the filler (diatomaceous earth) was determined to be the dominant source of PAE contamination (Figure 2(a)).

Consequently, two cleaning procedures were added to the adopted method: more effective cleaning of the glassware used to handle the samples, extracts, storage and preparation, and an addional prewash for the ASE extraction cells. The washing procedure for the relevant glassware included rinsing with dichloromethane, followed by acetone, then heating overnight at 200˚C prior to a supplemental rinse with n-hexane before use. To reduce the major contamination derived from the filler, it was prewashed by ASE before use. Adopting and continuously performing these cleaning stages significantly decreased the contaminating PAEs, as seen from the DBP and DEHP peak signals (Figure 2(b)).

Because the PAEs contamination could not be completely eliminated, real ascidians samples were prepared with different sample weights (0.5 and 1.0 g), spiked with 5.9 ng of the DEHP-d_4 surrogate and compared to evaluate the amount of PAE contamination relative to the results with the ascidian real samples (Figure 2). The obtained recoveries of the surrogate, using method C, were 95% and 74% for samples weighted 0.5 g and 1.0 g, respectively.

Table 2. Assessment of optimal ASE parameters based on recovery of PAE standards in diatomaceous earth filler.

	Method A	Method B	Method C	Method D
Temperature (˚C)	100	120	120	140
No. of cycles	3	3	2	2
Static time (min)	4	4	6	6

The obtained peak area ratio results (Figure 2) were improved using 1.0 g compared to blank. Therefore, the method was modified by increasing the static time from 6 to 10 minutes (Method E, Table 3), which increased the surrogate recovery from 74% (method C) to 89% (method E). The improvement of recovery was demonstrated by the following procedure: after ASE prewash (method E applied on a cell filled with diatomaceous earth), about 2/3 (v/v) of the washed filler was mixed homogeneously with about 1.0 g of dry sample in a glass beaker, spiked with 1.0 mL of DEHP-d_4 surrogate (5.9 ng) and reloaded for extraction. Since method E showed the best performance, it was adopted as the ASE extraction method for this study.

The combination of all of these procedures (increasing sample weight, pre-extraction cell wash and glassware rinsing and heating) reduced the PAE blank signal to sample signal ratio to less than 7%. This indicated that the results

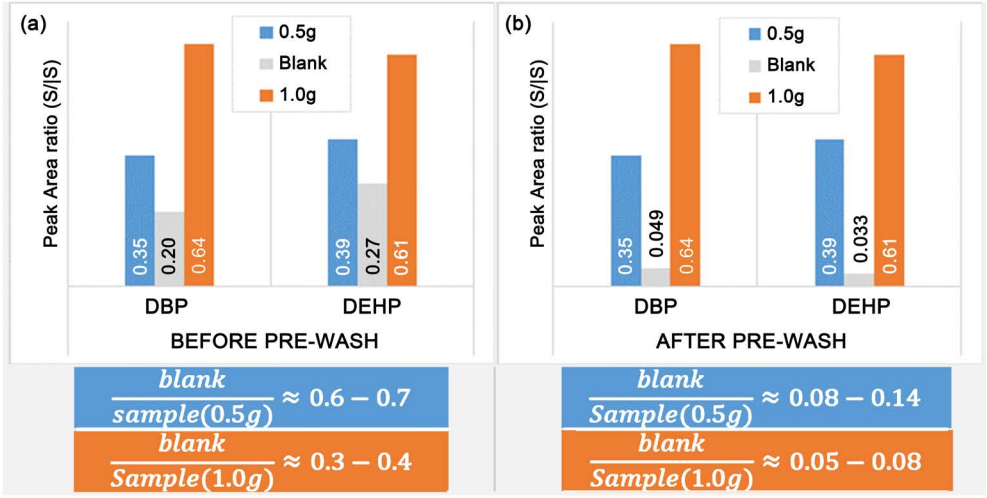


Figure 2. Reducing contributions to PAE contamination along the procedure. (a) Effect of sample amount on the ratio of PAEs extracted from the blank (diatomaceous earth extracted by hexane), to PAEs derived from the sample; (b) Impact of diatomaceous earth prewash procedure on this ratio.

Table 3. Optimized ASE method based on recovery of surrogate standard in sample.

ASE conditions	Method E
Sample dry weight (g)	1.0
Cell	10 mL with glass-fiber filter
Filler	Diatomaceous earth
Temperature (˚C)	120
Cycles	2
Static time (min)	10
Solvent	n-Hexane
Rinse volume (%)	60%
Purge time (sec)	60
Volume obtained (mL)	~20

of the suggested method would represent PAE concentrations in the samples with a deviation of ±7% potentially contributed by the laboratory procedures (Figure 2).

3.2. Method Validation

3.2.1. Specificity, Linearity, LOD and LOQ

Specificity was demonstrated by running 12 blank (filler) samples (Figure 3(a)), which indicated compatible interference values up to 0, 10 and 32 ng/g for DnOP, DBP and DEHP, respectively. In addition, all tested samples gave acceptable qualifier ion abundance ratios.

Adequate linearity was obtained in the range of 5.4 to 269 ng/mL, with a correlation coefficient $R^2 \geq 0.998$ for the selected PAEs (Figure 4). The equivalent concentrations were calculated by multiplying the concentration by the dilution factor, 25, and dividing by the sample weight (1 g). Thus, the linearity range (5.4 - 269 ng/mL) was equivalent to the actual PAE concentrations of 135 to 6725 ng/g dry sample. S/N above 3 and 9 were considered for LOD and LOQ, respectively, and were calculated according to:

$$\begin{aligned} \mathrm{LOD}_{(\mathrm{DBP})} &= 3.3\times\left(0.042/0.0284\right)\times 25 = 122; \\ \mathrm{LOQ}_{(\mathrm{DBP})} &= 10\times\left(0.042/0.0284\right)\times 25 = 370 \end{aligned} \tag{4.1}$$

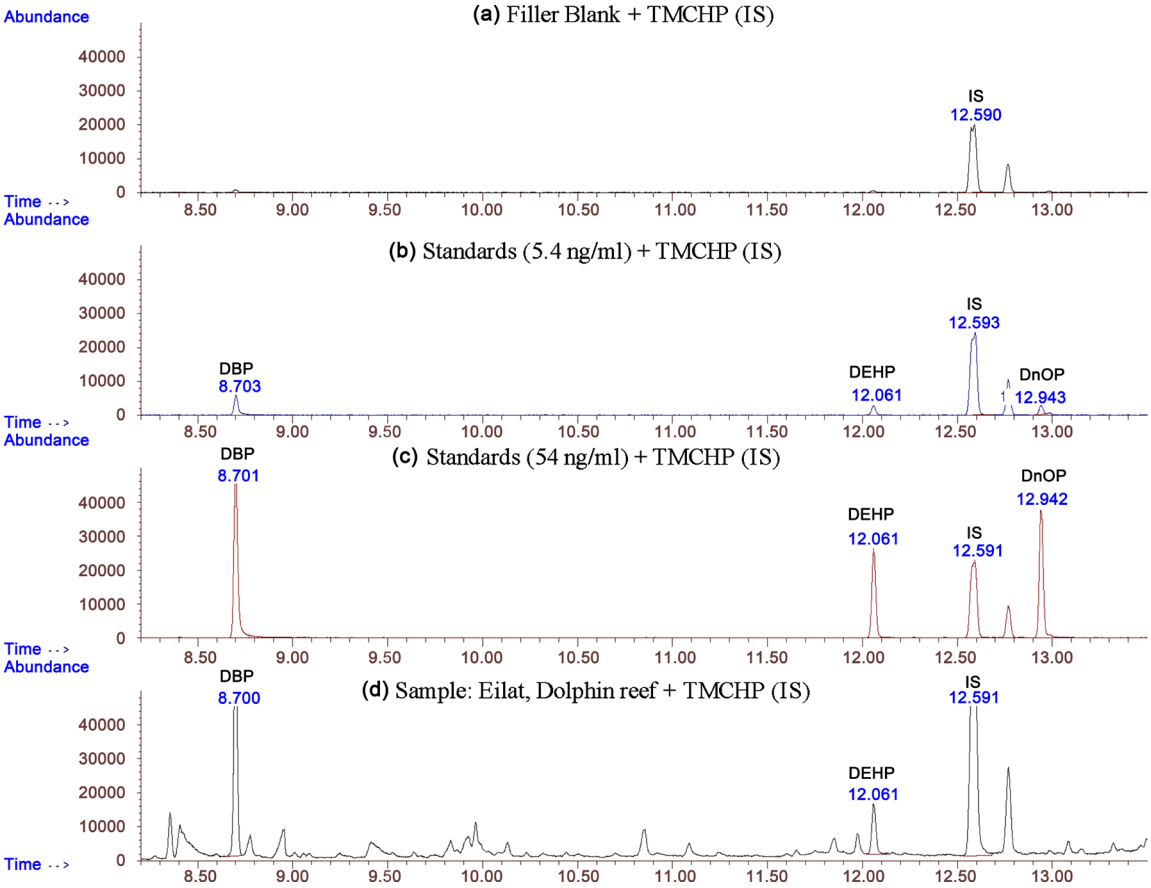


Figure 3. GC-MS chromatograms. (a) Filler blank sample + IS; (b) PAE standards (5.4 ng/mL) + IS; (c) PAE standards (54 ng/mL) + IS; (d) Chromatogram of a representative sample (Eilat, Dolphin Reef) + IS.

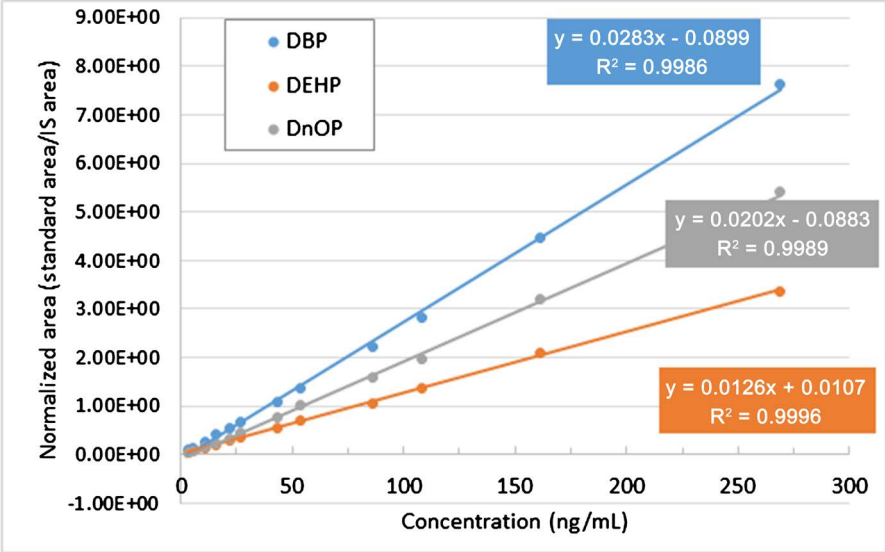


Figure 4. Representative linearity ranges for DnOP, DBP and DEHP.

$$\mathrm{LOD}_{(\mathrm{DEHP})} = 3.3\times(0.020/0.0127)\times 25 = 130; \quad \mathrm{LOQ}_{(\mathrm{DEHP})} = 10\times(0.020/0.0127)\times 25 = 394 \tag{4.2}$$

$$\mathrm{LOD}_{(\mathrm{DnOP})} = 3.3\times(0.022/0.0203)\times 25 = 89; \quad \mathrm{LOQ}_{(\mathrm{DnOP})} = 10\times(0.022/0.0203)\times 25 = 270 \tag{4.3}$$

3.2.2. Accuracy, Repeatability and Stability

Accuracy was determined by recovery of the isotope-labeled surrogate DEHP-d_4 in each sample at an amount of 5.9 μg. The average recovery obtained from 52 samples was 89.2% with RSD = 10.3%, indicating acceptable accuracy.

Method repeatability was examined by preparing one set of six individual samples from pooled homogenized powder of milled dried ascidians. The average concentration and RSD were calculated (Table 4). The obtained RSD values for DBP and DEHP were lower than 5.0%, and no DnOP was detected. These RSD values indicate good repeatability of the proposed method.

Sample stability was evaluated by the calculated RD values of the target PAE concentrations in samples after the extraction and dilution procedure. On-machine stability was tested by leaving samples (n = 6) in GC vials in the autosampler for 22 h. Average RD values of 8% ± 3% and 4% ± 3% were obtained for DBP and DEHP, respectively. A storage stability test was conducted by placing sample solutions (n = 4) in volumetric flasks at 4˚C for 72 h. Average RD values of 2% ± 2% and 8% ± 7% were obtained for DBP and DEHP, respectively. Therefore, an average RD of less than 10% was considered acceptable.

3.3. Method Application to Ascidian Samples

The method was applied for two different invasive ascidian species. Samples were collected from different sites along the Israeli Mediterranean and Red Sea coasts to identify and quantify the selected PAEs. As expected, while processing

and treating the environmental samples, several undefined signals appeared on the chromatograms (Figure 3(d)). However, the obtained signals for the target PAEs were much more prominent, due to method optimization. The average concentrations of DBP and DEHP were relatively high and varied among the different sites (Figure 5). No DnOP was identified in any of the samples [3].

Furthermore, the obtained results showed high uptake of PAEs by the ascidians. This suggests the high potential of these organisms, as natural filters, to act as bioaccumulators and to serve as bioindicators for PAE occurrence and contamination in the marine environment [3].

4. Summary and Conclusion

A method to identify and quantify three common PAEs—DEHP, DBP and DnOP extracted from a biological matrix (solitary ascidians) was developed and presented. The method includes extraction of PAEs from ascidian tissues using ASE technique and GC-MS chromatographic method. The extraction method parameters were optimized. LODs and LOQs were obtained from the S/N ratio and the statistical calculations using multiple calibration curves, while a good

Table 4. Average concentration of DBP and DEHP detected in invasive ascidians dry samples.

Replicate #	DBP (ng/g)	DEHP (ng/g)	DnOP (ng/g)	Sample dry weight (g)
1	585.6	1220.8	<40	1.0060
2	612.7	1115.2	<40	1.0375
3	649.2	1084.7	<40	1.0739
4	669.8	1185.1	<40	1.0151
5	643.1	1152.4	<40	1.0685
6	647.8	1178.5	<40	1.0455
Average	634.7	1156.1		
RSD (%)	4.8	4.3		

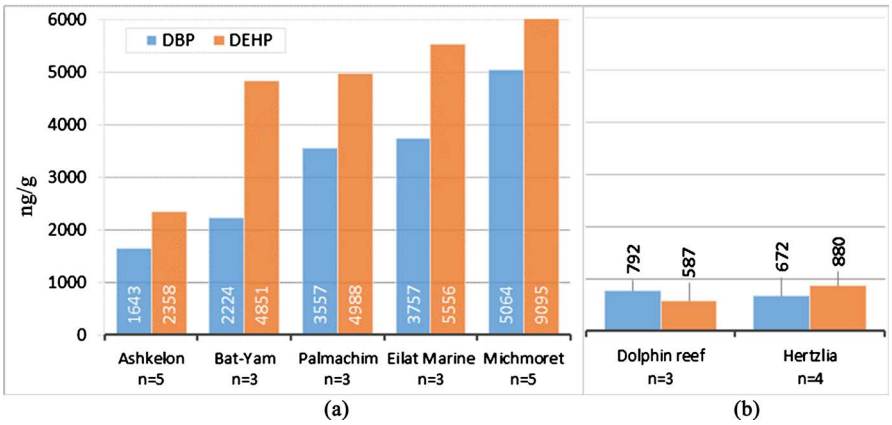


Figure 5. Average concentrations of DBP and DEHP detected in samples of dried invasive ascidians. (a) Samples containing relatively high concentrations of the target PAEs; (b) Samples containing relatively low concentrations of the target PAEs.

linear correlation was demonstrated (total range of 50). Method accuracy and repeatability were excellent indicating method quality. Special attention was paid to the cleaning process and to avoidance of cross-contamination from plastics during the procedure. These satisfactory results demonstrate the method's applicability for the detection of PAEs specifically in ascidians, and potentially also, with minor modifications, in other marine organisms. The use of more sensitive equipment, such as a triple quadrupole mass spectrometer, and additional sample preparation procedures (concentration and cleanup), is expected to increase the method's sensitivity, allowing its use for even smaller organisms with less PAE uptake. Having shown their ability to accumulate PAEs in their body, ascidians might be used as bioindicators for future monitoring programs and studies on plastic contamination in the marine environment.

Funding

This research was funded by a Moshe Mirilashvili Fellowship.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

References

[1] Calvo-Flores, F.G., Isac-Garcéa, J. and Dobado, J.A. (2017) Industrial Chemicals as Emerging Pollutant. In: *Emerging Pollutants*: *Origin*, *Structure and Properties*, Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim, Germany, 265-340. https://doi.org/10.1002/9783527691203.ch9

[2] Chen, X., Xu, S., Tan, T., Lee, S.T., Cheng, S.H., Lee, F.W.F., Xu, S.J.L. and Ho, K.C. (2014) Toxicity and Estrogenic Endocrine Disrupting Activity of Phthalates and Their Mixtures. *International Journal of Environmental Research and Public Health*, **11**, 3156-3168. https://doi.org/10.3390/ijerph110303156

[3] Vered, G., Kaplan, A., Avisar, D. and Shenkar, N. (2019) Using Solitary ascidians to Assess Microplastic and Phthalate Plasticizers Pollution among Marine Biota: A Case Study of the Eastern Mediterranean and Red Sea. *Marine Pollution Bulletin*, **138**, 618-625. https://doi.org/10.1016/j.marpolbul.2018.12.013

[4] Hermabessiere, L., Dehaut, A., Paul-Pont, I., Lacroix, C., Jezequel, R., Soudant, P. and Duflos, G. (2017) Occurrence and Effects of Plastic Additives on Marine Environments and Organisms: A Review. *Chemosphere*, **182**, 781-793. https://doi.org/10.1016/j.chemosphere.2017.05.096

[5] Zimmermann, S., Gruber, L., Schlummer, M., Smolic, S. and Fromme, H. (2012) Determination of Phthalic Acid Diesters in Human Milk at Low PPB Levels. *Food Additives & Contaminants*: *Part A*, **29**, 1780-1790. https://doi.org/10.1080/19440049.2012.704529

[6] Lin, S., Ku, H.-Y., Su, P.-H., Chen, J.-W., Huang, P.-C., Angerer, J. and Wang, S.-L. (2011) Phthalate Exposure in Pregnant Women and Their Children in Central Taiwan. *Chemosphere*, **82**, 947-955. https://doi.org/10.1016/j.chemosphere.2010.10.073

[7] Jambeck, J.R., Geyer, R., Wilcox, C., Siegler, T.R., Perryman, M., Andrady, A., Narayan, R. and Law, K.L. (2015) Plastic Waste Inputs from Land into the Ocean.

Science, **347**, 768-771. https://doi.org/10.1126/science.1260352

[8] Browne, M.A., Niven, S.J., Galloway, T.S., Rowland, S.J. and Thompson, R.C. (2013) Microplastic Moves Pollutants and Additives to Worms, Reducing Functions Linked to Health and Biodiversity. *Current Biology*, **23**, 2388-2392. https://doi.org/10.1016/j.cub.2013.10.012

[9] Avio, C.G., Gorbi, S., Milan, M., Benedetti, M., Fattorini, D., d'Errico, G., Pauletto, M., Bargelloni, L. and Regoli, F. (2015) Pollutants Bioavailability and Toxicological Risk from Microplastics to Marine Mussels. *Environmental Pollution*, **198**, 211-222. https://doi.org/10.1016/j.envpol.2014.12.021

[10] Chen, F.-P., Chien, M.-H., Chen, H.-Y. and Ng, Y.-T. (2018) Effects of Phthalates on Normal Human Breast Cells Co-Cultured with Different Fibroblasts. *PLoS ONE*, **13**, e0199596. https://doi.org/10.1371/journal.pone.0199596

[11] Radke, E.G., Braun, J.M., Meeker, J.D. and Cooper, G.S. (2018) Phthalate Exposure and Male Reproductive Outcomes: A Systematic Review of the Human Epidemiological Evidence. *Environment International*, **121**, 764-793. https://doi.org/10.1016/j.envint.2018.07.029

[12] Dirtu, A.C., Van den Eede, N., Malarvannan, G., Ionas, A.C. and Covaci, A. (2012) Analytical Methods for Selected Emerging Contaminants in Human Matrices—A Review. *Analytical and Bioanalytical Chemistry*, **404**, 2555-2581. https://doi.org/10.1007/s00216-012-6053-0

[13] Guo, Y. and Kannan, K. (2012) Challenges Encountered in the Analysis of Phthalate Esters in Foodstuffs and Other Biological Matrices. *Analytical and Bioanalytical Chemistry*, **404**, 2539-2554. https://doi.org/10.1007/s00216-012-5999-2

[14] Lehotay, S.J., Mastovska, K., Amirav, A., Fialkov, A.B., Martos, P.A., de Kok, A. and Fernández-Alba, A.R. (2008) Identification and Confirmation of Chemical Residues in Food by Chromatography-Mass Spectrometry and Other Techniques. *TrAC Trends in Analytical Chemistry*, **27**, 1070-1090. https://doi.org/10.1016/j.trac.2008.10.004

[15] André, F., De Wasch, K.K.G., De Brabander, H.F., Impens, S.R., Stolker, L.A.M., Van Ginkel, L., Stephany, R.W., Schilt, R., Courtheyn, D., Bonnaire, Y., Fürst, P., Gowik, P., Kennedy, G., Kuhn, T., Moretain, J.P. and Sauer, M. (2001) Trends in the Identification of Organic Residues and Contaminants: EC Regulations under Revision. *TrAC Trends in Analytical Chemistry*, **20**, 435-445. https://doi.org/10.1016/S0165-9936(01)00085-1

[16] Kruve, A., Rebane, R., Kipper, K., Oldekop, M.L., Evard, H., Herodes, K., Ravio, P. and Leito, I. (2015) Tutorial Review on Validation of Liquid Chromatography-Mass Spectrometry Methods: Part II. *Analytica Chimica Acta*, **870**, 8-28. https://doi.org/10.1016/j.aca.2015.02.016

[17] Net, S., Delmont, A., Sempéré, R., Paluselli, A. and Ouddane, B. (2015) Reliable Quantification of Phthalates in Environmental Matrices (Air, Water, Sludge, Sediment and Soil): A Review. *Science of the Total Environment*, **515-516**, 162-180. https://doi.org/10.1016/j.scitotenv.2015.02.013

[18] Fossi, M.C., Panti, C., Guerranti, C., Coppola, D., Giannetti, M., Marsili, L. and Minutoli, R. (2012) Are Baleen Whales Exposed to the Threat of Microplastics? A Case Study of the Mediterranean Fin Whale (*Balaenoptera physalus*). *Marine Pollution Bulletin*, **64**, 2374-2379. https://doi.org/10.1016/j.marpolbul.2012.08.013

[19] Hu, X., Gu, Y., Huang, W. and Yin, D. (2016) Phthalate Monoesters as Markers of Phthalate Contamination in Wild Marine Organisms. *Environmental Pollution*, **218**, 410-418. https://doi.org/10.1016/j.envpol.2016.07.020

[20] Gao, D., Li, Z., Wen, Z. and Ren, N. (2014) Occurrence and Fate of Phthalate Esters in Full-Scale Domestic Wastewater Treatment Plants and Their Impact on Receiving Waters along the Songhua River in China. *Chemosphere*, **95**, 24-32. https://doi.org/10.1016/j.chemosphere.2013.08.009

[21] Guerranti, C., Cau, A., Renzi, M., Badini, S., Grazioli, E., Perra, G. and Focardi, S.E. (2016) Phthalates and Perfluorinated Alkylated Substances in Atlantic Bluefin Tuna (*Thunnus thynnus*) Specimens from Mediterranean Sea (Sardinia, Italy): Levels and Risks for Human Consumption. *Journal of Environmental Science and Health*, *Part B*, **51**, 661-667. https://doi.org/10.1080/03601234.2016.1191886

[22] Murphy, B., Lingam, S., Richter, B. and Carlson, R. (2012) Simultaneous Extraction of PAHs and PCBs from Environmental Samples Using Accelerated Solvent Extraction. 1-5.

[23] Ademollo, N., Patrolecco, L., Rauseo, J., Nielsen, J. and Corsolini, S. (2018) Bioaccumulation of Nonylphenols and Bisphenol A in the Greenland Shark *Somniosus microcephalus* from the Greenland Seawaters. *Microchemical Journal*, **136**, 106-112. https://doi.org/10.1016/j.microc.2016.11.009

[24] Huerta, B., Jakimska, A., Gros, M., Rodriguez-Mozaz, S. and Barcelo, D. (2013) Analysis of Multi-Class Pharmaceuticals in Fish Tissues by Ultra-High-Performance Liquid Chromatography Tandem Mass Spectrometry. *Journal of Chromatography A*, **1288**, 63-72. https://doi.org/10.1016/j.chroma.2013.03.001