

Development of dispersive liquid-liquid microextraction coupled to UPLC-DAD for detection and determination of phthalic acid esters in mouthwash

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ABSTRACT

A dispersive liquid-liquid micro extraction (DLLME) coupled with ultra performance liquid chromatography-diode array detector (UPLC-DAD) method for extraction and pre-concentration of four phthalic acid esters (PAEs), consisting diethyl phthalate (DEP), Bis-2-Ethyl Hexyl Phthalate (BEHP), dicyclohexyl phthalate (DCHP), and di-n-butyl phthalate (DnBP) in mouthwash have been described. Excellent linearity ($r=0.9998$) was observed over the concentration range of 1-1000 ng. mL⁻¹ for the PAEs. The RSD% ranged from 0.3 to 2.4% (n= 5). BEHP was the most common type of PAEs encountered in mouthwash with a concentration of more than 99.2 ng. mL⁻¹.

Keywords: PAEs, DLLME, Mouthwash, UPLC-DAD.

1. INTRODUCTION

Toxicity, persistence, and bioaccumulation of PAEs afforded much attention to the development of effective sample preparation and analytical methods for the analysis of food containers and toys which may use PAEs as plasticizer [1-3]. AEs are a group of industrial chemicals widely used in consumer products such as solvents, additives, packages, clothes, films, toys, paints and plasticizers (beverage bottles, food covers, detergent holders and mouthwashes) [4-6]. They can be leached from the products and find a way to various environmental matrices [6-8]. Moreover, certain PAEs, as well as their metabolites and degradation products, can cause adverse effects to human health (particularly on the liver, kidney and testicles), [9]. Potential endocrine disrupting properties were also reported [10]. Certain phthalates are endocrine disruptors and have been linked to adverse health outcomes [11-13]. Due to their potential risks to human health and the environment, several of them have been included on the priority list of pollutants of different national and supranational organizations [14]. According to Section 307 of the US Clean Water Act, DEP and DnBP should be considered as priority toxic pollutants [15]. Evaluation and monitoring of traces of these compounds in different environmental matrices are imperative for human health protection and the environmental control [16]. The low concentration of PAEs and the complex matrix in many environmental samples make sample preparation necessary for the reliable determination of such compounds [17]. So far, various methods such as liquid-liquid extraction (LLE) [18, 19], solid-phase extraction (SPE) [20, 21], and solid phase microextraction (SPME) [22, 23] have been employed as sample pretreatment procedures for the determination of PAEs. However, the traditional pretreatment processes (LLE and SPE) are time-consuming and need a large amount of organic solvents, which are dangerous to human and the environment. Among all methods, SPME is proven as a simple, fast, solventless and efficient sample preparation technique that enables the determination of PAEs at

low concentrations of ng.mL⁻¹. However, it also suffers from some drawbacks including the fiber is expensive and fragile, and samples carry over is beside a problem [24]. Consequently, liquid phase micro extraction was developed as a solvent-minimized sample pretreatment procedure that is inexpensive, and since the very little solvent is used, there is minimal exposure to toxic organic solvents [25]. However, this method suffers from some disadvantage: fast stirring would tend to form air bubble, meanwhile, extraction is time-consuming and equilibrium could not be attained after a long time in most cases [26].

Recently, a novel microextraction technique termed as dispersive liquid-liquid microextraction (DLLME) was suggested in 2006 by Assadi, et al [27-29]. The method is based on the ternary component solvent system. A mixture of water immiscible extraction solvent dissolved in a water miscible disperser solvent is injected rapidly into an aqueous sample [30]. A cloudy solution formed consists of fine droplets of the extraction solvent dispersed into the aqueous phase. Due to the considerably large surface area between the extraction solvent and the aqueous sample, the extraction of the analytes is achieved quickly. After extraction, phase separation is performed by centrifugation and the enriched analyte in the sedimented phase is determined by chromatography or spectrometric methods [31-33]. DLLME is simple to operate and is an especially rapid, inexpensive extraction method with high pre-concentration enrichment and low sample volume requirements [34, 35]. DLLME was applied for pre-concentration of polyaromatic hydrocarbons [36], chlorophenols [37], PAEs [19, 38], and triazine herbicides [39, 40]. Simultaneous DLLME and derivatization were suggested for chlorophenols [41, 42].

The aim of this research was to determine four PAEs which are mostly encountered including DCHP, BEHP, DEP and DnBP in mouthwash, by an optimized and validated DLLME method followed by UPLC-DAD.

2. EXPERIMENTAL SECTION

2.1. Reagents and standards. DEP (99.0%), DCHP (99.0%), BEHP (98.0%) and DnBP (99.0%) were purchased from the local market made by Sigma-Aldrich (Missouri, USA). Acetone, methanol, n-heptane, dichloromethane, chloroform, carbon tetrachloride, acetonitrile (HPLC grade) and sodium chloride (99.5%) were purchased from the local market made by Merck (Darmstadt, Germany). The individual standard stock solutions shall were prepared in methanol at a concentration of 100 mg.L⁻¹ and stored at 4 °C. The standard working solutions shall were prepared daily by dilution of the standard stock solution with deionized water to the required concentrations. Water utilized in all procedures was deionized water prepared by Milli-Q5TM (Millipore, Milford, MA, USA). All solvents and solutions for UPLC analysis were filtered through a Millipore filter (pore size 0.45µm) to remove particulate matter before investigation. Special care was taken to avoid the contact of reagents and solvents with plastic materials. In order to reduce background contamination, all glassware was cleaned prior to the analysis. Then, glassware was sealed with foil of aluminum and stored in a clean environment to avoid adsorption of PAEs from the air.

2.2. UPLC conditions. Chromatographic analysis was performed on an Agilent® 1200 liquid chromatography system, equipped with a quaternary pump and degasser, a thermo stated autosampler, column compartment (40°C), a diode array detector, a personal computer equipped with an Agilent® ChemStation® program for LC was used to process chromatographic data. A reversed-phase Agilent® XDB-C18 column (4.6×50 mm, 1.8 µm) was used for separations. An Eclipse XDB-C18 analytical guard column (4.6×12.5 mm) from Agilent® was used to protect the column. The mobile phase was a mixture of acetonitrile–water (90:10, v/v), the flow rate was 0.5 mL.min⁻¹, the preferential detection wavelength was 280 nm, and the injection volume was 5 µL. An ALC 4232 centrifuge (Milano, Italy) was used for centrifugation.

2.3. Evaluation of peak purity. Spectral wavelengths of chromatographic peaks were monitored continuously from 200 to 400 nm. The purity of peaks in chromatograms of sample preparations was evaluated online from the spectral data by analysis of peak spectral identity with that of a reference standard, and by measurement of peak purity parameters. Spectral searches were carried out during the chromatographic run to determine the degree of identity of a peak area spectrum with that of a reference

standard stored in a library in the computer, using multicomponent analysis. Identity was expressed under the form of similarity and dissimilarity indices, with identical spectra having similarity and dissimilarity indices of 1 and 0, respectively. The purity parameter of a chromatographic peak represents the average wavelength throughout the peak spectrum within a specified wavelength range. In pure peaks, upslope, apex, and down slope purity parameters are identical. Apex purity parameters were compared with those of standards under same chromatographic conditions. Agreement within a range of ±1 nm was considered evidence of a pure peak. Purity parameters and similarity- dissimilarity indices were printed in post run reports. Purity data was gotten from the spectral analysis report and a peak purity value greater than 990 shows a homogeneous peak.

2.4. Extraction method. For the DLLME, 5.0 mL of deionized water spiked with the DEP, DnBP, DCHP, and BEHP at 1 mg L⁻¹ the mixture of analytes was put into a 10 mL screw-cap glass test tube with a conical bottom, and the sample was heated to 40 °C in a water bath. Then, 800 µL of a solution containing 750 µL of acetonitrile (as disperser solvent) and 50 µL of carbon tetrachloride (as extraction solvent) were quickly injected into the above-mentioned sample solution by using 50 µL syringes (it was injected in 50 ml aliquots, 16 times). A cloudy solution that contains of very tiny droplets of CCl₄ dispersed into sample solution was formed, and therefore the analytes were extracted into the tiny droplets. After centrifugation for 10 min at 2000 rpm, and fine droplets of extraction solvent settled at the bottom of the tube. The sedimented phase was taken with a microsyringe (Hamilton, Reno, NV, USA), dried under a gentle stream of nitrogen, and in the next step, it was again dissolved in 200 µL of methanol; and 5 µL of this solution was injected into the UPLC for analysis.

All the solvents and water samples were filtered through a 0.45 µm membrane, to eliminate particulate matter, before analysis.

2.5. Sample preparation. The sample was bought from the native market. From every Mouthwash in plastic containers, a sample of 10 mL was prepared. This technique of preparation was used, as a result of the aim was to know that in normal conditions, how much of the PAEs present in the Mouthwash will leach to the surrounding media. All the subsequent steps for extraction were similar to the procedure described in section 2.4.

3. RESULTS SECTION

3.1. Optimization of UPLC conditions.

The parameters in UPLC analysis of PAEs were optimized through investigating the influence of the mobile phase combination, flow rate and detection wavelength because these parameters play important roles in resolution and sensitivity. In this work, three solvent including water, methanol and acetonitrile with different ratios were used as the mobile phase. Results revealed that the acetonitrile-water system was the optimum mobile phase with the most chromatographic peaks, the strong peak strength and the finest abilities of separation and resolution.

System suitability of the method was evaluated using peak purity and resolution factor that have been summarized for each analyte in Table 1.

Table 1. Peak purity and resolution factor of different analyte peaks in the chromatogram at optimum conditions.

Sample	Peak purity	Resolution Factor
DEP	999.38	-
DnBP	999.98	9.51
DCHP	999.99	2.78
BEHP	1000.00	3.23

Results show all the analyte peaks in the chromatogram have high purity and resolved from the adjacent peaks. The detection wavelength was investigated with DAD in the range of 200–400 nm in order to obtain a large amount of detectable peaks in the UPLC chromatogram of the studied samples. Meanwhile, the UV absorption information of PAEs was also considered. In the end, using 3-D-plot, the wavelength of 280 nm was chosen to achieve chromatographic fingerprint profiles and used as the detection wavelength by comprehensive consideration.

Chromatograms of analytes separately and in combination have been shown in Figure 1. The picture clearly shows that the system was used to analyze PAEs is suitable.

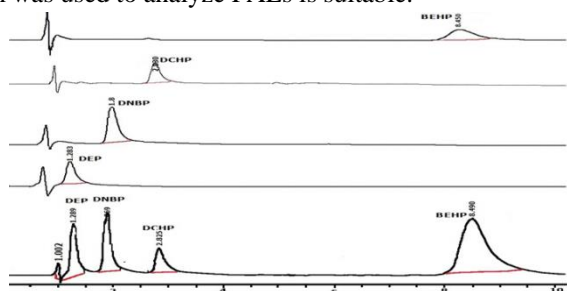


Fig. 1 Chromatograms of standard samples after DLLME in optimum conditions.

3.2. Optimization of DLLME

To achieve the optimized extraction requirement, enrichment factor (EF) and extraction recovery (ER) were applied to assess the extraction efficiency in different conditions.

3.2.1. Selection of extraction solvent. To develop DLLME method, first select the appropriate extraction solvent. The extraction solvent must have the following characteristics: higher density than water, fine chromatographic behavior, extraction ability of interesting combinations, Poorly soluble in water, and form a two-phase system with a dispersive solvent when injected into an aqueous solution. Nearly all of the halogenated solvents respond these characteristics, thus among the solvents with density higher than water (mainly chlorinated solvents), dichloromethane, chloroform and carbon tetrachloride were studied. In this research, all combinations of n-heptane, dichloromethane, chloroform and carbon tetrachloride (75 μ L) as an extraction solvent and methanol, acetonitrile, and acetone (1.0 mL) as dispersive solvents were analyzed. n-Heptane as extraction solvent was rejected instantly as it could not create a separate phase with any dispersive solvents of this research. chromatographic peaks of standard Solutions after DLLME Indicated that the retention time of dichloromethane is very close to that of DEP, and its Chromatogram is not separated from the analyte peaks, so it was eliminated. Chloroform and carbon tetrachloride with acetonitrile as dispersive solvent made a two-phase system which was good stability. Figure 2a shows that carbon tetrachloride has higher ER for all the PAEs investigated; Therefore carbon tetrachloride was used as a solvent extraction in all of the following steps.

3.2.2. Effect of extraction solvent volume. To investigate the effect of extraction solvent volume on the EF, variable volume ranges of CCl_4 (20, 40, 50, 75 and 100 μ L) and a fixed volume of dispersive solvent (acetonitrile, 1.0 mL) were Examined. Figure 2b illustrates EF versus volume of extraction solvent. Findings indicate that the highest ER was specified in 50 μ L.

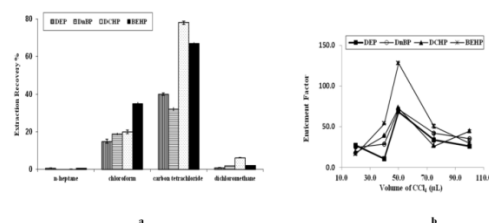


Fig. 2. a) Effect of different extraction solvent on the extraction recovery (ER %) of PAEs. Extraction conditions: sample volume, 5.0 mL; Extraction solvent volume, 75 μ L; dispersive solvent, acetonitrile; 1.0 mL; b) Effect of the volume of extraction solvent (CCl_4) on the Enrichment Factor (EF) of PAEs. Extraction conditions: sample volume, 5.0 mL; dispersive solvent (acetonitrile) volume, 1.0 mL.

3.2.2. Selection of dispersive solvent. Miscibility, toxicity and price were considered as main agents for the selection disperser solvent. The miscibility directly influences the formation of a cloudy solution for DLLME method in the extraction solvent and the aqueous sample. Based on these requirements, acetone, acetonitrile, and methanol were tested as dispersive solvents. The effect of various dispersive solvents on the EF of PAEs was illustrated in Figure 3a. Findings indicate, the highest EF was specified by acetonitrile as dispersive solvent.

3.2.4. Effect of dispersive solvent volume. To obtain the optimum volume of dispersive solvent, various experiments were performed by different volumes of acetonitrile (500, 750, 1000, and 1500 μ L) containing a constant volume of extraction solvent (CCl_4 , 50 μ L). The results were shown in Figure 3b. As it can be seen, EF increased by increasing the volume of acetonitrile when it was less than 0.75 mL. Reduction in EF was observed after the volume of acetonitrile exceeded 750 μ L. Because at low volume, acetonitrile cannot disperse CCl_4 properly and the cloudy solution is not formed completely, and at higher volumes, the solubility of PAEs in water will be increased, so, 750 μ L acetonitrile was chosen as optimum volume.

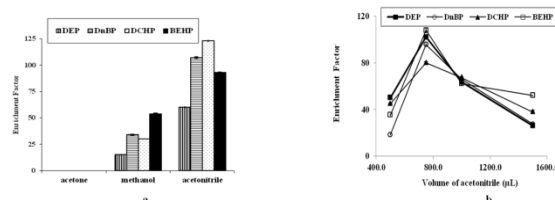


Fig. 3. a) Effect of different dispersive solvent on the EF of PAEs. Extraction conditions: sample volume, 5.0 mL; dispersive solvent volume, 1.0 mL; extraction solvent, CCl_4 , 75 μ L; b) Effect of the volume of dispersive solvent (acetonitrile) on the enrichment factor of PAEs. Extraction conditions: sample volume, 5.0 mL; Extraction solvent (CCl_4) volume: 50 μ L.

3.2.5. Effect of extraction time. In DLLME, extraction time, defined as duration between injecting the mixture of disperser solvent (acetonitrile) and extraction solvent (CCl_4), and starting the centrifuge, was Examined the range of 0–20 minutes with same procedure. findings indicated that peak area differences are less than 5% that can be negligible. It can be concluded that the DLLME for extraction of PAEs at the conditions used was time-independent. It can be due to the extremely large surface area between the extraction solvent and aqueous phase and equilibrium state can be achieved quickly. This is main advantage of DLLME method.

3.2.6. Effect of ionic strength. Adding salt to the aqueous sample is usually used to increase the extraction of analytes because it generally decreases the solvation power of the solution. To study the effect of salt on the efficiency of DLLME of the PAEs, the extraction was done with different concentrations of NaCl (0–1.5% (w/v)). The results obtained in Figure 4 indicate that the addition of NaCl significantly decreased the ER% of BEHP, rather decrease the ER% of DEP and DCHP, while has no effect on the ER% of DnBP. Thus, it seems that not only salt could not remove analytes from aqueous solutions to the organic solvent, but in some cases increasing salt concentration decrease ER% of the analytes, significantly. according to the results, extraction trials achieved without the addition of salt.

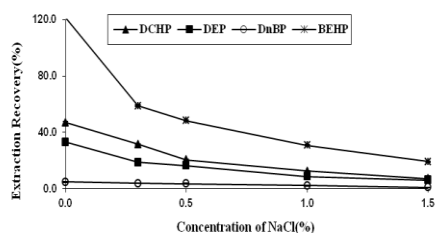


Fig. 4. Effect of salt addition on the ER % of PAEs. Extraction conditions: sample volume: 5.0mL; extraction solvent (CCl₄), 50 μL, dispersive solvent (acetonitrile), 750 μL; temperature, 40 °C.

3.2.7. Effect of temperature. To evaluate the effect of temperature on the extraction method, it was performed at different temperature ranges from 15 to 50 °C. The results are shown in Figure 5. The best extraction temperature was obtained at 40 °C.

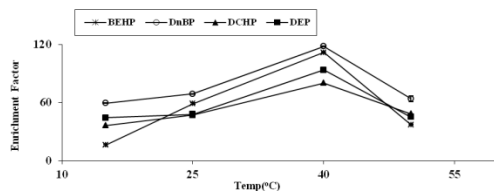


Fig. 5. Effect of temperature on the EF of PAEs. Extraction conditions: sample volume, 5.0mL; extraction solvent (CCl₄), 50 μL, dispersive solvent (acetonitrile), 750 μL.

4. CONCLUSIONS

The developed method (DLLME/UPLC-DAD) comprise reliable and efficient alternatives for PAEs determination of low concentrations in mouthwash. The method is truly simple, precise and demonstrates desirable characteristics for routine analyses, such as very low LOD, fast, sensitive, cheap, environmentally

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3.3. Validation of the method

Under optimum conditions, linearity, LOD, LOQ, enrichment factor, accuracy and repeatability of the DLLME method for the PAEs were investigated and have been listed in Table 2.

Table 2. The characteristics of the DLLME method for the PAEs examined in this research.

Compound	Linear range (ng. mL ⁻¹)	R ²	RSD (%)	LOD (ng. mL ⁻¹)	LOQ (ng. mL ⁻¹)	EF
DEP	1-1000	0.9998	2.4	0.9	1.1	89
DnBP	10-1000	0.9999	1.2	9.0	10.1	110
DCHP	10-1000	0.9999	0.3	1.4	2.3	96
BEHP	10-1000	0.9999	1.6	6.9	9.8	160

As it can be seen, all the figures of merit including linearity, repeatability, LOD, LOQ and EF are in the acceptable range and level and confirmed that the proposed method has very high sensitivity and stability, and tremendous potential exists for the application of the method to the analysis of PAEs at trace levels in aqueous media.

3.4. Real sample analysis

The practical applicability of the recommended method was evaluated by extracting DEP, DnBP, DCHP and BEHP from mouthwash. The result is given in Table 3.

Table 3. Concentration of PAEs in mouthwash.

Real Sample (ng. mL ⁻¹)	Real Sample (ng. mL ⁻¹)			
	DEP	DnBP	DCHP	BEHP
mouthwash	37.6	50.1	39.0	99.2

Chromatograms obtained for real sample under the optimum conditions was shown in Figure 6. Result depict that, Mouthwash contains the highest level of PAEs, that it can be dangerous to the infant's health.



Fig. 6. Chromatograms of mouthwash after DLLME in optimum conditions.

friendly and suitable. The linearity, precision and accuracy have been investigated. The accuracy test is satisfactory for the sample, thus indicating that the proposed methods are very interesting and applicable for environmental and toxicological analyses.

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