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Analytical Methods

Determination of phthalic acid esters and di(2-ethylhexyl) adipate in fish and squid using the ammonium formate version of the QuEChERS method combined with gas chromatography mass spectrometry

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ABSTRACT

In the present study, the ammonium formate version of the QuEChERS method, considered highly advantageous in relation to instrument maintenance and other issues, was applied for the first time to extract a group of twelve phthalic acid esters (PAEs, *i.e.* dipropyl phthalate, DPP; diisobutyl phthalate, DIBP; dibutyl phthalate, DBP; diisopentyl phthalate, DIPP; di-*n*-pentyl phthalate, DNPP; dihexyl phthalate, DHP; butyl benzyl phthalate, BBP; dicyclohexyl phthalate, DCHP; di(2-ethylhexyl) phthalate, DEHP; di-*n*-octyl phthalate, DNOP; diisononyl phthalate, DINP; and diisodecyl phthalate, DIDP) and one adipate (di(2-ethylhexyl) adipate, DEHA) from two species of fish (*Scomber colias* and *Katsuwonus pelamis*) and one of squid (*Loligo gahi*). The method was validated in terms of linearity, trueness and matrix effects. Determination coefficients (R^2) for matrix-matched calibration curves were higher than 0.99 in all cases, being the lowest calibration levels in the range 0.5–10 ng/g. Mean recovery values were between 70 and 117% with relative standard deviation values ≤20%. Matrix effects were soft (between −20 and +20%) for most analytes and matrices, except in squid samples, which was mostly medium with a moderate ion suppression. The analysis of 10 samples of each type showed the presence of DIBP, DBP and DEHP at concentrations up to 44.2 ± 2.1 ng/g of wet weight in some of the samples and species, still not representing concerning values when considering the daily intake of such species of seafood in the human diet (tolerable daily intake -TDI- values were not exceeded). Results demonstrated that the ammonium formate version of the QuEChERS method can be applied with success for the extraction and determination of the selected PAEs and DEHA in fish and squid samples.

1. Introduction

Phthalic acid esters (PAEs) are manufactured chemicals which were first introduced in the 1920s. They are widely used as plasticizers in the plastic industry, mainly -but not limited to- in the production of polyvinyl chloride, to increase plastic plasticity by reducing intermolecular

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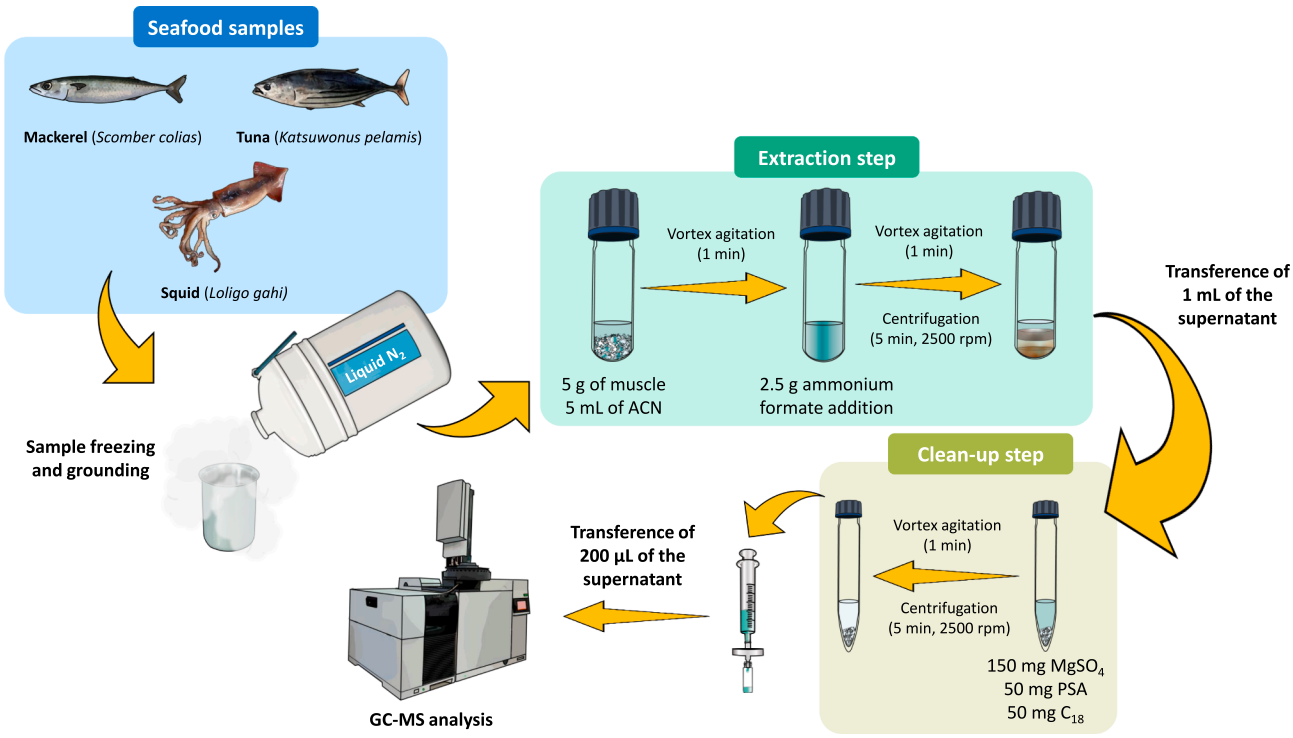

Fig. 1. General scheme of the sample pre-treatment and QuEChERS extraction method applied in this work.

forces and, therefore, to facilitate its moulding. Some of them are also used as solvents and fragrances fixers, as well as additives in medical devices, household, cosmetics and personal care products (Katsikantami et al., 2016). Since they are not chemically bonded to the polymeric matrix, they easily migrate to their surrounding environment and, as a result, they are considered ubiquitous chemicals (Fasano, Cirillo, Esposito, & Lacorte, 2015; Katsikantami et al., 2016).

PAEs are the main type of plasticizers used nowadays; in fact, they accounted for 55% world production of plasticizers in 2020 (IHS Markit, 2021). As a result of their wide application and also of their ubiquitous presence in the environment, in the last years, important concern has arisen regarding the negative effects of PAEs and their metabolites on human health, which include their capacity to mimic the actions of natural hormones in the organism, producing several endocrine system disorders (Chang, Herianto, Lee, Hung, & Chen, 2021; Huang et al., 2021; Yang et al., 2015). Nevertheless, further research is still needed to effectively evaluate their toxic potential, especially the long-term effects. As a consequence, many public organizations/administrations have initiated actions to control/limit their use. This is the case of the EU, which banned di(2-ethylhexyl) phthalate (DEHP), dibutyl phthalate (DBP) and benzyl butyl phthalate (BBP) in all toys and childcare articles, and diisononyl phthalate (DINP), diisodecyl phthalate (DIDP) and di-*n*-octyl phthalate (DNOP) in those articles that children could take to their mouth (The European Comission, 2006). More recently, the EU through its REACH regulation restricted the use of the four phthalates DEHP, DBP, diisobutyl phthalate (DIBP), and BBP coming into force in July 2020 (The European Comission, 2018), due to their demonstrated endocrine disrupting properties with effects on human health (Endocrine, 2019). According to it, the four PAEs are "restricted to a concentration equal to or below 0.1% by weight individually or in any combination in any plasticized material in articles used by consumers or in indoor areas". Earlier, in 2011 and 2012, these PAEs were identified as substances of very high concern (SVHCs) and added to the EU authorization list for being toxic and affecting reproduction mechanisms (European Chemicals Agency, 2021).

Regarding food contact materials, the use of DBP, DEHP, BBP, DINP and DIDP has been limited to certain situations also establishing specific migration limits (The European Commission, 2007). In this sense, on February 2019, EFSA panel on Food Contact Materials, Enzymes, and Processing Aids (CEP Panel) published their updated draft opinion on the risk assessment of such five PAEs, in which they established a group tolerable daily intake (TDI) of 50 µg/kg of body weight (b.w.) per day for DBP, BBP, DEHP and DINP, and for DIDP its own TDI of 150 µg/kg of b.w. per day (Silano & Baviera, 2019). The World Health Organization (WHO) has also established a TDI for DEHP of 25 µg/kg of b.w. and recommends not to exceed a concentration of 8 µg/L in drinking water (World Health Organization, 2003). In this regard, it should also be remarked that DEHP has also been included in the watch list given in Directive 2013/38/EU as a priority substance in the field of water policy (The European Commission, 2013).

In this context, it is more than evident that the determination of the presence of PAEs in food products is of high importance since the major route of exposure for the human beings is food ingestion (Yang et al., 2015). Several studies have already reported the presence of PAEs in fishery products (Castro-Jiménez & Ratola, 2020; Hidalgo-Serrano, Borrull, Marcé, & Pocurull, 2021; Xu et al., 2018). Such contamination can either be a result of the migration of PAEs from plastic packaging or of their absorption from the aquatic environment (Abdel daiem et al., 2012; Hahladakis, Velis, Weber, Iacovidou, & Purnell, 2018). In fact, the ubiquitous presence of these compounds in marine waters and their lipophilic properties, might facilitate their accumulation in marine organisms (Hahladakis et al., 2018). Under this last respect, it should be indicated that PAEs have been recently proposed as plastic tracers in the marine environment, since despite that there are many restrictions on the manufacture and application of PAEs, these chemicals are still prevalent in the aquatic environments (Baini et al., 2017; Vered, Kaplan, Avisar, & Shenkar, 2019).

The complexity of fishery products requires the application of reliable and effective sample preparation methods for their analysis, which, following the current trends in the field, also demand simple and sustainable procedures with a minimum risk for humans. In this sense, the QuEChERS method (standing for *Quick*, *Easy*, *Cheap*, *Effective*, *Rugged*

Table 1
Matrix-matched calibration data of the selected PAEs and DEHA and matrix effect (ME) percentage in mackerel, squid and tuna (DBP-d_4 was used as IS of DPP, DBP and BBP, DNPP-d_4 was used as IS of DIBP, DIPP and DNPP, DHP-d_4 was used as IS of DHP, DEHA and DCHP, while DEHP-d_4 was used as IS of DEHP, DNOP, DINP and DIDP).

Analyte	Sample	Studied linear range (μg/L)*	Regression equation (n = 8)		$s_{y/x}$	R^2	ME (%)**
			$b \pm s_b \cdot t_{(0.05;6)}$	$a \pm s_a \cdot t_{(0.05;6)}$			
DPP	Mackerel	1–150	$5.43 \cdot 10^{-3} \pm 4.66 \cdot 10^{-4}$	$4.82 \cdot 10^{-3} \pm 3.72 \cdot 10^{-2}$	$2.73 \cdot 10^{-2}$	0.9945	−35
	Squid	1–150	$2.10 \cdot 10^{-3} \pm 5.08 \cdot 10^{-5}$	$3.59 \cdot 10^{-4} \pm 4.05 \cdot 10^{-3}$	$2.97 \cdot 10^{-3}$	0.9996	−75
	Tuna	0.5–150	$5.02 \cdot 10^{-3} \pm 1.90 \cdot 10^{-4}$	$3.01 \cdot 10^{-3} \pm 1.42 \cdot 10^{-2}$	$1.24 \cdot 10^{-2}$	0.9986	−40
DIBP	Mackerel	5–150	$5.94 \cdot 10^{-3} \pm 2.06 \cdot 10^{-4}$	$-9.73 \cdot 10^{-3} \pm 1.78 \cdot 10^{-2}$	$1.03 \cdot 10^{-2}$	0.9994	−27
	Squid	1–150	$2.49 \cdot 10^{-3} \pm 1.93 \cdot 10^{-5}$	$1.39 \cdot 10^{-2} \pm 1.54 \cdot 10^{-3}$	$1.13 \cdot 10^{-3}$	1.0000	−69
	Tuna	1–150	$5.71 \cdot 10^{-3} \pm 2.17 \cdot 10^{-4}$	$2.76 \cdot 10^{-2} \pm 1.73 \cdot 10^{-2}$	$1.27 \cdot 10^{-2}$	0.9989	−29
DBP	Mackerel	1–150	$8.94 \cdot 10^{-3} \pm 9.65 \cdot 10^{-4}$	$3.35 \cdot 10^{-2} \pm 7.69 \cdot 10^{-2}$	$5.65 \cdot 10^{-2}$	0.9913	18
	Squid	5–150	$3.59 \cdot 10^{-3} \pm 5.47 \cdot 10^{-5}$	$-1.33 \cdot 10^{-2} \pm 4.71 \cdot 10^{-3}$	$2.72 \cdot 10^{-3}$	0.9999	−53
	Tuna	10–150	$8.15 \cdot 10^{-3} \pm 1.18 \cdot 10^{-3}$	$9.85 \cdot 10^{-2} \pm 1.11 \cdot 10^{-1}$	$4.52 \cdot 10^{-2}$	0.9938	7
DIPP	Mackerel	0.5–150	$5.27 \cdot 10^{-3} \pm 6.34 \cdot 10^{-5}$	$-8.13 \cdot 10^{-5} \pm 4.73 \cdot 10^{-3}$	$4.12 \cdot 10^{-3}$	0.9999	10
	Squid	1–150	$2.37 \cdot 10^{-3} \pm 2.89 \cdot 10^{-5}$	$2.12 \cdot 10^{-5} \pm 2.30 \cdot 10^{-3}$	$1.69 \cdot 10^{-3}$	0.9999	−51
	Tuna	0.5–150	$5.09 \cdot 10^{-3} \pm 7.79 \cdot 10^{-5}$	$4.98 \cdot 10^{-3} \pm 5.81 \cdot 10^{-3}$	$5.07 \cdot 10^{-3}$	0.9998	6
DNPP	Mackerel	0.5–150	$8.44 \cdot 10^{-3} \pm 8.10 \cdot 10^{-5}$	$-6.26 \cdot 10^{-4} \pm 6.04 \cdot 10^{-3}$	$5.27 \cdot 10^{-3}$	0.9999	11
	Squid	1–150	$3.75 \cdot 10^{-3} \pm 3.96 \cdot 10^{-5}$	$1.17 \cdot 10^{-3} \pm 3.16 \cdot 10^{-3}$	$2.32 \cdot 10^{-3}$	0.9999	−51
	Tuna	0.5–150	$8.01 \cdot 10^{-3} \pm 1.21 \cdot 10^{-4}$	$1.10 \cdot 10^{-3} \pm 9.05 \cdot 10^{-3}$	$7.89 \cdot 10^{-3}$	0.9998	5
DHP	Mackerel	5–150	$8.46 \cdot 10^{-3} \pm 1.47 \cdot 10^{-4}$	$1.95 \cdot 10^{-3} \pm 1.27 \cdot 10^{-2}$	$7.34 \cdot 10^{-3}$	0.9998	7
	Squid	5–150	$4.13 \cdot 10^{-3} \pm 6.00 \cdot 10^{-5}$	$3.45 \cdot 10^{-4} \pm 5.16 \cdot 10^{-3}$	$2.99 \cdot 10^{-3}$	0.9999	−48
	Tuna	5–150	$8.62 \cdot 10^{-3} \pm 2.04 \cdot 10^{-4}$	$4.91 \cdot 10^{-3} \pm 1.76 \cdot 10^{-2}$	$1.02 \cdot 10^{-2}$	0.9997	9
BBP	Mackerel	5–150	$3.17 \cdot 10^{-3} \pm 1.74 \cdot 10^{-4}$	$1.12 \cdot 10^{-3} \pm 1.50 \cdot 10^{-2}$	$8.67 \cdot 10^{-3}$	0.9984	68
	Squid	5–150	$1.72 \cdot 10^{-3} \pm 4.37 \cdot 10^{-5}$	$-2.01 \cdot 10^{-4} \pm 3.77 \cdot 10^{-3}$	$2.18 \cdot 10^{-3}$	0.9997	−9
	Tuna	5–150	$3.72 \cdot 10^{-3} \pm 1.64 \cdot 10^{-4}$	$-3.05 \cdot 10^{-3} \pm 1.41 \cdot 10^{-2}$	$8.16 \cdot 10^{-3}$	0.9990	97
DEHA	Mackerel	5–150	$2.97 \cdot 10^{-3} \pm 5.50 \cdot 10^{-5}$	$4.44 \cdot 10^{-3} \pm 4.74 \cdot 10^{-3}$	$2.74 \cdot 10^{-3}$	0.9998	15
	Squid	5–150	$1.42 \cdot 10^{-3} \pm 7.36 \cdot 10^{-5}$	$3.46 \cdot 10^{-3} \pm 6.34 \cdot 10^{-3}$	$3.67 \cdot 10^{-3}$	0.9986	−45
	Tuna	5–150	$2.88 \cdot 10^{-3} \pm 6.14 \cdot 10^{-5}$	$3.07 \cdot 10^{-3} \pm 5.29 \cdot 10^{-3}$	$3.06 \cdot 10^{-3}$	0.9998	11
DCHP	Mackerel	5–150	$6.02 \cdot 10^{-3} \pm 1.32 \cdot 10^{-4}$	$1.02 \cdot 10^{-3} \pm 1.14 \cdot 10^{-2}$	$6.60 \cdot 10^{-3}$	0.9997	17
	Squid	5–150	$3.08 \cdot 10^{-3} \pm 4.27 \cdot 10^{-5}$	$3.01 \cdot 10^{-5} \pm 3.67 \cdot 10^{-3}$	$2.13 \cdot 10^{-3}$	0.9999	−40
	Tuna	5–150	$6.26 \cdot 10^{-3} \pm 1.04 \cdot 10^{-4}$	$9.30 \cdot 10^{-4} \pm 8.93 \cdot 10^{-3}$	$5.17 \cdot 10^{-3}$	0.9999	22
DEHP	Mackerel	5–150	$7.57 \cdot 10^{-3} \pm 1.59 \cdot 10^{-4}$	$-1.46 \cdot 10^{-2} \pm 1.37 \cdot 10^{-2}$	$7.92 \cdot 10^{-3}$	0.9998	5
	Squid	5–150	$3.98 \cdot 10^{-3} \pm 4.33 \cdot 10^{-5}$	$-2.95 \cdot 10^{-2} \pm 3.73 \cdot 10^{-3}$	$2.16 \cdot 10^{-3}$	0.9999	−45
	Tuna	5–150	$7.69 \cdot 10^{-3} \pm 2.20 \cdot 10^{-4}$	$2.69 \cdot 10^{-2} \pm 1.90 \cdot 10^{-2}$	$1.10 \cdot 10^{-2}$	0.9996	6
DNOP	Mackerel	5–150	$1.23 \cdot 10^{-2} \pm 2.79 \cdot 10^{-4}$	$-1.82 \cdot 10^{-3} \pm 2.40 \cdot 10^{-2}$	$1.39 \cdot 10^{-2}$	0.9997	10
	Squid	5–150	$5.57 \cdot 10^{-3} \pm 4.05 \cdot 10^{-4}$	$-1.45 \cdot 10^{-2} \pm 3.49 \cdot 10^{-2}$	$2.02 \cdot 10^{-2}$	0.9973	−50
	Tuna	5–150	$1.29 \cdot 10^{-2} \pm 1.88 \cdot 10^{-4}$	$-5.10 \cdot 10^{-3} \pm 1.62 \cdot 10^{-2}$	$9.38 \cdot 10^{-3}$	0.9999	15
DINP	Mackerel	5–150	$8.60 \cdot 10^{-3} \pm 2.86 \cdot 10^{-4}$	$-8.79 \cdot 10^{-3} \pm 2.47 \cdot 10^{-2}$	$1.43 \cdot 10^{-2}$	0.9994	3
	Squid	5–150	$3.70 \cdot 10^{-3} \pm 2.88 \cdot 10^{-4}$	$-9.69 \cdot 10^{-3} \pm 2.48 \cdot 10^{-2}$	$1.43 \cdot 10^{-2}$	0.9969	−56
	Tuna	5–150	$9.51 \cdot 10^{-3} \pm 6.88 \cdot 10^{-5}$	$2.99 \cdot 10^{-2} \pm 5.92 \cdot 10^{-3}$	$3.43 \cdot 10^{-3}$	1.0000	14
DIDP	Mackerel	5–150	$7.28 \cdot 10^{-3} \pm 2.57 \cdot 10^{-4}$	$-8.11 \cdot 10^{-3} \pm 2.21 \cdot 10^{-2}$	$1.28 \cdot 10^{-2}$	0.9994	−16
	Squid	5–150	$3.18 \cdot 10^{-3} \pm 4.15 \cdot 10^{-4}$	$-1.03 \cdot 10^{-2} \pm 3.58 \cdot 10^{-2}$	$2.07 \cdot 10^{-2}$	0.9912	−63
	Tuna	–	–	–	–	–	–

b: slope; s_b: standard deviation of the slope; a: intercept; s_a: standard deviation of the intercept; R^2: determination coefficient; $s_{y/x}$: standard deviation of the estimate. *Also equivalent to ng/g in real samples. **Calculated following the equation used by Kwon et al. (Kwon et al., 2012).

and *Safe*) is nowadays considered as a *mega* method, since it has demonstrated to be effective in the extraction of a wide variety of analytes and matrices after some adaptations (González-Curbelo et al., 2015; Socas-Rodríguez, González-Sálamo, Herrera-Herrera, Hernández-Borges, & Rodríguez-Delgado, 2017; Varela-Martínez, González-Sálamo, González-Curbelo, & Hernández-Borges, 2020). It is also considered a green method, as a result of the low amounts of solvents, reagents and energy required and their low toxicity (Varela-Martínez et al., 2020). Regarding the specific extraction of PAEs from fish and squid sample, the QuEChERS method has been applied in a very reduced number of occasions as shown in Table S1 of the Supplementary Material. Some of those works have also found some of the selected PAEs in the target samples by applying different versions of the QuEChERS method, though the most common has been the original/classical version in which acetonitrile (ACN) is used in the extraction step together with NaCl to promote partitioning as well as to produce a salting out effect, and $MgSO_4$ to also promote partitioning and to heat the mixture around 40 °C as a result of the exothermic hydration process (Varela-Martínez et al., 2020). Despite the advantages of the use of both salts, trace amounts of them make necessary to intensify the periodic maintenance of the chromatographic systems like liners replacements in gas chromatography (GC) or the cleaning of the ion source in liquid chromatography-mass spectrometry (LC-MS), as well as in this last case contribute to the formation of sodium adducts. In 2014, González-Curbelo *et al.* proposed a modification of the method using ammonium formate instead of NaCl and $MgSO_4$ since it is also able to induce phases separation and it minimizes the disadvantages of the use of magnesium and sodium salts in MS analysis and also enhances the ionization of the analytes (González-Curbelo, Lehotay, Hernández-Borges, & Rodríguez-Delgado, 2014). Furthermore, the use of ammonium formate has also shown to have a similar performance as previous versions and to reduce the amount of co-extracted materials, leading to cleaner extracts and to a lower matrix effect (ME) (González-Curbelo et al., 2014; Han, Matarrita, Sapozhnikova, & Lehotay, 2016; Varela-Martínez, González-Curbelo, González-Sálamo, & Hernández-Borges, 2020). However, and despite its clear advantages, the ammonium formate version has not been fully

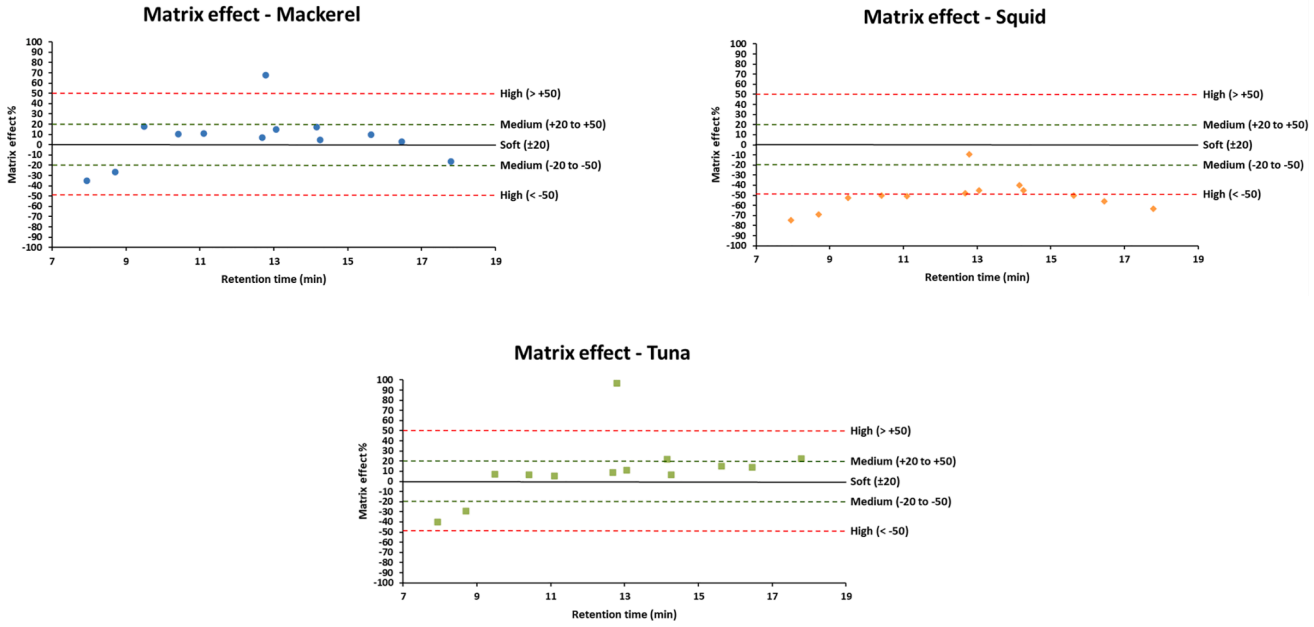

Fig. 2. Distribution of the ME (%) *vs* the retention time (min) of each PAE and DEHA for mackerel, squid and tuna matrix after the application of the QuEChERS-GC–MS method.

explored as other versions of the QuEChERS method have been, probably as a result of the commercialization of a good number of QuEChERS kits under "classical" formulations to facilitate its application. Therefore, it is still necessary to study in depth this highly advantageous version and to extent its application to a wide variety of matrices and analytes.

In this context, the aim of this study was to apply for the first time the ammonium formate version of the QuEChERS method to the extraction of a group of 12 PAEs and one adipate (di(2-ethylhexyl) adipate, DEHA) from fish and squid samples (*Scomber colias*, *Katsuwonus pelamis* and *Loligo gahi*) in order to evaluate its performance as well as to study the ME. Ten samples of each type bought in local markets were analysed to check the possible presence of these compounds in the three selected species. This work represents the first application of the ammonium formate version of the QuEChERS method to these types of samples and the first report of the presence of PAEs and DEHA in seafood species consumed in the Canary Islands.

2. Materials and methods

2.1. Chemicals

The analytical standards that were used were dipropyl phthalate (DPP, CAS 131-16-8), DIBP (CAS 84-69-5), DBP (CAS 84-74-2), diisopentyl phthalate (DIPP, CAS 605-50-5), di-*n*-pentyl phthalate (DNPP, CAS 131-18-0), dihexyl phthalate (DHP, CAS 84-75-3), BBP (CAS 85-68-7), DEHA (CAS 103-23-1), dicyclohexyl phthalate (DCHP, CAS 84-61-7), DEHP (CAS 117-81-7), DNOP (CAS 117-84-0), DINP (CAS 20548-62-3) and DIDP (CAS 89-16-7). In addition, DBP-3,4,5,6-d_4 (DBP-d_4, CAS 93952-11-5), DNPP-3,4,5,6-d_4 (DNPP-d_4, CAS 358730–89-9), DHP-3,4,5,6-d_4 (DHP-d_4, CAS 1015854-55-3) and DEHP-3,4,5,6-d_4 (DEHP-d_4, CAS 93951–87-2) were used as internal standards (ISs). All of them had a purity greater than 97.0% and were acquired from Sigma-Aldrich (Madrid, Spain) and Dr. Ehrenstorfer (Augsburg, Germany). Table S2 of the Supplementary Material shows the chemical structures and properties of the studied PAEs and DEHA.

Individual stock solutions of each compound of interest and each IS were prepared at concentrations between 900 and 1100 mg/L in cyclohexane and stored in the darkness at −18 °C. Mix working solutions of all analytes and ISs were prepared at different concentrations in cyclohexane and stored at −18 °C in the darkness. All chemicals were used without further purification.

Tap water was purified with an Elix Essential water purification system, and then it was deionized using a Milli-Q gradient system A10 from Millipore (Burlington, MA). ACN of LC-MS grade and ammonium formate (purity 98.0%) were from VWR International Eurolab (Barcelona, Spain). Primary secondary amine (PSA) and C_{18} were from Agilent Technologies (Santa Clara, CA, USA), and $MgSO_4$ monohydrate (purity 97%) was from Sigma-Aldrich (Madrid, Spain).

2.2. Apparatus and software

An 8860 GC system provided with an autosampler was used for analytes separation, which was coupled to a 5977B single quadrupole (Q) mass spectrometer for analytes detection, both from Agilent Technologies. The carrier gas was helium at a flow rate of 1.2 mL/min. Separation was performed in a HP-5 ms Ultra Inert column ((5%-phenyl)-methylpolysiloxane, 30 m × 250 μm × 0.25 μm) from Agilent Technologies. The temperature gradient program was as follows: temperature was increased from 60 to 170 °C at 40 °C/min, and finally increased to 310 °C at 10 °C/min and held for 3 min reaching a total run time of 20.75 min. Injection was carried out in the splitless mode (the split was opened after 0.75 min with a purge flow of 40 mL/min) at 280 °C and the injection volume was 2 μL. Other parameters that were established were the temperature of the ion source at 230 °C, the temperature of the transfer line at 280 °C and an ionization energy of −70 eV. In addition, single ion monitoring (SIM) mode was picked out. Enhanced MassHunter software from Agilent Technologies was used to control the GC–MS system.

A vortex was used to shake and mix the samples with the solvent or salts, and a Mega Star 3.0R centrifuge was used to separate the different layers from the homogeneous solution, both of them from VWR International. A 224i-1S analytical balance was also employed from Sartorius (Goettingen, Germany).

2.3. Samples

Patagonian squid (*Loligo gahi*, hereafter squid), Atlantic chub

Table 2
Relative recovery and RSD values of the target analytes in mackerel, squid, and tuna (n = 5 at each spiking level).

Analytes	Sample	Level 1	Level 2	Level 3	Mean
		Recovery % (RSD %)	Recovery % (RSD %)	Recovery % (RSD %)	Recovery % (RSD %)
DPP	Mackerel	106 (5)	110 (6)	103 (4)	107 (5)
	Squid	**131** (6)	105 (6)	**122** (2)	117 (11)
	Tuna	**136** (4)	106 (2)	110 (4)	114 (11)
DIBP	Mackerel	**69** (12)	106 (7)	107 (3)	94 (20)
	Squid	72 (14)	100 (6)	93 (1)	89 (15)
	Tuna	86 (5)	99 (3)	107 (5)	97 (10)
DBP	Mackerel	89 (6)	96 (2)	97 (4)	94 (6)
	Squid	79 (6)	101 (1)	85 (11)	90 (13)
	Tuna	**59** (13)	96 (3)	98 (0)	87 (20)
DIPP	Mackerel	99 (2)	98 (4)	97 (4)	98 (3)
	Squid	103 (5)	104 (2)	99 (1)	102 (3)
	Tuna	83 (3)	104 (1)	111 (1)	100 (13)
DNPP	Mackerel	96 (1)	96 (3)	95 (4)	96 (3)
	Squid	94 (3)	102 (1)	95 (1)	97 (4)
	Tuna	77 (2)	104 (1)	109 (1)	97 (15)
DHP	Mackerel	95 (1)	95 (4)	94 (4)	95 (3)
	Squid	93 (2)	103 (2)	100 (5)	99 (5)
	Tuna	81 (3)	103 (1)	108 (1)	97 (13)
BBP	Mackerel	97 (1)	91 (5)	96 (4)	95 (4)
	Squid	**68** (1)	96 (5)	86 (13)	86 (16)
	Tuna	**64** (1)	102 (3)	107 (3)	95 (19)
DEHA	Mackerel	90 (4)	79 (5)	81 (7)	83 (8)
	Squid	84 (10)	92 (2)	88 (1)	88 (6)
	Tuna	85 (6)	94 (2)	96 (2)	92 (6)
DCHP	Mackerel	97 (2)	95 (5)	97 (4)	96 (4)
	Squid	93 (3)	104 (1)	100 (5)	99 (6)
	Tuna	78 (3)	104 (2)	109 (2)	97 (15)
DEHP	Mackerel	114 (7)	101 (5)	97 (4)	104 (9)
	Squid	72 (6)	102 (2)	100 (6)	93 (16)
	Tuna	77 (10)	103 (2)	114 (8)	98 (18)
DNOP	Mackerel	92 (2)	90 (4)	92 (5)	91 (4)
	Squid	89 (3)	108 (2)	96 (1)	98 (9)
	Tuna	82 (4)	105 (3)	109 (2)	99 (13)
DINP	Mackerel	92 (6)	80 (5)	83 (6)	85 (8)
	Squid	77 (4)	99 (2)	87 (2)	88 (11)
	Tuna	78 (4)	93 (3)	98 (2)	90 (10)
DIDP	Mackerel	112 (5)	79 (6)	80 (6)	89 (18)
	Squid	**55** (11)	83 (4)	71 (2)	70 (18)
	Tuna	–	–	–	–

Level 1: 5 ng/g of w.w. except for DBP in tuna which was 10 ng/g of w.w.; level 2: 75 ng/g of w.w.; level 3: 150 ng/g w.w. for all the analytes. Data outside the 70–120% range for recovery values and 0–20% for RSD values are in bold.

mackerel (*Scomber colias*, hereafter mackerel) and skipjack tuna (*Katsuwonus pelamis*, hereafter tuna) were purchased from local markets in Tenerife (Canary Islands, Spain). When purchased, they were all covered in aluminium foil and immediately taken to the laboratory where they were cleaned and dissected. Dorsal muscles of fishes and mantles and tentacles of squids were extracted, triturated and frozen with liquid nitrogen. Afterwards, the frozen samples were grounded in a metal laboratory homogenizer to obtain a homogeneous powder. Once the sample reached room temperature, it was spiked with the analytes and/or ISs and allowed to stand for at least 20 min before the application of the ammonium formate version of the QuEChERS method.

For recovery studies, samples were spiked to yield 5, 10, 75 and 150 ng/g of wet weight (w.w.) depending on the analyte and matrix and 125 ng/g of w.w. for the ISs. Water content was determined by weighing 5 g of each sample in triplicate in porcelain capsules and letting it dry in an oven at 120 °C for 1.5 h, after which they were cooled in a desiccator and weighted until constant weight.

2.4. QuEChERS method

Five grams of homogenized tissue sample and 5 mL of ACN were introduced into a round bottom glass tube of 25 mL with a screw cap and was vigorously vortexed for 1 min. Then, 2.5 g of ammonium formate were added, and the sample was vortexed again for 1 min more and centrifugated for 5 min at 2500 rpm. Afterwards, 1 mL of the supernatant was transferred to a 15 mL round bottom glass tube containing 150 mg of $MgSO_4$ monohydrate, 50 mg of PSA and 50 mg of C_{18}. Then, the tube was vortexed for 1 min and centrifuged for 5 min at 2500 rpm. The resulting supernatant was filtered using a 0.2 μm polyvinylidene fluoride (PVDF) filter from Whatman™ (GE Healthcare, United States). Finally, 200 μL were transferred to a vial for injection in the GC–MS system.

2.5. Minimization and control of contamination

Volumetric glassware was cleaned with a sulphuric acid (95%, w/w, VWR International) solution of Nochromix® from Godax Laboratories (Maryland, USA) for 24 h. Non-volumetric glassware was cleaned by heating up to 550 °C for 4–5 h (Muffle Carbolite CWF 11/13). High purity solvents were used in all cases as well as PAEs free pipette tips and gloves. Procedural blanks (analysis without sample) were carried out with every batch of samples.

3. Results and discussion

3.1. GC–MS determination and application of the ammonium formate version of the QuEChERS method

In this work, GC–MS equipped with a single quadrupole analyser was used for the separation and detection of the 12 target PAEs and DEHA. The selected PAEs include those phthalates currently regulated by the EU in its different legislative actions, in particular, DBP, BBP, DEHP and DINP for which the EU and the WHO (only in the case of DEHP) have established TDIs. Table S2 of the Supplementary Material compiles the physicochemical properties of the selected analytes. In general, the length of the alkyl chains determines their different properties, like their hydrophobicity which increases with the increase of the chains (Yang et al., 2015). This also influences their chromatographic behaviour since long chain PAEs like DNOP, DINP and DIDP are eluted last in either GC or LC. Among the selected analytes, DEHA has also been included, since it is one of the most applied and studied alternative plasticisers to PAEs (Bui et al., 2016). As an example, it is among the plasticisers with the highest annual production in the EU, between 10,000 and 100,000 tonnes/year (Bui et al., 2016).

Though PAEs have also been determined by LC, they are more frequently determined by GC since they have enough volatility and thermal stability (González-Sálamo, Socas-Rodríguez, & Hernández-Borges, 2018; Martín-Pozo, Gómez-Regaladodel, Moscoso-Ruiz, & Zafra-Gómez, 2021). In our case, the thermal gradient described in the Experimental Section was applied, obtaining a complete separation of the target analytes in less than 18 min. Concerning the ISs, isotopically labelled ISs were used. In particular, DBP-d_4 was used as IS of DPP, DBP and BBP, DNPP-d_4 of DIBP, DIPP and DNPP, DHP-d_4 of DHP, DEHA and DCHP, while DEHP-d_4 was used as IS of DEHP, DNOP, DINP and DIDP, the longer chain PAEs. The MS system was operated in the SIM mode. Table S3 of the Supplementary Material shows the quantifier and the two qualifier ions selected as well as the retention time of each analyte. Relative ion intensities with a ± 20% maximum permitted tolerance as well as the retention time were also considered as identification points (The European Commission, 2002). It should be remarked that the MS or tandem mass spectrometry (MS/MS) fragmentation pathways of most PAEs with alkyl side chains are similar, giving the *m/z* 149 as the most intensive parent ion, which corresponds to the protonated phthalic anhydride as the result of the fragmentation of the aliphatic side chains

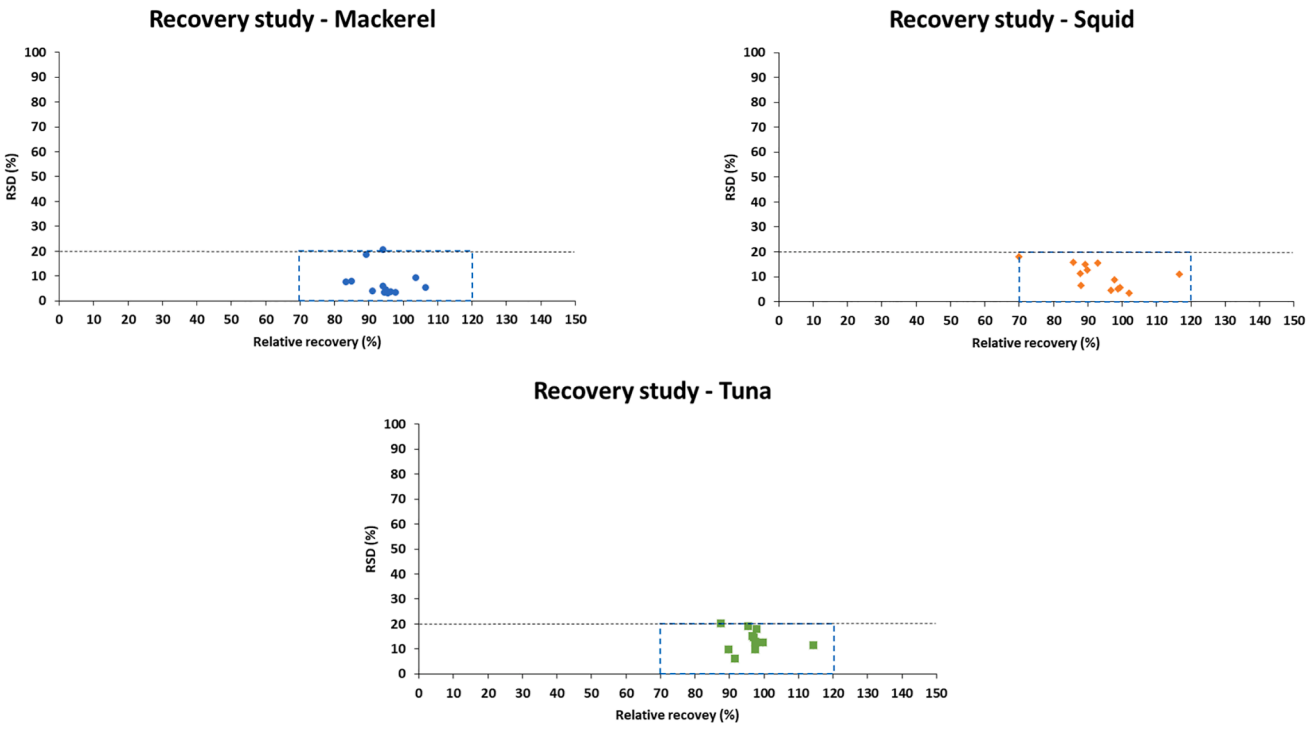

Fig. 3. Overall RSD values (%) *vs* relative recovery (%) of each PAE and DEHA in each matrix after the application of the QuEChERS-GC–MS method. Compounds with RSD less than 20% and relative recovery values in the 70–120% range are in the indicated box.

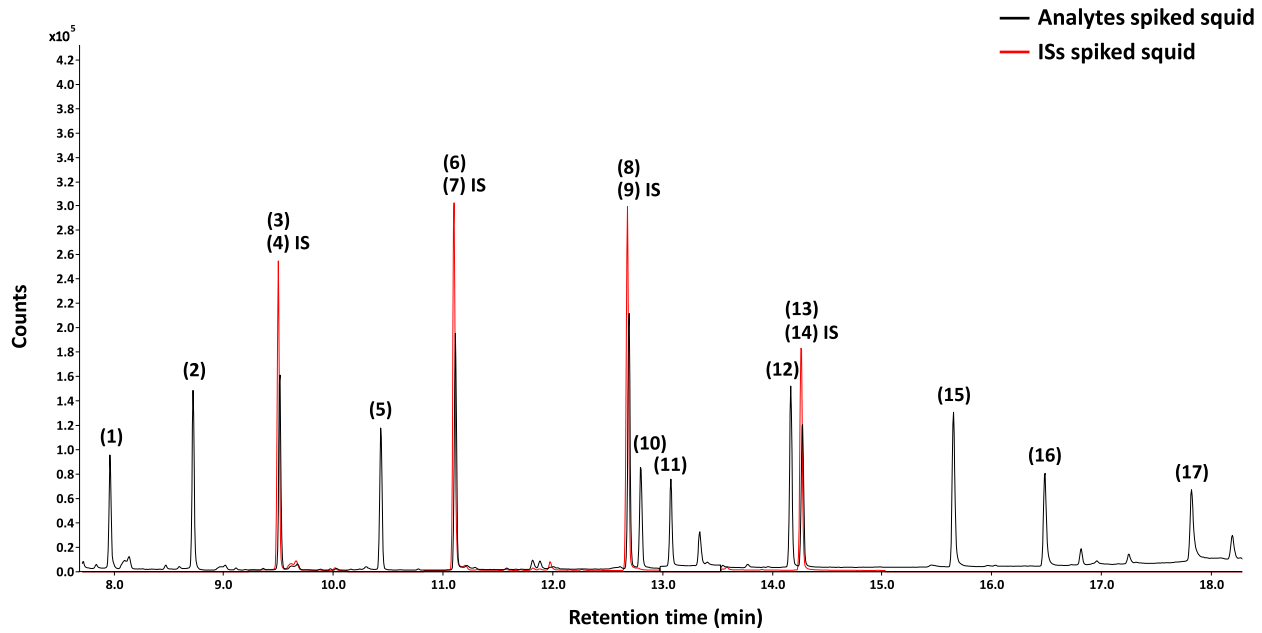

Fig. 4. GC–MS chromatogram of a spiked squid sample at 75 ng/g level after the application of the ammonium formate version of the QuEChERS method. Peak identification: DPP (1), DIBP (2), DBP (3), DBP-d_4 (4, IS), DIPP (5), DNPP (6), DNPP-d_4 (7, IS), DHP (8), DHP-d_4 (9, IS), BBP (10), DEHA (11), DCHP (12), DEHP (13), DEHP-d_4 (14, IS), DNOP (15), DINP (16), DIDP (17).

(Yin et al., 2014). As a consequence, the selection of *m/z* 149 for the quantification can make the determination of PAEs a very difficult task due to its low selectivity, being necessary a good resolution between peaks. To overcome this lack of selectivity, two qualifiers were monitored.

Apart from the previous consideration, it should also be taken into account that PAEs are ubiquitous in analytical laboratories, being necessary to minimize and control PAEs contamination. For this purpose, glassware should be used as much as possible, as well as PAEs free plastics (if employed) which should be carefully checked before being used. High purity solvents and reagents should also be selected since they contain less amounts of plasticizers and, what is more important, procedural blanks should be analysed on a daily basis. All these precautions have been taken into consideration, as indicated in the Experimental Section, in particular, the analysis of procedural blanks with each batch of samples.

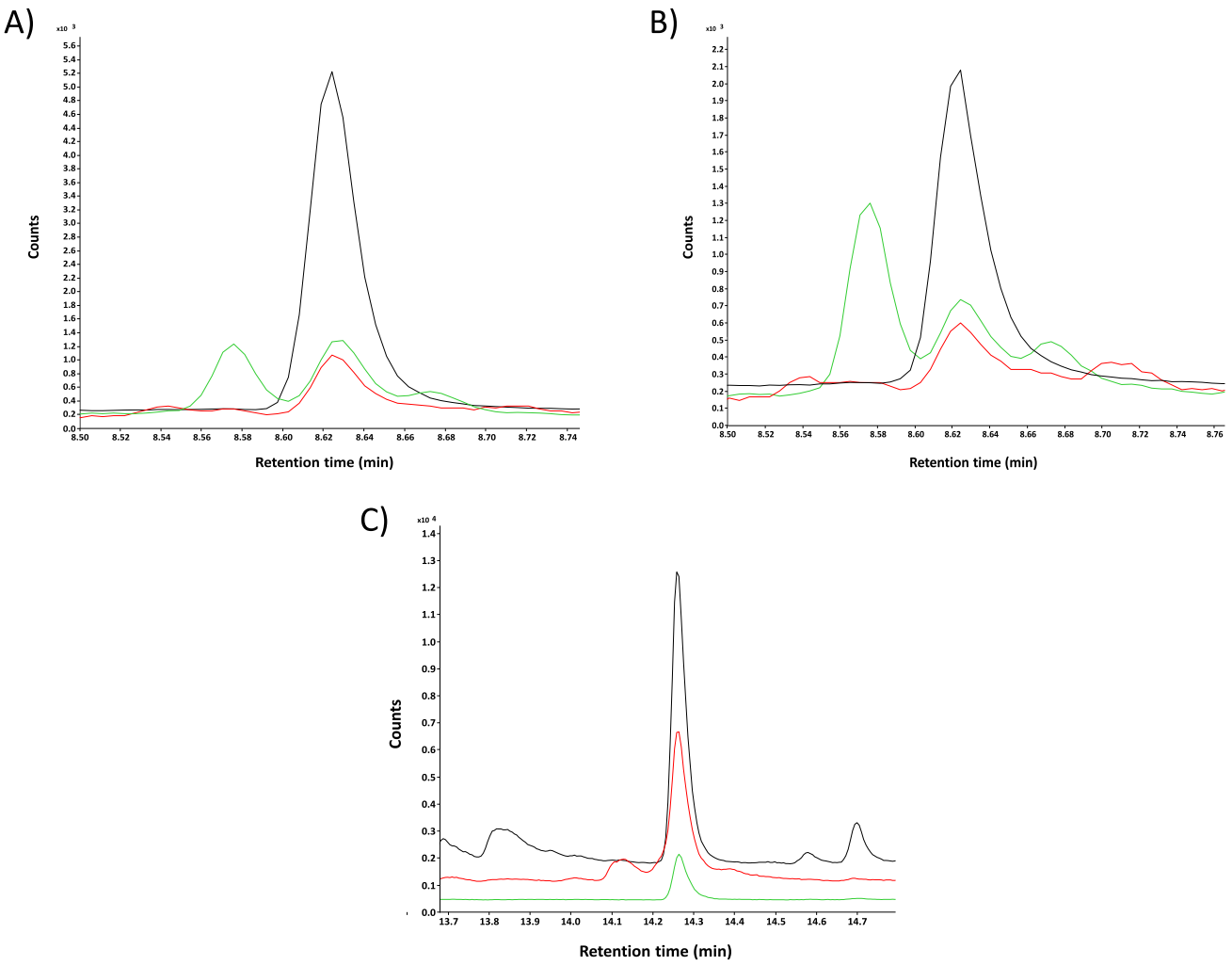

Fig. 5. GC–MS chromatogram of A) DIBP in a squid sample, B) DBP in a squid sample, and C) DEHP in a mackerel sample after the application of the ammonium formate version of the QuEChERS method. All three PAEs have *m/z* 149 as the quantification ion (black line). For DIBP and DBP the qualifier ions are 205 (red line) and 223 (green line), while for DEHP they are 167 (red line) and 279 (green line). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

As previously pointed out, the ammonium formate version of the QuEChERS method was studied by applying the experimental conditions indicated in Section 2.4, which is also summarized in Fig. 1, in which ammonium formate was added instead of NaCl and $MgSO_4$. In this case, 5 g of homogenized fish (*Scomber colias* and *Katsuwonus pelamis*) or squid (*Loligo gahi*) were accurately weighted and 5 mL of ACN were added, followed by 1 min of vortex agitation. Then, 2.5 g of ammonium formate were added and 1 min more of vortex was applied. Phase separation was quickly achieved with relatively clean extracts after centrifugation, and 1 mL of the ACN layer was transferred to a glass tube containing 150 mg $MgSO_4$, 50 mg PSA (for polar matrix interferences removal, *i.e.* organic acids) and 50 mg C_{18} (for fat removal). Once vortex agitated and centrifuged, the supernatant was filtered and directly injected in the GC system avoiding any evaporation or further step that may yield to analyte loses. To facilitate sample comminution and to enhance analyte extraction, samples were frozen with liquid nitrogen and homogenized in a metal laboratory homogenizer. A fine powder was obtained in each case and, once at room temperature, 5 g of each sample were weighted and the QuEChERS method was applied. At the same time, 5 g of each sample were weighted in triplicate in porcelain capsules and their water content was determined in order to provide the final content of each PAE in dry weight (d.w.) (see Experimental Section for details).

3.2. Matrix-matched calibration and matrix effect evaluation

In order to evaluate the existence of ME, which should be assessed at an initial method validation stage, matrix-matched calibration curves were obtained, by spiking the final extracts with the ISs (at 125 ng/g) and the target analytes (at eight concentration levels). Each matrix-matched standard was injected in triplicate and the GC liner was changed between the calibration of different matrices in order to correctly evaluate the ME. Non-spiked samples were also analysed and the ISs were added before the extraction in order to check/correct the possible presence of PAEs in the samples; in case a PAE or DEHA was found, the signal was subtracted for the calculations. Method performance acceptability criteria proposed by the SANTE Guidelines (SANTE/12682/2019, 2020) were also adopted in this study.

Table 1 shows the full calibration curves, including the studied linear range, the confidence intervals of the slope and intercept, as well as the determination coefficients (R^2) for all the target analytes, considering the IS previously indicated for each analyte. As can be seen in the table, R^2 values were higher than 0.99 in all cases. Regarding the lowest calibration levels (LCLs), they ranged between 0.5 and 10 μg/L (equivalent to 0.5–10 ng/g, respectively) being the signal-to-noise (S/N) ratio in all cases equal or higher than 10. Regarding DIDP in tuna samples, an important interference precluded the correct quantification of the

Table 3
Results of the analysis of mackerel, squid, and tuna samples after the application of the QuEChERS-GC–MS method.

	Sample	Sampling date	Analytes (ng/g) wet weight		
			DIBP	DBP	DEHP
Mackerel	1	June 21st, 2021	n.d.	n.d.	n.d.
	2	June 21st, 2021	n.d.	n.d.	n.d.
	3	June 22nd, 2021	n.d.	n.d.	n.d.
	4	June 22nd, 2021	10.2 ± 3.4	n.d.	n.d.
	5	June 23rd, 2021	7.24 ± 3.39	n.d.	44.2 ± 2.1
	6	June 23rd, 2021	5.82 ± 3.40	n.d.	43.2 ± 2.1
	7	June 24th, 2021	n.d.	n.d.	n.d.
	8	June 24th, 2021	n.d.	n.d.	n.d.
	9	June 24th, 2021	n.d.	n.d.	n.d.
	10	June 24th, 2021	n.d.	n.d.	n.d.
Squid	1	July 10th, 2021	2.95 ± 0.80	n.d.	<LCL
	2	July 10th, 2021	6.70 ± 0.80	<LCL	<LCL
	3	July 10th, 2021	2.92 ± 0.80	<LCL	<LCL
	4	July 10th, 2021	1.17 ± 0.80	n.d.	<LCL
	5	July 10th, 2021	n.d.	<LCL	<LCL
	6	July 10th, 2021	n.d.	n.d.	5.32 ± 1.06
	7	July 10th, 2021	n.d.	<LCL	n.d.
	8	July 10th, 2021	n.d.	10.9 ± 1.5	<LCL
	9	July 10th, 2021	n.d.	<LCL	<LCL
	10	July 10th, 2021	n.d.	<LCL	<LCL
Tuna	1	July 21st, 2021	<LCL	n.d.	24.5 ± 2.8
	2	July 23rd, 2021	<LCL	<LCL	n.d.
	3	July 23rd, 2021	<LCL	n.d.	n.d.
	4	July 23rd, 2021	<LCL	n.d.	n.d.
	5	July 23rd, 2021	<LCL	n.d.	n.d.
	6	July 23rd, 2021	n.d.	n.d.	n.d.
	7	July 23rd, 2021	n.d.	n.d.	<LCL
	8	July 23rd, 2021	<LCL	n.d.	n.d.
	9	July 23rd, 2021	n.d.	n.d.	<LCL
	10	July 23rd, 2021	n.d.	n.d.	n.d.

analyte and, therefore, matrix-matched calibration curves could not be obtained. LCLs were taken as the limits of quantification of the method, which, once proper calculation was made taken into account the solid nature of the samples, were less than 5 ng/g for all the analytes in the matrices analysed, except for DBP in tuna which was 10 ng/g.

Table S4 of the Supplementary Material also shows the analogous data obtained for solvent calibration which was obtained in order to calculate the ME using the following equation: ME (%) = (slope of matrix-matched calibration curve − slope of pure solvent-based calibration curve)/(slope of pure solvent-based calibration curve) × 100 (Kwon, Lehotay, & Geis-Asteggiante, 2012). ME values are also shown in Table 1, though they have also been represented for each matrix in Fig. 2 *vs* the retention time of each PAE. Negative ME values mean that a signal suppression is taking place, while positive values correspond to a signal enhancement. When the percentage ranges between −20 and 20%, a soft ME takes place and matrix-matched calibration is not required. However, ME in the ranges between −20 and −50% or between 20 and 50% correspond to a medium ME while values higher than 50 or lower than −50% correspond to a strong/significant ME. In both cases, matrix-matched calibration is necessary. From the figure, it is clear that for both mackerel and tuna samples a soft ME takes place for most of the selected PAEs, though for few of them ME is medium and mainly caused by signal suppression. On the contrary, for squid samples, an important signal suppression (strong/significant in most cases) can be observed which clearly indicates the need to develop matrix-matched calibration, though, in general, ME percentages are not extremely high.

3.3. Trueness

In order to evaluate the trueness of the method, a recovery study was carried out at three concentration levels by developing five consecutive extractions at each level. Samples were spiked with the analytes and ISs and let to stand for at least 20 min at room temperature before the application of the QuEChERS method. Concentration of level 1 was 5 ng/g (except for DBP in tuna which was 10 ng/g), 75 ng/g for level 2 and 150 ng/g for level 3 in the three types of samples. The three levels covered low, medium, and high concentrations of the linearity range of the target compounds. Table 2 shows the relative recovery values obtained at each level in which it can clearly be seen that acceptable recovery values, between 70 and 120% with relative standard deviation (RSD) values below 20% were obtained for most of the target PAEs and levels, similar values were also obtained for absolute recovery values, which clearly shows the high extraction efficiency and precision of the method. Though, as can be seen in Table 2, few of those values are outside this range (which have been marked in the table in bold), RSD values are also consistent, since they are lower than 20%. Moreover, if mean recovery values of the three levels are considered for each sample, it can be seen that they range between 70 and 117% with RSD values ≤ 20%, which are also acceptable criteria according to SANTE guidelines (SANTE/12682/2019, 2020). To better appreciate this issue, mean RSD values have been plotted versus mean recovery values as shown in Fig. 3, and the range 0–20% for RSD values and 70–120% for recovery values have been marked.

Fig. 4 shows a chromatogram of the separation of a squid sample spiked at the medium concentration level, while Fig. 5 shows a GC–MS chromatogram of (Fig. 5A) DIBP in a squid sample, (Fig. 5B) DBP in a squid sample, and (Fig. 5C) DEHP in a mackerel sample. As can be seen, in all cases, the analytes could be perfectly identified and quantified. Similar chromatograms were obtained for the rest of the samples except, as previously mentioned for DIDP in tuna.

3.4. Real sample analysis

Once the method was validated, it was applied to the analysis of ten samples of each type. For this purpose, the complete muscle of each individual was cut, frozen with liquid nitrogen and homogenized until a fine powder was obtained. Afterwards, a portion of 5 g of each sample was analysed in duplicate. At the same time, the water content of each sample was determined, which ranged between 65.7 and 75.2% for mackerel, between 83.9 and 87.8% for squid and between 70.5 and 71.9% for tuna samples. Table 3 shows the results of the analysis of the 30 samples expressed as ng/g of w.w.

As can be seen in the table, only DIBP and DEHP were found above the LCL in some mackerel samples, as well as DIBP, DBP and DEHP in some squid samples and DEHP in tuna samples. Since some of the samples were collected in the same date as they were analysed, the concentration for some of them are quite similar, being DEHP the one with the highest variability. Among the PAEs found, TDIs have only been established for DEHP while no maximum residue limits have been stablished in Europe for these compounds and matrices. If an average consumption of 125–150 g of fish fillets or 200–250 g of whole fish with 2–4 servings/week is considered (advisable dietary intake in Spain (Guidelines, 2021)), the TDI of DBP and DEHP of 50 μg/kg of b.w. is not exceeded in any case, neither individually nor considering the group TDI established for DBP, BBP, DEHP and DINP. As an example, the ingestion of 150 g of the mackerel with the highest concentration of DEHP (44.2 ± 2.1 ng/g of w.w.) means a single ingestion of less than 7 μg per person.

Besides, in some of the squid samples, also the PAEs DBP and DEHP were found below the LCL while for some tuna samples DIBP, DBP, and DEHP were found below such levels too. Among them, TDI values have only been established for DBP, but since the concentration are below the

LCLs, its TDI is not exceeded either.

Regarding previous works in which similar samples have been analysed, similar concentration ranges of PAEs were also found. Castro-Jiménez and Ratola (Castro-Jiménez & Ratola, 2020) found total PAEs concentrations of 19–83 ng/g for Atlantic bonito *Sarda sarda* and European hake *Merluccius merluccius*. Xu *et al.* (Xu et al., 2018) found in different fish samples (species were not indicated) similar PAEs to the ones of our work, in particular, DIBP, DBP and DEHP in the range 38.47–763.22 ng/g of w.w. Very recently, Hidalgo-Serrano *et al.* (Hidalgo-Serrano et al., 2021) analysed different samples of European squid (*Loligo vulgaris*) and fish (Atlantic salmon *Salmo salar*, Atlantic mackerel *Scomber scombrus* and sole *Solea solea*) finding concentrations up to 978 ng/g of d.w. In this case, DEHP, DBP and BBP together with other PAEs not in common with this work were also found though in most cases below the limits of quantification of the method.

4. Conclusions

The application of the ammonium formate version of the QuEChERS method to extract 12 PAEs and the adipate DEHA from two species of fish (*Scomber colias* and *Katsuwonus pelamis*) and one of squid (*Loligo gahi*) resulted satisfactory in terms of linearity (matrix-matched calibration) and recovery values, except in the case of DIDP which could not be perfectly quantified in tuna samples as a result of an important chromatographic interference. ME was negligible for most analytes in the case of fish samples, though for squid a moderate/high signal suppression was found. The analysis of 10 samples of each species revealed the presence of DIBP, DBP and DEHP above the LCLs in some of the samples, as well as other PAEs below such level. Even though, the TDIs for those PAEs for which such limit has been established were not exceeded in any case. The application of this version of the method, as previously reported, is highly advantageous from an instrumental point of view, being still so simple and easy to apply as expected for the QuEChERS method.

CRediT authorship contribution statement

Annalisa Sambolino: Validation, Formal analysis, Investigation, Data curation, Writing – original draft, Writing – review & editing, Visualization. **Cecilia Ortega-Zamora:** Validation, Formal analysis, Investigation, Data curation, Writing – original draft, Writing – review & editing, Visualization. **Javier González-Sálamo:** Conceptualization, Methodology, Validation, Formal analysis, Data curation, Writing – original draft, Writing – review & editing, Visualization, Supervision. **Ana Dinis:** Conceptualization, Resources, Writing – original draft, Writing – review & editing, Supervision, Project administration, Funding acquisition. **Nereida Cordeiro:** Conceptualization, Resources, Writing – original draft, Writing – review & editing, Supervision, Project administration, Funding acquisition. **João Canning-Clode:** Conceptualization, Resources, Writing – original draft, Writing – review & editing, Supervision, Project administration, Funding acquisition. **Javier Hernández-Borges:** Conceptualization, Methodology, Validation, Formal analysis, Resources, Data curation, Writing – original draft, Writing – review & editing, Visualization, Supervision, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2022.132174.

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