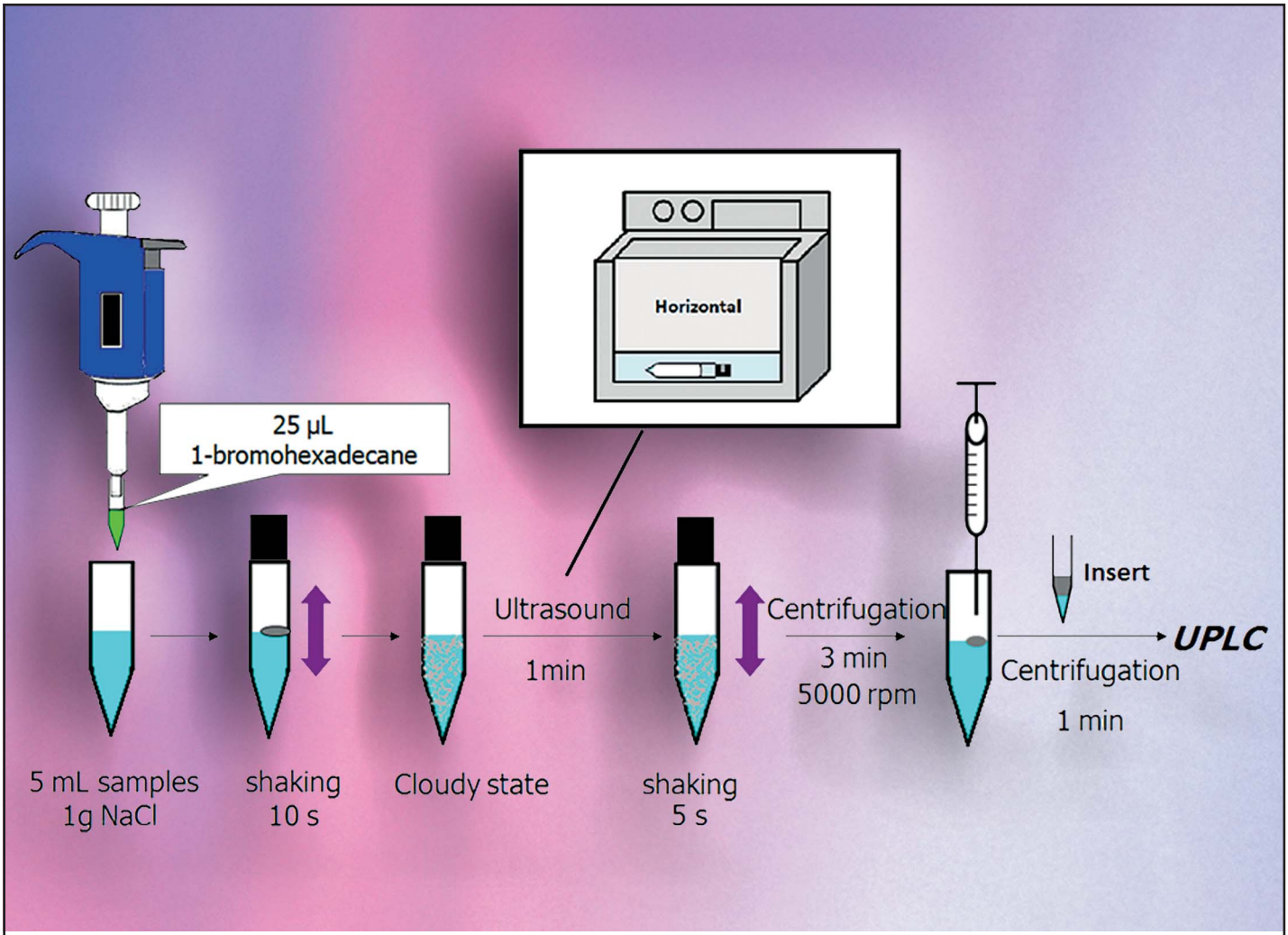

Showcasing research on manual shaking-enhanced, ultrasound-assisted emulsification microextraction from the research groups of Professor Ming-Ren Fuh (Department of Chemistry, Soochow University) and Professor Shang-Da Huang (Department of Chemistry, National Tsing Hua University) in Taiwan.

Title: Determination of endocrine-disrupting phenols in water samples by a new manual shaking-enhanced, ultrasound-assisted emulsification microextraction method

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PAPER

Determination of endocrine-disrupting phenols in water samples by a new manual shaking-enhanced, ultrasound-assisted emulsification microextraction method

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Manual shaking-enhanced, ultrasound-assisted emulsification microextraction (MS-USAEME) combined with ultraperformance liquid chromatography (UPLC) with UV detection has been developed for the determination of five endocrine-disrupting phenols (EDPs) in seawater samples and detergent samples: 4-*tert*-butylphenol (4-t-BP), 4-cumylphenol (4-CP), 4-*tert*-octylphenol (4-t-OP), 2,4-di-*tert*-butylphenol (2,4-di-t-BP) and 4-nonylphenol (4-NP). Optimum conditions were found to be: 25 μL 1-bromohexadecane as extraction solvent, 5 mL of aqueous sample and 1 g of NaCl to control the ionic strength; manual shaking for 10 s; ultrasonication for 1 min; centrifugation for 3 min at 5000 rpm (speed). For MS-USAEME, manual shaking for 10 s is essential for effective extraction when the ultrasonic extraction time is as brief as 1 min. The small volume of aqueous sample enhances the effect of manual shaking significantly. For seawater samples, the limit of detection (LOD) was 0.5–2.8 ng mL^{-1}, the limit of quantification (LOQ) was 1.8–9.3 ng mL^{-1} with the relative standard deviation (RSD) in the range 4.2–10.3%. For detergent samples, the LOD was 0.4–2.4 ng mL^{-1}, LOQ was 1.6–8.2 ng mL^{-1} and RSD 4.7–10.0%. The relative recovery was 96–109% for seawater samples and 81–106% for the detergent samples.

1. Introduction

Endocrine-disrupting chemicals (EDCs) are environmental hormones and can interfere with the function of the endocrine system, which is an increasingly important issue. EDCs disturb the normal endocrine system, affecting the reproductive and hormonal control of development of animals. Alkylphenols (APs), EDCs with estrogenic effects, are common in aqueous environments. APs are generated mainly from the biodegradation of alkylphenol ethoxylates (APEOs), which are non-ionic surfactants widely used as emulsifiers in paints and pesticides as well as in industrial and household detergents.[1] Endocrine-disrupting phenols (EDPs) are a group of EDCs that include 4-*tert*-butylphenol (4-t-BP), 4-cumylphenol (4-CP), 4-*tert*-octylphenol (4-t-OP), 2,4-di-*tert*-butylphenol (2,4-di-t-BP) and 4-nonylphenol (4-NP), and have been linked to physiological and reproductive effects in fish and other wildlife[2–5] and to effects on human fertility *via* the food chain (bioaccumulation).[6] These findings have raised public concern over the effects of EDPs on the environment in general and human health in particular.[7]

Analytical microextraction is an important development in the methodology of sample preparation. The commonest extraction techniques used in environmental analysis were solid-phase extraction (SPE)[8] and liquid–liquid extraction (LLE); however, both are time-consuming, labor-intensive and tedious. Moreover, LLE uses large amounts of toxic and environmentallyunfriendly organic solvent.

A few years later, solid-phase microextraction (SPME)[9] and liquid-phase microextraction (LPME) methods appeared in the literature. SPME is a solvent-free technique but the fiber used is fragile and expensive. Several techniques have been developed for LPME, including single-drop microextraction (SDME),[10,11] static and dynamic LPME,[12] hollow fiber LPME,[13] liquid–liquid–liquid microextraction (LLLME)[14] and solvent-bar microextraction (SBME),[15] a miniaturization of the traditional liquid–liquid extraction (LLE) procedure in which the solvent to aqueous phase ratio is greatly reduced. All of these extraction methods are known to be toxic and damaging to the environment and are hazardous to human health.

In general, lengthy extraction times are required to obtain good extraction efficiency (the extracted amount of the extraction analytes) and dispersive liquid–liquid microextraction (DLLME) has been developed to overcome this disadvantage.[16,17] However, the frequent use of a disperser solvent (500–1000 μL) is incompatible with the concept of green chemistry.

Recently, the use of ultrasound for dispersion of an extraction solvent within an aqueous sample has been explored. Ultrasonication accelerates the homogenization/emulsification step

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and the formation of submicrometer droplets greatly increases the area of the contact surface between the two liquids, facilitating rapid and efficient transfer of analyte from the aqueous sample to the organic solvent.[18–21] Some studies have used vortex mixing[22–24] to disperse an extraction solvent within an aqueous sample in order to reduce the amount of organic solvent used. The other studies used DLLME to form small droplets of the organic solvent within the sample solution by manually shaking the tube containing the two liquids.[25,26] Lin and Fuh used ultrasonication with occasional manual shaking to give a cloudy aqueous sample.[27] Fontana *et al.* developed a coacervative microextraction ultrasound-assisted back-extraction technique (CME-UABE) in which hydrochloric acid and Triton X-114 were added and mixed manually.[28] The main objective of this work was to develop a simple and rapid equilibrium liquid-phase microextraction method using a combination of manual shaking-enhanced, ultrasound-assisted emulsification microextraction (MS-USAEME) with ultraperformance liquid chromatography (UPLC) and UV detection for the analysis of five EDPs. The extraction time of this method was shorter than some of the previous microextraction methods.[29–36] Many phenol compounds were analyzed by HPLC[37–39] and UPLC[40] in the previous studies. Manual shaking of the glass centrifuge tube containing the aqueous sample and the organic solvent produced an emulsion with small droplets of the extraction solvent. The tube was immersed in an ultrasonic bath and ultrasonication for 1 min reduced the size of the droplets, resulting in rapid transport of analytes from the aqueous phase into the extraction solvent droplets.

2. Materials and methods

2.1 Reagents and materials

The EDPs 4-*tert*-butylphenol (4-t-BP, 99%), 4-cumylphenol (4-CP, 99%), 4-*tert*-octylphenol (4-t-OP, 97%) and 2,4-di-*tert*-butylphenol (2,4-di-t-BP, 99%) were purchased from Sigma-Aldrich (Steinheim, Germany) and 4-nonylphenol (4-NP, 98%) was purchased from Alfa Aesar (MA, USA). Deionized water (DI water) was purified by a Milli-Q water-purification system (Millipore, Bedford, MA, USA). A stock solution of each EDP was prepared at a concentration of 1000 μg mL^{-1} in methanol. Equal volumes of each EDP stock solution were combined to form a methanolic mixed standard solution containing each EDP at a concentration of 100 μg mL^{-1}. All stock and standard solutions were stored at 4 °C. Working standard solutions of each EDP at a concentration of 100 ng mL^{-1} were prepared by diluting the standard solution (100 μg mL^{-1}) with DI water. Working standard solutions at the concentration levels of interest were prepared daily. 1-Dodecanol (95%) was purchased from Fluka (Buchs, Switzerland). Hexadecane was purchased from TCI (Japan). 1-Bromohexadecane (97%) was purchased from Acros (Geel, Belgium). Methanol and acetonitrile were obtained from ECHO (Miaoli, Taiwan). 1-Decanol (99%) and sodium chloride (NaCl) were purchased from Merck (Darmstadt, Germany). All chemicals were of reagent grade or HPLC grade and were used without further purification.

Samples of seawater were collected at the Nanliao coastline in Hsinchu, Taiwan. The detergent samples (diluted dish-washing detergents, Taiwan) were diluted with DI water to a concentration of 0.25 g L^{-1}. These environmental samples were used to evaluate the analytical performance of the method. Environmental samples were stored at 4 °C and were filtered through a 0.45 μm pore size membrane filter (Millipore) before analysis.

2.2 Instrumentation

In this study, an ACQuity UPLC system equipped with a photodiode array detector (Waters, Manchester, UK) was used for the separation and quantification of 5 EDPs in seawater and detergent samples on an ACQuity UPLC® BEH C_{18} column (5 cm × 2.1 mm, 1.7 μm (film thickness)), at 30 °C. The photodiode array detector wavelength was set at 275 nm. A 50 μL injection loop and an autosampler were used. The mobile phase separation gradient started with 30% (v/v) acetonitrile in DI water at a flow rate of 0.4 ml min^{-1} for 1 min. The flow rate of the mobile phase was increased to 55% acetonitrile over 3 min, kept at 55% for 6 min, increased to 75% acetonitrile over 5 min and, finally, increased to 100% over 1 min. The total analysis time was 16 min. All mobile phases were filtered through a 0.22 μm pore size membrane (Millipore, Bedford, MA, USA). A micropipette (Branson, 20–200 μL) was used to inject the extraction solvent into the aqueous sample. A microsyringe (Hamilton, 25 μL) was used to collect and transfer extraction solvent into an insert (Waters, ACQuity UPLC). Centrifugation was done with a Hsiangtai (CN-2200) system and a desktop centrifuge (miVac, DVC-12060-C00). We used a Branson ultrasonic bath (model no. 3510; 335 W, 42 kHZ). A vortex mixer (Vortex-Genie 2 model no. G560; Scientific Industries, Bohemia, NY, USA) was used to mix aqueous samples with the extraction solvent. An insert was used to separate the aqueous and organic phases.

2.3 Extraction process

A working standard (5 mL) or an environmental sample (5 mL) spiked with all target analytes (each at a concentration of 100 μg L^{-1}) was placed into a round-bottomed glass centrifuge tube with the Teflon caps (to prevent cause of loss of the extraction solvent) and 1 g of NaCl was added (Fig. 1).

Organic extraction solvent (25 μL of 1-bromohexadecane) was injected into the centrifuge tube contents by microsyringe. The tube was shaken manually for 10 s and small droplets of the extraction solvent formed an emulsion with the sample solution. The tube was immersed in an ultrasonic water bath and sonicated

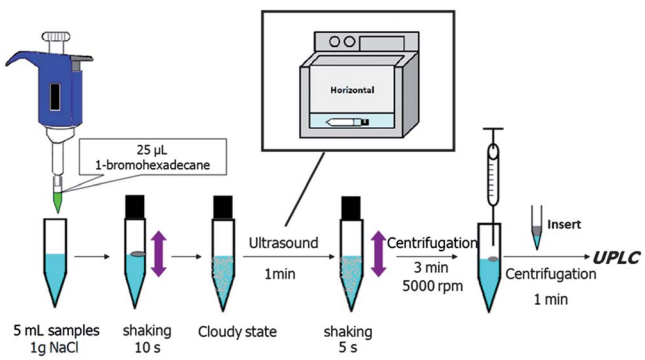

Fig. 1 A flow chart of the proposed method.

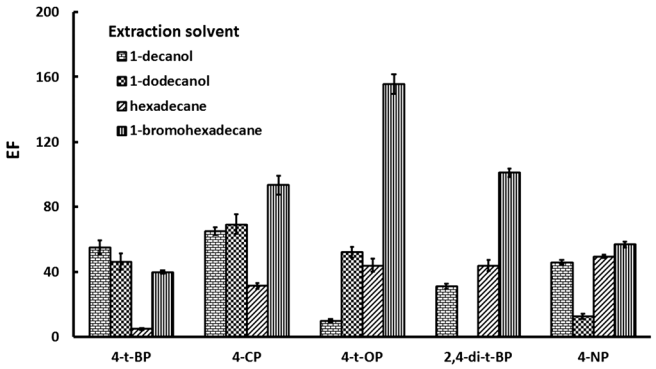

Fig. 2 Effect of the type of extraction solvent ($n = 3$). Samples were spiked with 100 ng mL^{-1} of each analyte and the tubes were immersed in the center of an ultrasonic bath. Extraction solvent volume, 28 μL; ultrasonication time, 5 min; amount of NaCl, 0.5 g. The tube was shaken manually for 10 s before ultrasonication and for 5 s after ultrasonication.

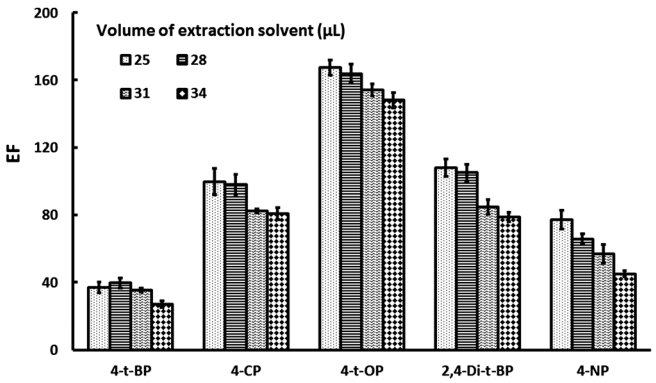

Fig. 3 Effect of the volume of extraction solvent ($n = 3$) Samples were spiked with 100 ng mL^{-1} of each analyte. Extraction solvent, 1-bromohexadecane; orientation, horizontal; location, center, ultrasonication time, 5 min; amount of NaCl, 0.5 g. Manual shaking for 10 s before and for 5 s after ultrasonication.

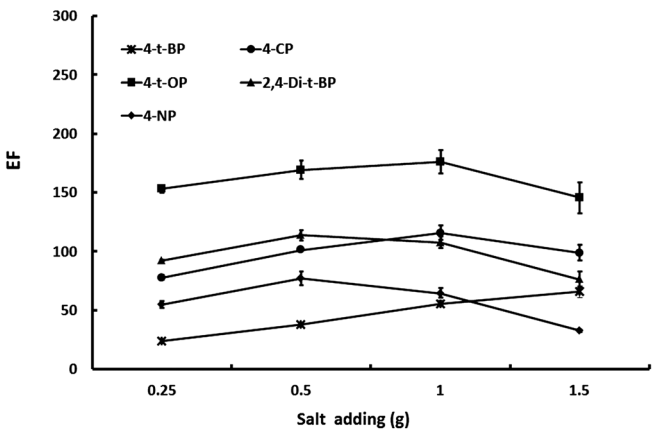

Fig. 4 Effect of ionic strength ($n = 3$). Samples were spiked with 100 ng mL^{-1} of each analyte. Extraction solvent, 1-bromohexadecane; extraction solvent volume, 25 μL; orientation, horizontal; location, center; ultrasonication time, 1 min. Manual shaking for 10 s before and for 5 s after ultrasonication.

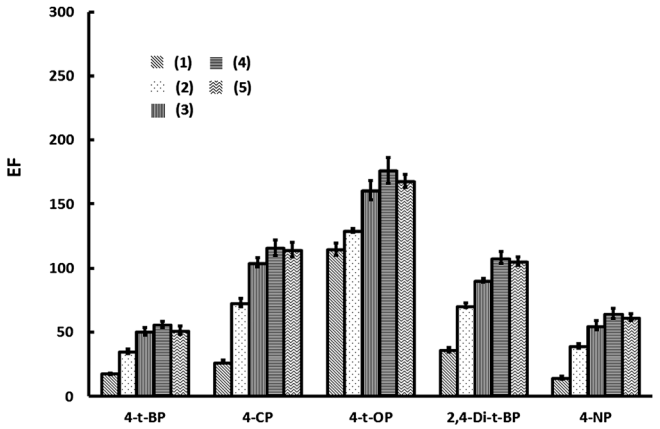

Fig. 5 Operational parameters ($n = 3$). Samples were spiked with 100 ng mL^{-1} of each analyte. Extraction solvent, 1-bromohexadecane; extraction solvent volume, 25 μL; orientation, horizontal; location, center; ultrasonication time, 1 min; amount of NaCl, 1 g. Manual shaking for 10 s before and for 5 s after ultrasonication. Method (1): no manual shaking before ultrasonication, manual shaking for 5 s after ultrasonication; method (2): manual shaking for 10 s before ultrasonication, no manual shaking after ultrasonication; method (3): no manual shaking before or after ultrasonication; method (4): manual shaking for 10 s before ultrasonication and for 5 s after ultrasonication; method (5): vortex mixing for 10 s before ultrasonication, manual shaking for 5 s after ultrasonication.

for 1 min. Formation of these tiny droplets increases the contact surface area between the two phases, which enhances the extraction efficiency. Shaking manually for 5 s after sonication prevented the extraction solvent being left on the wall of the centrifugation tube. After centrifugation for 3 min at 5000 rpm (speed), the extraction solvent floating on the surface was collected with a Hamilton microsyringe and transferred to an insert. The organic and aqueous phases separated in the insert after centrifugation for 1 min in a desktop centrifuge. The water phase in the lower portion of the liquid in the insert was removed and the volume of the remaining organic solution was ~20 μL. The insert was placed into the UPLC automatic injector system and 10 μL of extraction solvent was withdrawn for analysis.

3. Results and discussion

3.1 Optimization of the experimental conditions

In order to obtain high levels of sensitivity and precision with manual shaking-enhanced, ultrasound-assisted emulsification liquid–liquid microextraction, several parameters of the method were considered and optimized. These included the effects of: extraction solvent; ultrasound location; extraction solvent volume; ultrasonication time; ionic strength; operational parameters; and manual shaking time.

3.2 Selection of extraction solvent

Selection of the appropriate extraction solvent is important for obtaining a high level of extraction efficiency. The extraction solvent needed to meet the following requirements: (1) a low level of toxicity; (2) immiscibility with water; (3) a high level of extraction efficiency; and (4) good chromatographic behavior.

Table 1 The linearity (LR), limit of detection (LOD), limit of quantification (LOQ), relative standard deviation (RSD), and relative recovery (RR) of the proposed method

Compounds	LR/ng mL^{-1}	r^2	LOD[a]/ng mL^{-1}	LOQ/ng mL^{-1}	RSD[b] (%)	RR[c] (%)	LOD[d]/ng mL^{-1}	LOQ/ng mL^{-1}	RSD[e] (%)	RR[f] (%)
4-t-BP	5–1000	0.9982	0.6	2.0	10.3	96	0.4	1.6	9.7	81
4-CP	5–1000	0.9954	0.5	1.8	7.8	102	0.4	1.6	10.0	87
4-t-OP	5–1000	0.9962	1.0	3.4	5.3	106	0.5	1.8	5.4	97
2,4-di-t-BP	5–1000	0.9987	0.7	2.6	6.1	103	0.6	2.1	8.3	106
4-NP	10–1000	0.9985	2.8	9.3	4.5	109	2.4	8.2	5.6	102

[a] Seawater sample spiked with 2.0 ng mL^{-1} for 4-t-BP, 4-CP, 4-t-OP and 2,4-di-t-BP; 5.0 ng mL^{-1} for 4-NP, respectively ($n = 7$). [b] Seawater sample spiked with 2.0 ng mL^{-1} for 4-t-BP and 4-CP; 3.5 ng mL^{-1} for 4-t-OP, 2.5 ng mL^{-1} for 2,4-di-t-BP and 9 ng mL^{-1} for 4-NP, respectively ($n = 7$). [c] Seawater sample spiked with 5.0 ng mL^{-1} for 4-t-BP, 4-CP, 4-t-OP and 2,4-di-t-BP; 10.0 ng mL^{-1} for 4-NP, respectively ($n = 3$). [d] Detergent sample spiked with 2.0 ng mL^{-1} for 4-t-BP, 4-CP, 4-t-OP and 2,4-di-t-BP; 5.0 ng mL^{-1} for 4-NP, respectively ($n = 7$). [e] Detergent sample spiked with 2.0 ng mL^{-1} for 4-t-BP and 4-CP; 2.5 ng mL^{-1} for 4-t-OP and 2,4-di-t-BP, 10 ng mL^{-1} for 4-NP, respectively ($n = 7$). [f] Detergent sample spiked with 5.0 ng mL^{-1} for 4-t-BP, 4-CP, 4-t-OP and 2,4-di-t-BP; 10.0 ng mL^{-1} for 4-NP, respectively ($n = 3$).

Table 2 Relative standard deviation (RSD) of EDPs in environmental samples at three different concentrations

	Sea water sample (RSD, %)		
Compounds	LOQ	10 × LOQ	100 × LOQ
4-t-BP	10.3	7.9	4.2
4-CP	7.8	6.2	5.9
4-t-OP	5.3	5.6	4.3
2,4-di-t-BP	6.1	5.8	5.2
4-NP	4.5	6.5	6.1
	Detergent sample (RSD, %)		
Compounds	LOQ	10 × LOQ	100 × LOQ
4-t-BP	9.7	7.4	6.5
4-CP	10.0	6.8	5.2
4-t-OP	5.4	6.3	5.8
2,4-di-t-BP	8.3	6.7	6.0
4-NP	5.6	5.2	4.7

On the basis of these requirements, we chose 1-decanol, 1-dodecanol, hexadecane and 1-bromohexadecane as potential extraction solvents for this study. Comparison of the enrichment factor (EF) obtained with these extraction solvents is shown in Fig. 2.

Because the polarity of 1-bromohexadecane is lower than that of 1-decanol and 1-dodecanol, it has a high level of extraction efficiency for the low polar analytes of 4-CP, 4-t-OP, 2,4-di-t-BP and 4-NP, but a low level of extraction efficiency for the high polar analyte 4-t-BP. The elution time of these analytes are not distinguished in the chromatogram. Moreover, because the polarity of hexadecane is much lower than that of 1-bromohexadecane it has a low level of extraction efficiency for the high polar analyte 4-t-BP. Accordingly, we chose 1-bromohexadecane as the extraction solvent in subsequent experiments. (Note, the LD_{50} of 1-bromohexadecane has not been reported.)

3.3 Effect of ultrasound location

We asked whether different orientations (vertical, leaning and horizontal) of the centrifuge tube in the ultrasonic bath are associated with different levels of extraction efficiency. Further studies were done with the orientation and location of the tube used at the beginning of the study, *i.e.* horizontal in the middle of the bath. The extraction efficiency was greatest when the tube was horizontal (Fig. 1). Because the density of the organic extraction solvent used in this study is 0.99 g cm^{-3}, it floated on the surface of water after injection. When the centrifuge tube was vertical, ultrasonication did not disperse the organic extraction solvent evenly in the aqueous sample because the level of fluid in the tube was too high. After ultrasonication, the upper part of the aqueous sample had more organic solvent than the lower part resulting in poor extraction efficiency. The fluid level was decreased when the centrifuge tube was horizontal and ultrasonication dispersed the organic extraction solvent uniformly, leading to greater extraction efficiency.

It is likely that different orientations of the centrifuge tube in the ultrasonic bath alter the intensity of the ultrasound vibration, resulting in different levels of extraction efficiency. Six different orientations were tested (four vertical and two horizontal) to investigate the symmetry of the ultrasonic bath system. No significant effect of the orientation of the tube on extraction efficiency was found.

3.4 Effect of extraction solvent volume

As shown in Fig. 3, the effect of the volume of extraction solvent from 25 to 34 μL was investigated. In this method, the extraction recovery (ER) is influenced by the amount of all extractants. A higher volume of extraction solvent may increase the ER, because of high analyte recovery. In the optimization, the recoveries of the selected volume of extraction solvent are slightly different. But in this method, not all the floated extraction phase was injected. The volume of the floating phase increased with increasing volume of extraction solvent and the concentration of analytes in the collected solvent decreased, because of the dilution effect. The results show that smaller volumes of extraction solvent give a greater EF. The EF is defined as the ratio between the analyte concentration in the floated phase ($C_{floated}$) and the initial concentration of analyte (C_0). The volume of extraction solvent injected into the liquid chromatography column for analysis was set at 10 μL because of the decreasing amount of organic solvent as the column was developed. The point of withdrawal was at 2 mm above the bottom of the insert to take

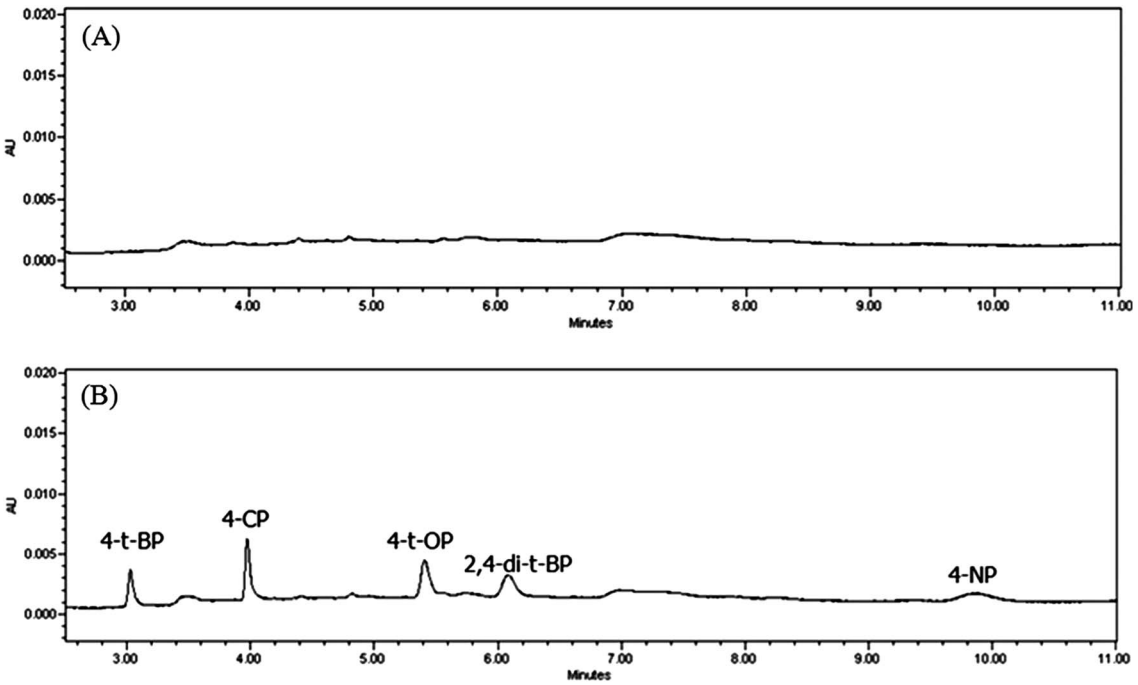

Fig. 6 Chromatogram of (A) an unspiked seawater sample and (B) a seawater sample spiked with 5 ng mL^{-1} of each analyte.

account of the position of the autosampler needle, *i.e.* the residual volume in the insert was 10 μL. We found that using 25 μL of extraction solvent led to ~20 μL suspended extraction solvent after centrifugation and this volume (25 μL) of extraction solvent was used for subsequent study.

3.5 Effect of ultrasound extraction time

The ultrasound extraction time, one of the main factors in MS-USAEME, is defined as the sum of (i) the shaking time after addition of the extraction solvent and (ii) the ultrasound

Table 3 Comparison with other extraction methods

Method (Ref.)	Instrument	Extractant	Extraction time/min	LOD/ng mL^{-1}	EF
PLE + SPE[a] (35)	LC–ESI-MS	Methanol + Oasis HLB	30	BP: 3	—
				NP: 12	—
SPME[b] (36)	GC-MS	Polyacrylate fiber	40	OP,NP: 0.001–0.003	—
DLLME[c] (26)	GC-MS	Chloroform: 50 μL	5	OP: 0.002	—
				NP: 0.03	—
LPME[d] (37)	HPLC-FLD	$[C_6MIM][PF_6]$: 10 μL	40	OP: 0.7	130
				NP: 0.3	163
DLLME[e] (38)	HPLC-FLD	Trichloroethylene: 50 μL	Few seconds	OP: 0.03	—
				NP: 0.1	—
DLLME + VALLME[f] (39)	HPLC-UV	Decanol: 150 μL	4	BP: 0.8	130
				CP: 0.2	137
				OP: 0.8	163
				NP: 1.6	100
SDME[g] (39)	HPLC-UV	Decanol: 2.5 μL	60	BP: 6	149
				CP: 4	140
				OP: 9	68
				NP: 9	44
VALLME[h] (22)	HPLC-FLD	Octanol: 50 μL	2	OP: 0.01	690
				NP: 0.07	150
HS-USAEME (This study)	UPLC-UV	1-Bromohexadecane:25 μL	~1	BP: 0.6	56
				CP: 0.5	116
				OP: 1.0	176
				NP: 2.8	64

[a] Pressurized liquid extraction and solid-phase extraction applied for 8 g corn cereals. [b] Solid-phase microextraction applied for 2 mL water sample (chlorinated tap water, detergent water, lake water). [c] Dispersive liquid–liquid microextraction applied for 5 mL ultrapure water (river water). [d] Liquid-phase microextraction applied for 15 mL sample solution (tap water, river water and effluent from sewage treatment plant). [e] Dispersive liquid–liquid microextraction applied for 5 mL water sample (tap water, river water and well water). [f] Dispersive liquid–liquid microextraction applied for 5 mL seawater. [g] Single drop microextraction applied for 5 mL seawater. [h] Vortex-assisted liquid–liquid microextraction applied for 20 mL water sample (tap water, river and wastewater).

extraction time. Variation of the extraction time affected emulsification and the mass transfer process, which influences the extraction efficiency for the analytes. The EF of the analytes increased from zero to maximum over 1 min (0–7 min). Note that this time (1 min) is much shorter than many other ultrasound-assisted LPME methods, presumably because the tube was shaken manually for 10 s before ultrasonic extraction. Effective dispersion is perhaps the most important step in MS-USAEME. Ultrasonication decreases the size of the droplets and disperses the organic extraction solvent uniformly within the aqueous solution, resulting in excellent enrichment, because the large contact surface area between the organic extraction solvent and the aqueous sample results in very rapid transport of analytes from the aqueous phase to the organic phase. Therefore, the short extraction time (~1 min) was used in subsequent studies.

3.6 Effect of ionic strength

Addition of salt increases the ionic strength of the sample solution, which improves the extraction of analytes in conventional liquid–liquid microextraction because of the salting-out effect. Further, increasing the ionic strength decreases the solubility of analytes in water and, therefore, they are extracted more easily by the organic solvent.

To evaluate the effect of the amount of salt added, different amounts of NaCl (0.25, 0.5, 1 and 1.5 g) were added to the aqueous sample. Fig. 4 shows that the EF for 4-t-BP was increased as the amount of salt was increased from 0.25 to 1.5 g. The EF for 2,4-di-t-BP and 4-NP increased as the amount of NaCl was increased up to 0.5 g and then decreased as the amount of salt was increased above 0.5 g. The EF for 4-CP and 4-t-OP increased as the amount of NaCl was increased up to 1 g and then decreased as the amount of NaCl was further increased. The solution was near saturation at 1.5 g of NaCl, so the effect of greater amounts was not studied. On the basis of these results, 1 g of NaCl was added to the aqueous sample in subsequent work.

3.7 Operational parameters

Shaking the tubes manually for 10 s before the ultrasonication dispersed the organic extraction solvent in aqueous samples. The organic extraction solvent used in this study is highly viscous and shaking the tubes manually for 5 s after the ultrasonication step prevented the organic solvent from remaining on the tube wall and the inner surface of the tube cap.

Five modes of operation were tested (Fig. 5):

Method (1) No manual shaking before ultrasonication, manual shaking for 5 s after ultrasonication.

Method (2) Manual shaking for 10 s before ultrasonication, no manual shaking after ultrasonication.

Method (3) No manual shaking before or after ultrasonication.

Method (4) Manual shaking for 10 s before ultrasonication and for 5 s after ultrasonication.

Method (5) Vortex mixing for 10 s before ultrasonication, manual shaking for 5 s after ultrasonication.

The results showed that:

Method (1) The organic extraction solvent was not well dispersed without manual shaking. The extraction efficiency was poor.

Method (2) A milky white cloudy emulsification was formed after manual shaking for 10 s at a frequency of 3 shakes s^{-1}. The extraction efficiency was decreased slightly by omitting manual shaking after ultrasonication.

Method (3) The extraction efficiency was poor without manual shaking before ultrasonication but was improved slightly by manual shaking after ultrasonication.

Method (4) This operational mode gave the highest level of extraction efficiency. Manual shaking before and after ultrasonication was essential for effective extraction.

Method (5) No cloudy emulsification was formed after vortex mixing continuously for 10 s. The extraction efficiency of vortex mixing was poorer than that of manual shaking.

3.8 Effect of shaking time

Most extraction methods use a large amount of solvent to achieve rapid extraction. By contrast, the method described here uses manual shaking, by using ultrasonication to emulsify and disperse the organic extraction solvent in aqueous samples. Emulsification is well-dispersed step, it reduces the ultrasonication time and increases extraction efficiency. Emulsification of the organic extraction solvent can be achieved with a combination of a dispersive solvent, vortex mixing and manual shaking. In recognition of the concept of green chemistry, this study was designed to reduce the amount of organic solvents used and, therefore, did not consider the use of a dispersive solvent. The effect of vortex mixing was poorer than that of the manual shaking results shown in the operational parameters. Finally, manual shaking achieved emulsification of the organic extraction solvent, which facilitated dispersion.

The time and frequency of manual shaking are important factors in the novel method described here: a maximum frequency of 3 shakes s^{-1} for 0, 3, 10, 20 and 40 s was used to estimate the effect of different lengths of shaking time. For all analytes, a shaking time of 10 s achieved an almost equilibrium effect and this time was chosen as the optimum length of time for manual shaking.

3.9 Ultrasound *versus* vortex mixing

Ultrasonication-assisted emulsification with manual shaking was compared to vortex mixing-assisted emulsification. All analytes reached constant extraction efficiency after 3–5 min of vortex mixing with an extraction efficiency similar to that of ultrasonication for 1 min. This study, therefore, used ultrasonication-assisted emulsification to maximize extraction efficiency.

3.10 Method validation

The calibration curve range, correlation coefficient, limit of detection (LOD), limit of quantification (LOQ) and relative standard deviation (RSD) were investigated under the optimized experimental conditions and the results are summarized in Table 1. The linear calibration of targeted EDPs was examined in the concentration range of 5–1000 ng mL^{-1}, except for 4-NP (10–1000 ng mL^{-1}). The correlation coefficients of the calibration

curves range were >0.995 and good linear behavior was observed. The LOD was calculated as three times the standard deviation of seven replicate runs of seawater samples and seven replicate runs of detergent samples spiked with each analyte at a low level. The LOD values were in the range of 0.5–2.8 ng mL^{-1} for seawater samples and in the range of 0.4–2.4 μg mL^{-1} for detergent samples. The RSD of the method obtained from the analysis of seawater samples and detergent samples spiked with three different concentrations of EDPs, based on the peak areas for seven replicate runs for this extraction and expressed as a percentage, was in the range 4.2–10.3% (Table 2).

3.11 Environmental sample analysis

The method was used for the determination of targeted EDCs in seawater samples and detergent samples. Fig. 6 shows typical chromatograms for aqueous samples unspiked and spiked with different concentrations of seawater samples. No analyte was found in unspiked aqueous samples under the optimized experimental conditions. In order to estimate the influence of the matrix, all of the aqueous samples were spiked with different concentrations of analytes to determine the relative recovery of the targeted analytes. The relative recoveries for the spiked aqueous samples were 96–109% for seawater samples and 81–106% for detergent samples (Table 1). These results show that the MS-USAEME method is suitable for the analysis of seawater samples and detergent samples and is not affected significantly by the sample matrix. Table 3 gives a summary of values in the literature for the analysis of EDPs in a variety of samples. This study is the first to use 1-bromohexadecane as the organic extraction solvent for the analysis of EDPs. The consumption of organic solvent in this method is approximately equal to or even lower than that for other methods in the literature. DLLME uses large amounts of dispersive solvent to achieve complete extraction immediately, whereas the extraction time in the method described here is ~1 min. The current method uses a small amount of organic solvent and ultrasound-induced emulsification to achieve complete extraction, which is much faster than traditional time-consuming extraction methods (SPME, LPME, SDME and vortex-assisted liquid–liquid microextraction). The measurement of EDPs in the current method is done by HPLC-UV, which has a higher LOD than that achievable by HPLC-FLD or GC-MS. In addition, the current method uses 5 mL aqueous samples, which minimizes the amount of waste solution generated and contributes to the higher LOD and lower EF values of this method compared to those in the literature for other methods.

4. Conclusions

A rapid, simple equilibrium liquid-phase microextraction method based on manual shaking-enhanced, ultrasound-assisted emulsification microextraction (MS-USAEME) and measurement by UPLC-UV was developed for the determination of five EDPs in seawater samples and in detergent samples. This extraction of trace analytes can detect low concentrations of EDPs and is faster (~1 min) than traditional liquid–liquid microextraction methods. No dispersive solvent is used in this method, and the amount of organic solvent is minimized, both of which contribute to the low level of pollution (~50% of the amount generated by traditional methods). In this method, the glass centrifuge tube containing the aqueous sample and the organic solvent is shaken manually for 10 s to form an emulsion of the sample solution and small droplets of the extraction solvent. The tube is then immersed in an ultrasonic water bath and ultrasonication for 1 min changes the small droplets of extraction solvent into tiny droplets. As a result, the contact surface area between the two phases is increased, which increases the extraction efficiency.

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