JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

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pubs.acs.org/JAFC

Effects of Push/Pull Perfusion and Ultrasonication on the Extraction Efficiencies of Phthalate Esters in Sports Drink Samples Using On-line Hollow-Fiber Liquid-Phase Microextraction

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Supporting Information

ABSTRACT: In previous studies, we developed a process, on-line ultrasound-assisted push/pull perfusion hollow-fiber liquid-phase microextraction (UA-PPP-HF-LPME), combining the techniques of push/pull perfusion (PPP) and ultrasonication with hollow-fiber liquid-phase microextraction (HF-LPME), to achieve rapid extraction of acidic phenols from water samples. In this present study, we further evaluated three more-advanced and novel effects of PPP and ultrasonication on the extraction efficiencies of neutral high-molecular-weight phthalate esters (HPAEs) in sports drinks. First, we found that inner-fiber fluid leakage occurs only in push-only perfusion-based and pull-only perfusion-based HF-LPME, but not in the PPP mode. Second, we identified a significant negative interaction between ultrasonication and temperature. Third, we found that the extraction time of the newly proposed system could be shortened by more than 93%. From an investigation of the factors affecting UA-PPP-HF-LPME, we established optimal extraction conditions and achieved acceptable on-line enrichment factors of 92−146 for HPAEs with a sampling time of just 2 min.

KEYWORDS: *push/pull perfusion, hollow fiber, on-line microextraction, ultrasonication, phthalate esters*

INTRODUCTION

When measuring trace levels of contaminants in a food sample, pretreatment is usually necessary prior to instrumental analysis. Liquid-phase microextraction (LPME) is a fairly new method with which to perform food sample pretreatment;[1] it is a miniaturized version of conventional liquid−liquid extraction (LLE). In 1999, Pedersen-Bjergaard and Rasmussen developed hollow-fiber LPME (HF-LPME), based on the use of a single porous hollow fiber (HF) made of polypropylene supporting a hydrophobic solvent in the pores [a so-called supported liquid membrane (SLM)] for extraction of target analytes.[2] Because HF-LPME involves placing the hydrophilic extracting phase inside the lumen of an HF, it is highly compatible with high-performance liquid chromatography (HPLC).[3] The principles and the analytical applications of HF-LPME have been described in several reviews.[3−6] Nevertheless, as with regular microextraction methods, most HF-LPME approaches still suffer from the common drawbacks of requiring considerable time and manual operation, with few essential solutions having been developed until recently.[3]

In recent years, HP-LPME methods exhibiting high extraction yields as well as efficient extraction kinetics have been realized by applying three major techniques: a pH gradient, carrier transport, and electromembrane extraction (EME).[6] EME is an electrokinetic migration-based technique that can provide high analyte recoveries within very short times, relative to conventional passive diffusion-based HF-LPME. Notably, however, the extraction mode of EME, as well as those of the other two modified approaches, is limited to basic or acidic analytes featuring ionizable functionalities.[6] Thus, there remains an urgent need to develop generic HF-LPME methods for the analyses of neutral molecules to minimize their extraction times. Ultrasonic radiation is a powerful tool that can accelerate various steps in a sample pretreatment process. Although a few ultrasound-assisted HF-LPME methods have been developed as alternatives to conventional LLE to accelerate the extraction kinetics of acidic or basic analytes,[7−11] their extraction capabilities toward neutral analytes remain limited, and possible interaction effects may occur with other HF-LPME variables.

On-line systems generally lessen the degree of manual handling of samples and, thereby, minimize the risk of errors, such as transfer losses and contamination of the samples.[12] A few recent efforts at modifying the HF-LPME technique have focused on the development of on-line methods.[10,11,13] In 2012, Yamini and co-workers developed the first automated on-line HF-LPME system.[13] They used an automated syringe pump to load the supported liquid membrane and acceptor solvents, a platform lift to move the sample vial, a sampling loop for on-line injecting of the extract to the HPLC system, along with an electronic board with an AVR microcontroller to store the data and instrument programs. This system allowed sample extraction and extract injection to be performed automatically. In this automatic system, the extraction occurred in the static (stop flow) acceptor phase, much like that in traditional off-line

Received: April 19, 2013
Revised: July 22, 2013
Accepted: July 23, 2013
Published: August 14, 2013

HF-LPME; the optimized extraction time for the extraction of the target analyte was 40 min. After that paper appeared, our group reported a rapid on-line HF-LPME module that operates in flow-through mode: ultrasound-assisted push/pull perfusion hollow-fiber liquid-phase microextraction (UA-PPP-HF-LPME).[10,11] In this new system, the extraction occurs in a flowing acceptor phase. By using an ultrasonic probe and a push/pull syringe pump to accelerate the mass transfer and dispense the acceptor phase to the HF, the optimized extraction time for the extraction of acidic phenols from water samples decreased to only 2 min, with enrichment factors (EFs) from 82 to 279.[10,11]

The on-line coupling of a regular HF-LPME device requires two important factors to be considered: (i) the thick contact wall of the HF will potentially necessitate long extraction times, which would result in low sample throughput in on-line applications, for target analytes; and (ii) the acceptor-to-donor/donor-to-acceptor fluid pressure caused by the flowing acceptor phase stream in the HF lumen can potentially lead to fluid loss or fluid gain across the porous membrane, thereby decreasing the collection of the extracted acceptor phase or diluting the extracted acceptor phase.[10,11] Although a few papers describe the problem,[10,11] none have reported the extent of inner-fiber fluid leakage in HF-LPME (especially for HF-LPME in nonstatic modes, such as dynamic HF-LPME and on-line HF-LPME); nevertheless, some suspected SLM leakage has been found in previous studies of nonstatic HF-LPME.[13,14] Further efforts will be required to clarify these phenomena.

In this study, we employed three high-molecular-weight phthalate esters (HPAEs) as model neutral compounds to obtain a clear understanding of the effects of PPP on inner-fiber fluid stability (including those of both the SLM and the acceptor phase), the effects of ultrasonication on the extraction efficiency and extraction speed for HPAEs, and the interaction effects of both PPP and ultrasonication toward other UA-PPP-HF-LPME extraction variables. The wider application of this method would potentially expand the application of HF-LPME as a routine technique for food sample analyses.

MATERIALS AND METHODS

Chemicals and Reagents. Di-*n*-octyl phthalate (DNOP, CAS number 117-84-0, >98%), diisononyl phthalate (DINP, CAS number 20548-62-3, >98%), and diisodecyl phthalate (DIDP, CAS number 89-16-7, >98%) were purchased from Toronto Research Chemicals (Alberta, Canada). *n*-Docane, *n*-dodecane, *n*-tetradecane, hexadecane, 1-octanol, 1-nonanol, 1-dodecanol, and 1-undecanol were purchased from Sigma–Aldrich (St. Louis, MO). Lichrosolv gradient-grade MeCN and MeOH were supplied by Merck (Darmstadt, Germany). All other reagents were of the highest grade commercially available. Ultrapure water (resistivity: 18.3 MΩ cm) was obtained from a Barnstead nanopure water system (Dubuque, IA); it was used to prepare all mobile phases and other related solutions. All reagents and water were checked for contamination with phthalates prior to use.

Instrumentation. The HPLC system consisted of Beckman components including a 126 solvent delivery system, a vacuum degasser, a module 126 UV detector with a 20-μL flow cell, and a PC running Beckman 32 Karat integration software. The detection wavelength was set at 203 nm. A C18 column (Ascentis-ODS, 250 mm × 4.6 mm i.d., 5 μm; Supelco, Bellefonte, PA) was used for chromatographic separation. The mobile phase was MeCN. The flow rates (gradient elution) were programmed as follows: 1.5 mL min^{-1} from the beginning to 6.5 min, decreasing to 1.0 mL min^{-1} until 10.7 min, and then increasing to 1.5 mL min^{-1} until 20 min, ready for the next analysis. A CMA/160 on-line injector (CMA, Stockholm, Sweden) with a 20-μL sample loop was used as the interface between the UA-HF-LPME module and the HPLC system for sample introduction.

The UA-PPP-HF-LPME module comprised a KDS 120 push/pull syringe pump (Scientific Instrument Services, NJ), two 1.0-mL glass syringes equipped with Teflon-capped plungers and PTFE tubings (760 μm O.D., 300 μm i.d.; Hamilton, NV), an Accurel Q3/2 polypropylene HF (600 μm i.d., 200 μm wall thickness, 0.2 μm pore size, and 8 cm length; Membrana, Wuppertal, Germany), a Super-Nuova stirring plate (Thermo Scientific, NH), a heating mantle (Glas-Col, IN), a PTFE stir bar (25.4 × 7.9 mm), and an ultrasonic liquid processor Q125 (Qsonica, CT). For chromatographic determination, the outlet tubing of the UA-PPP-HF-LPME module was connected to the sample loop. The assembly of the UA-PPP-HF-LPME-HPLC system is illustrated elsewhere;[10] a photograph of the assembled UA-PPP-HF-LPME system is presented in Figure 1.

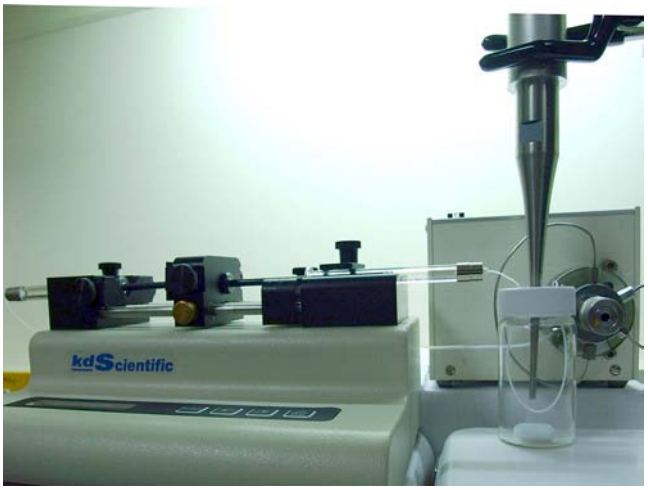

Figure 1. Photograph of the proposed UA-PPP-HF-LPME system.

Standard and Sample Solutions. Prior to analysis, all glassware was washed with hot water and then rinsed with ultrapure water, thoroughly rinsed with acetone, and finally thermally treated at 450 °C for at least 2 h in a muffle furnace. After being baked, the items were wrapped in aluminum foil and stored in a furnace. Prior to use, the glassware was rinsed again with a small portion of MeCN. The standard stock solutions of each HPAE were prepared in MeCN at a concentration of 1000 μg mL^{-1}. Working standard solutions were prepared daily through appropriate dilution of the stock solutions of the HPAEs with ultrapure water. The stock and working standard solutions were stored at 4 °C in a refrigerator. Sports drink samples were purchased from local supermarkets.

The blank values of the analytical procedure were determined by extracting an ultrapure water sample in the absence of HPAEs. The concentrations of the HPAEs in the samples were calculated after subtraction of the procedure blank value (procedure performed without a water or sports drink sample). Each sample was tested three times; the average of three measurements was taken as the result.

Inner-Fiber Fluid Stability Test. In three-phase HF-LPME, the inner-fiber fluid consists of the SLM in the wall of the HF and the acceptor phase in the lumen of the HF. In the inner-fiber fluid stability test, the SLM found in the donor or acceptor phase was used as an indicator of the SLM stability; the HPAEs found in the donor phase were used as an indicator of the acceptor phase stability. To evaluate the SLM stability, two tests were performed: an SLM inleakage test and an SLM outleakage test. In the SLM inleakage test (an indicator of inner-fiber fluid gain), the acceptor phase (MeCN) obtained after performing PPP-HF-LPME for 2 min was on-line-injected into the HPLC system for determination of the SLM (1-octanol). In the SLM outleakage test (an indicator of inner-fiber fluid loss), the donor phase (ultrapure water, 12 mL) obtained after performing PPP-HF-LPME for 10 min was off-line-injected into the HPLC system for

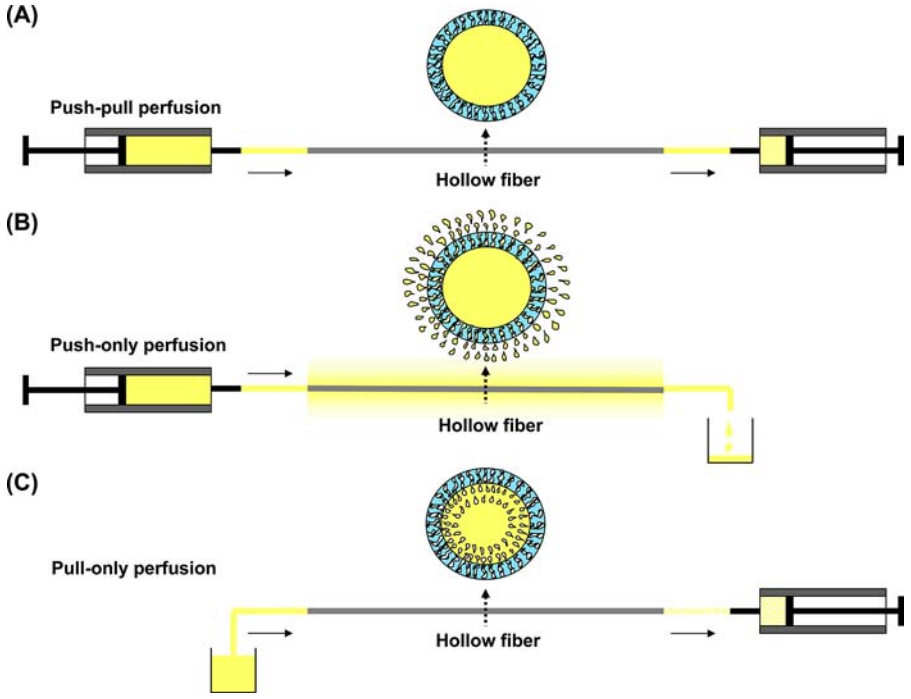

Figure 2. Effects of the type of perfusion pump on inner-fiber leakage.

determination of the SLM (1-octanol). In the acceptor phase (standard HPAEs solution) stability test, the donor phase was ultrapure water (12 mL). The acceptor phase after performing PPP-HF-LPME for 2 min was on-line-injected into the HPLC system for determination of the HPAEs. Three experimental setups were used in these stability tests: a PPP-based HF-LPME module, a push-only perfusion-based HF-LPME module, and a pull-only perfusion-based HF-LPME module.

PPP-Based HF-LPME Sampling. The assembly of the PPP-based HF-LPME-HPLC system was the same as that described previously,[10] except for the ultrasonic probe. To set up the PPP-based HF-LPME module, one end of an HF was inserted into PTFE needle tubing and the other into a single segment of PTFE tubing, and then the HF was immersed into the SLM (1-octanol) for 30 s to ensure that the pores of the HF membrane were filled with the intermediary. After SLM impregnation, the SLM that had diffused into the lumen of the fiber was removed by passage of air from a 5-mL syringe; the inlet PTFE needle tubing was then connected to the push syringe, which was filled with the acceptor solvent, whereas the outlet PTFE tubing was connected to an on-line injector. An empty pull syringe equipped with PTFE needle tubing was then inserted into the outlet of the on-line injector. Simultaneous infusion and withdrawal of the acceptor solvent at the same rates was conducted using a push/pull syringe pump with two opposing syringes on the same drive screw, with limited perfusion pressure produced during the perfusion process. Subsequently, the HF was placed in the sample solution. PPP-based HF-LPME sampling was performed by perfusing the HF with the acceptor solvent at a flow rate of 0.6 mL h^{-1}. To prevent memory effects, the whole PPP-based HF-LPME system (except for the HF, which was employed only once in each study) was cleaned sequentially with the acceptor solvent between runs.

Push-Only Perfusion-Based HF-LPME Sampling. Instead of the push/pull syringe pump used in the PPP-based HF-LPME module, an infusion syringe pump was used in the push-only perfusion-based HF-LPME module. Similar to the experimental setup described above (PPP-Based HF-LPME Sampling), after SLM (1-octanol) impregnation and removal of air from the inner fiber, the inlet PTFE needle tubing of the HF was connected to a push syringe, which was filled with the acceptor solvent, while the outlet PTFE tubing of the HF was connected to an on-line injector. The outlet of the on-line injector was then connected to an empty waste vial, rather than to an empty pull syringe. The perfusion of the acceptor solvent was conducted using an infusion syringe pump; a large acceptor-to-donor perfusion pressure was produced during the perfusion process.

Pull-Only Perfusion-Based HF-LPME Sampling. Instead of the push/pull syringe pump used in the PPP-based HF−LPME module, a withdraw syringe pump was used in the pull-only perfusion-based HF-LPME module. Similar to the experimental setup described above (PPP-Based HF-LPME Sampling), after SLM (1-octanol) impregnation and removal of air from the inner fiber, the inlet PTFE tubing of the HF was immersed in a test tube filled with the acceptor solvent, instead of a push syringe filled with the acceptor solvent; the outlet PTFE tubing of the HF was connected to an on-line injector. An empty pull syringe equipped with PTFE needle tubing was then inserted into the outlet of the on-line injector. The perfusion of the acceptor solvent was conducted using a withdraw syringe pump; a large donor-to-acceptor perfusion pressure was produced during the perfusion process.

Optimization of UA-PPP-HF-LPME. The experimental setup described above (Instrumentation) and a 51-mL glass sample vial (40 × 60 mm) were used in the optimization experiments. The extraction efficiencies were evaluated by the relative peak area ratio produced by comparing the found peak areas of HPAEs obtained after each optimization experiment with the peak area of DNOP obtained after applying the HF-LPME conditions described in Figure S8.

Multiple Effect Evaluation. Multiple effects, including main, interaction, and quadratic effects, between selected UA-PPP-HF-LPME variables were examined using a full factorial design. An analysis of variance (ANOVA) was performed on the design to assess these effects. The data were analyzed using Stata software (v. 12, for Windows).

Off-line HF-LPME Sampling. Similar to the experimental setup described in Optimization of UA-PPP-HF-LPME, but without the ultrasonic probe, a conventional medical syringe needle and a 25-μL HPLC syringe were used in the off-line HF-LPME module, rather than the inlet PTFE needle tubing connected to the push/pull syringe pump and the outlet PTFE tubing connected to the on-line injector used in Optimization of UA-PPP-HF-LPME. The 25-μL HPLC syringe served to introduce the acceptor solution into the HF prior to extraction and to collect this solution after extraction, while the conventional medical syringe needle was used to support the HF.

RESULTS AND DISCUSSION

Although the assistance of PPP and ultrasonication makes UA-PPP-HF-LPME a rapid and efficient approach for on-line extraction of acidic analytes featuring ionizable functionalities,[10,11] we have limited information regarding the effects of both PPP and ultrasonication on the stability of the extraction solvent or its extraction capability, including extraction speed, toward neutral compounds. On the other hand, although ultrasonic radiation is a powerful tool that can accelerate various steps during sample pretreatment, our understanding of its interaction effects with other effective HF-LPME variables remains limited. In this study, we investigated these advanced features by using three HPAEs as model neutral analytes, by using SLM/HPAEs in the acceptor/donor phase as solvent stability indicators, and by examining the time profiles of the extraction efficiencies of HPAEs from aqueous solutions using conventional off-line HF-LPME. Furthermore, by using full factorial design as a powerful evaluation tool, we hereby report the negative interaction between ultrasonication and temperature on the extraction efficiencies of neutral analytes when using on-line HF-LPME.

Effect of Perfusion Mode on Inner-Fiber Fluid Stability. The fiber commonly employed in most HF-LPME systems has been an Accurel Q3/2 HF membrane having a pore size of 0.2 μm; its wall thickness (200 μm) provides excellent mechanical stability and simplifies the preparation of the extraction units.[3] Nevertheless, the feasibility of extracting HPAEs with a regular HF-LPME device in a U-shaped configuration when using a conventional push- or pull-only syringe pump as the driving source has been impeded by the porous nature of this membrane.[10,11] During extraction using a push- or pull-only syringe pump, the acceptor-to-donor or donor-to-acceptor perfusion pressure caused by the flowing acceptor phase always leads to convective inner-fiber fluid loss or inner-fiber fluid gain across the HF's semipermeable membrane. The physical manifestation of the fluid loss is that the HF appears as if it were sweating (Figure 2B), whereas for fluid gain the HF appears as if it were inleaking during the extraction procedure (Figure 2C). Both the sweating and the inleaking effects decrease the target analyte recoveries and EFs, thereby attenuating the applicability of these approaches for on-line coupling to any analytical instrument. With the PPP-based HF-LPME system, however, inner-fiber fluid loss or gain is successfully minimized by using a simple push/pull syringe pump to decrease the acceptor-to-donor/donor-to-acceptor perfusion pressure, such that no visible sweating or inleaking occurs during the on-line sampling process (Figure 2A).

Although PPP-based HF-LPME, push-only perfusion-based HF-LPME, and pull-only perfusion-based HF-LPME are closely related in their extraction principles, the nature of the perfusion equipment and the perfusion pressure differ significantly. In this study, we examined the effects of the perfusion mode on the stability of the SLM and acceptor phase by varying the nature of the syringe pump. When we used the standard solution of HPAEs as the acceptor phase and ultrapure water as the sample solution (donor phase), Figure 3A and B reveals that the pull-only perfusion mode, which led to higher donor-to-acceptor fluid pressure and, thus, increased inner-fiber lumen fluid gain, including inleakage of the SLM to the acceptor phase, resulted in a greater amount of SLM (1-octanol) present in the acceptor phase (Figure 3A) relative to those in the push-only and push/pull perfusion modes (Figure 3B). Figure 3B also reveals that the push-only perfusion mode, which led to higher acceptor-to-donor fluid pressure and, thus, increased inner-fiber lumen fluid loss, including outleakage of the SLM to the donor phase, resulted in a greater amount of SLM (1-octanol) present in the donor phase relative to those in the pull-only and push/pull perfusion modes (Figure 3B). Figure 3C reveals that the PPP mode, which led to lower perfusion pressure (both the acceptor-to-donor/donor-to-

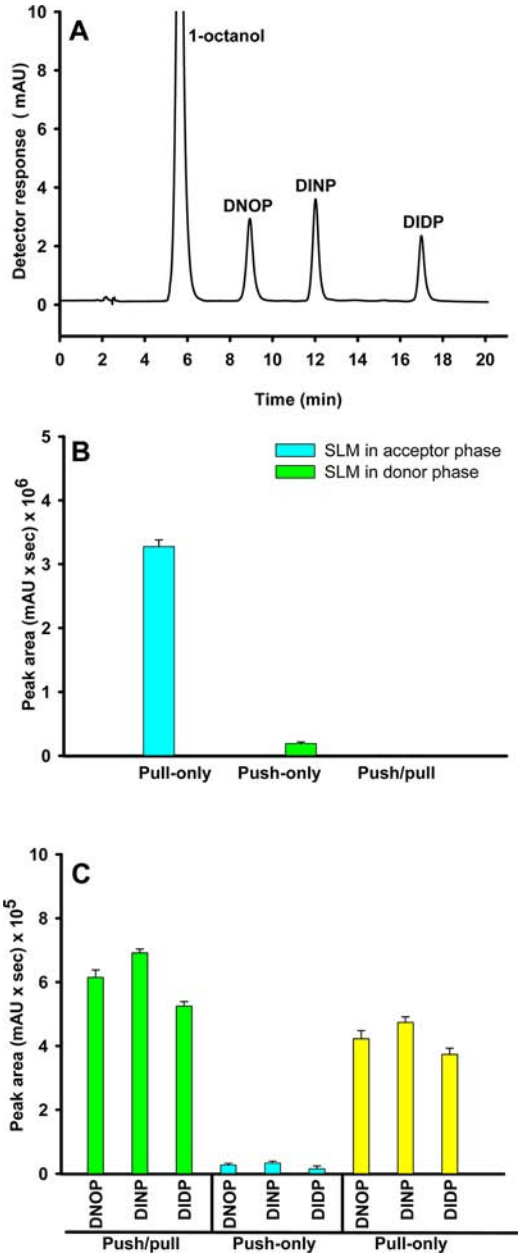

Figure 3. Effects of the type of perfusion pump on the stability of the inner-fiber fluid and the recoveries of the target analytes. (A) Chromatogram of 1-octanol after extraction using pull-only perfusion-based HF-LPME combined with HPLC-UV; (B) 1-octanol leakage after extraction using the on-line HF-LPME systems; and (C) recoveries of HPAEs after extraction using the on-line HF-LPME systems. On-line HF-LPME sampling: sample solution, ultrapure water; acceptor phase, DNOP (100 ng mL^{-1}), DINP (200 ng mL^{-1}), and DIDP (200 ng mL^{-1}) in ultrapure water at 0.6 mL h^{-1}; SLM, 1-octanol; agitation speed, 600 rpm; fiber length, 8 cm; sample temperature, 25 °C (ambient temperature).

dx.doi.org/10.1021/jf401734u | J. Agric. Food Chem. 2013, 61, 8063−8071

acceptor pressure) and, thus, no (or limited) inner-fiber lumen fluid loss/gain, provided much higher recoveries of the HPAEs than those in the push- and pull-only perfusion modes. In the push-only perfusion mode, the limited recoveries of the HPAEs indicated the leakage of both the standard solution of HPAEs (acceptor phase) and the SLM (1-octanol) into the sample solution. In the pull-only perfusion mode, the moderate recoveries of the HPAEs indicated a dilution effect, resulting from the increased inner-fiber lumen fluid gain, including the inleakage of both ultrapure water (sample solution) and the SLM (1-octanol) to the acceptor phase (the standard solution of HPAEs). This finding is consistent with the results in Figure 3A. Accordingly, we selected the push/pull syringe pump, which did not cause any problems relating to fluid loss or gain across the HF membrane, as the driving source for the proposed on-line HF-LPME system.

Optimization of UA-PPP-HF-LPME Conditions. To obtain the optimal conditions for the UA-PPP-HF-LPME of HPAEs from aqueous solutions and sports drink samples, we used a conventional one-variable-at-a-time method to evaluate the effects of eight parameters (the nature of the SLM and acceptor solvent, the addition of salt, the fiber length, the stirring rate, the sample temperature, the sonication amplitude, and the perfusion flow rate) and then used a full factorial design to further assess the novel effective factors of on-line HF-LPME.

Selection of SLM and Acceptor Solvent. The selection of the two immiscible organic solvents, the SLM immobilized in the pores of the HF and the acceptor filled in the lumen of the fiber, is a critical aspect of three-phase HF-LPME.[15] On the basis of some of the considerations described in our previous studies,[10,11] we selected four water-immiscible alkanes as organic membrane solvent candidates and water, MeOH, EtOH, and MeCN as the acceptor organic solvent candidates. Both of the sets of solvents have all of the previously mentioned required characteristics. We found that the variations in the extraction efficiencies of the SLM candidate solvents were not remarkable, whereas the extraction efficiency of aprotic MeCN was greater than those of the protic (water, MeOH, EtOH) acceptor phase candidates (Supporting Information, Figures SM1 and SM2). Thus, we selected *n*-tetradecane as the SLM solvent and MeCN as the most-suitable acceptor solvent.

Effect of Salt Addition. Addition of NaCl to the sample solution may have several effects on LPME.[16,17] On the basis of the results of previous studies,[18] we performed experiments in which we added various amounts of NaCl (0−5%) into the donor phase. We found that the addition of salt at a concentration greater than 1% restricted the extraction of the target analytes (Supporting Information, Figure SM3), either because it changed the physical properties of the Nernst diffusion film[16] or it increased the donor viscosity;[17] both factors would decrease the mass transfer of analytes to the acceptor phase. Hence, we decided not to alter the salt content of the sample solutions in subsequent extractions.

Effect of Fiber Length. As reported in the literature, the extraction efficiency of HF-LPME depends on the length of the HF.[19] In this study, we investigated the extraction efficiencies of the proposed HF-LPME system upon increasing the length of the fiber from 2 to 12 cm. The relative peak area of the HPAEs increased upon increasing the HF length from 2 to 8 cm; any further increase in the HF length decreased the relative peak area, due to a dilution effect (Supporting Information, Figure SM4). Accordingly, we selected an HF length of 8 cm for subsequent experiments.

Effect of Stirring Rate. Magnetic stirring can improve the extraction efficiency and decrease the time required to reach thermodynamic equilibrium by facilitating the mass transfer process.[15] In this study, we agitated samples having a volume of 50 mL at various stirring rates (0, 200, 400, 600, and 800 rpm). The relative peak areas of all of the analytes increased upon increasing the stirring rate up to 600 rpm. Higher stirring rates (>600 rpm) caused air bubbles to adhere to the HF membrane surface, conceivably hindering the partitioning of analytes and, inevitably, affecting the extraction efficiency and precision (Supporting Information, Figure SM5). Hence, for the following studies, we selected a stirring rate of 600 rpm.

Effect of Sample Temperature. Generally, increasing the sample solution temperature can increase the diffusion coefficients of the analytes, thereby facilitating the partitioning of the analytes from the aqueous solution to the extracting phase and, thereby, leading to more-rapid establishment of the equilibrium state.[20] To study the effect of the sample temperature on the extraction performance, we tested sample temperatures from 25 to 60 °C. In terms of the extraction efficiency of the HF-LPME process, increasing the sample temperature restricted the extraction of the HPAEs (Supporting Information, Figure SM6). Therefore, we performed our subsequent experiments under conditions of ambient temperature.

Effect of Sonication. For most on-line applications, sampling times shorter than the total chromatographic time are often chosen to ensure high sample throughput. In the three-phase HF-LPME technique, two liquid−liquid interfaces exist in the extraction system: the donor phase−SLM and the SLM−acceptor phase; therefore, analytes require a relatively long time to diffuse through both of these interfaces.[3] Analyses of acidic and basic analytes in HF-LPME systems have been accelerated through ultrasonication, as a result of more-effective mixing of the extracted boundary layers with the bulk of the sample.[7−11] To study the effect of the sonication conditions on the extraction performance, we examined the system's performance at amplitude waves set between 0 and 60%. Our results indicated that the extraction efficiencies of the target HPAEs reached their maxima at an amplitude wave of approximately 40%. At amplitude waves greater than 40%, the penetration of the target analytes increased, but the degree of SLM depletion also increased during the extraction process (Supporting Information, Figure SM7). Therefore, we selected an ultrasound amplitude wave of 40% as the optimal setting.

Effect of Flow Rate. Similar to microdialysis,[21] which also works in flow-through mode, the extraction efficiency and extraction time of the proposed HF-LPME system both depend on the perfusion flow rate. A low perfusion flow rate increases the extraction recovery, but it takes time to collect a sufficient amount of the perfusate to clear the eluent from the sample loop and for its injection into the chromatographic system. To obtain acceptable extraction efficiency within an acceptable time, we performed a series of tests using perfusion flow rates ranging from 0.1 to 0.9 mL h^{-1}. Our results revealed that the extraction efficiencies increased significantly upon increasing the perfusion flow rate to 0.6 mL h^{-1}. We suspect that less of the SLM was lost and fewer air bubbles formed within shorter extraction times. On the other hand, the extraction efficiency decreased slightly when we increased the flow rate to 0.9 mL h^{-1} (Supporting Information, Figure SM8), presumably

because insufficient time was available for extraction of the samples, leading to deteriorated recovery. Therefore, the optimal flow rate of perfusion appeared to be 0.6 mL h^{-1}.

Multiple Effect Evaluation. Among the factors evaluated in Optimization of UA-PPP-HF-LPME Conditions, three of them, ultrasonication, temperature, and flow rate, were first tested in on-line HF-LPME approaches.[13] To obtain more information regarding their impact on the extraction efficiencies of the HPAEs, we used a full factorial design to examine multiple effects, including main, interaction, and quadratic effects, among these selected factors. The extraction procedure was controlled using the optimized variables obtained from the optimization experiments, except for the ultrasonication conditions, the temperature, and the flow rate. In the full factorial design, we used three levels for each of the three factors (overall 27 experiments) to experimentally assess the multiple effects. Furthermore, we performed three replicates for each experiment to obtain the experimental variance. An ANOVA *p*-value of less than 0.05 indicates that an effect is statistically significant at a 95% level of confidence. Table 1 lists

Table 1. Independent Variables and Their Levels Used in the Full Factorial Design

		level		
variable	coded	−1	0	+1
sonication (%)	A	20	40	60
temp (°C)	B	25	40	60
flow rate (mL h^{-1})	C	0.3	0.6	0.9

Table 2. ANOVA Data for the Experimental Responses Obtained Using the Full Factorial Design

factor	coefficient	*F*-value	*p*-value
A − sonication	20.3946	574.0816	<0.001
B − temp	−2.7444	5.7121	0.020
C − flow rate	642.6511	128.3689	<0.001
AB	−0.1199	7.1824	0.009
AC	0.2121	0.2401	0.628
BC	−0.5642	1.2996	0.260
A^2	−0.2047	489.2944	<0.001
B^2	0.0178	2.0449	0.156
C^2	−488.6226	141.1344	<0.001

the variables and their levels considered; Table 2 provides a summary of the results from the ANOVA tests. We observed significant positive main effects for ultrasonication and the flow rate, with a negative main effect observed for the temperature. In addition, we also detected a notable quadratic effect for ultrasonication and the flow rate. Accordingly, the rate of increase in the extraction efficiency of the HPAEs decreased upon increasing the level of ultrasonication and the flow rate. These results are comparable with those obtained from our conventional optimization experiments. Furthermore, we identified a significant negative interaction (p for interaction = 0.009) between ultrasonication and the temperature, suggesting that the effect of increased ultrasonication on the extraction efficiency of the HPAEs decreased upon increasing the temperature. According to Li and co-workers,[22] this behavior can be explained by the decrease in the partition coefficients of the analytes between the SLM and the aqueous phase and by the increase in SLM depletion during the extraction process, both of which decrease the mass transfer rate of the analytes to the acceptor phase.

Conventional Off-line HF-LPME. To evaluate the net effect of ultrasonication and PPP on the rate of extraction of the HPAEs from aqueous solutions when using UA-PPP-HF-LPME, we examined the time profiles of conventional off-line HF-LPME for the extraction efficiencies of the HPAEs. We evaluated the extraction efficiencies from the relative peak area ratios, produced by comparing the found peak areas of the HPAEs obtained after conventional off-line HF-LPME without the help of ultrasonication and PPP with those obtained after the present on-line HF-LPME with the help of ultrasonication and PPP. In the three-phase HF-LPME technique, two liquid−liquid interfaces exist in the extraction system (donor phase−SLM and SLM−acceptor phase); therefore, analytes require a relatively long time to diffuse through both of these interfaces.[3] Figure 4 reveals that the extraction efficiencies using conventional off-line HF-LPME increased quickly upon increasing the extraction time up to 40 min; no substantial increase occurred thereafter. Notably, the extraction efficiency (relative peak area ratio of 100% for extracted HPAEs) reached after more than 30 min when using conventional off-line HF-LPME without the help of ultrasonication and PPP (Figure 4) was the same as that obtained within only 2 min when using the UA-PPP-HF-LPME system. Thus, with the assistance of ultrasonication and PPP, the rate of extraction of the proposed system was elevated, potentially cutting the extraction time by more than 93%.

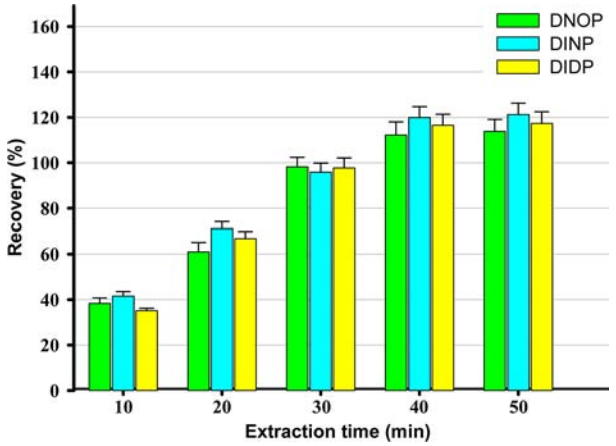

Figure 4. Extraction efficiencies of HPAEs plotted with respect to extraction time. Off-line HF-LPME sampling: sample solution, DNOP (50 ng mL^{-1}), DINP (100 ng mL^{-1}), and DIDP (100 ng mL^{-1}) in ultrapure water; SLM, *n*-tetradecane; acceptor phase, MeCN; fiber length, 8 cm; sample temperature, 25 °C (ambient temperature).

Method Evaluation. To evaluate the performance of the proposed UA-PPP-HF-LPME technique, we investigated its repeatability, linearity, and limit of detection (LOD) by analyzing standard solutions of HPAEs in ultrapure water. As summarized in Table 3, the calibration curves for the HPAEs at concentrations ranging from 0.5 to 500 ng mL^{-1} exhibited correlations of determination (r^2) of greater than 0.992, with LODs in the range 0.05−0.09 ng mL^{-1}, based on 3 times the standard deviation of seven replicate runs of water spiked with each analyte at about the lowest concentration of the calibration curve.[23] We calculated the relative standard deviations (RSDs) based on the peak areas for seven replicated

Table 3. Performance of the UA-PPP-HF-LPME System

analyte	linear range (ng mL^{-1})	LOD[a] (ng mL^{-1})	r^2	EF	RSD[b] % (n = 7)	RSD[c] % (n = 7)
DNOP	0.5−500	0.05	0.998	146	3.3	3.4
DINP	0.5−500	0.08	0.996	114	5.2	2.5
DIDP	0.5−500	0.09	0.992	92	6.1	3.2

[a]LODs were calculated as 3 times the standard deviation of seven replicated runs of the standard solution. Concentrations: DNOP, 0.5 ng mL^{-1}; DINP, 0.5 ng mL^{-1}; DIDP, 0.5 ng mL^{-1}. [b]Data obtained by extraction in seven replicates. Concentrations: DNOP, 0.5 ng mL^{-1}; DINP, 0.5 ng mL^{-1}; DIDP, 0.5 ng mL^{-1}. [c]Data obtained by extraction in seven replicates. Concentrations: DNOP, 5 ng mL^{-1}; DINP, 5 ng mL^{-1}; DIDP, 5 ng mL^{-1}.

runs; these values were less than 6.1%. Therefore, this new on-line technique provided good repeatability as a result of decreased manual handling of the sample and, therefore, decreased risk of errors, such as adsorption of the HPAE components onto glassware, transfer losses, exposure to air and the bulk extraction solvent, and contamination of the sample.

Table 4 compares the figures of merit of the proposed method with those of previously published methods using other microextraction approaches coupled with HPLC for the determination of HPAEs.[24−27] Clearly, our proposed method, involving both HF microfiltration and sonication, provides analytical performance better than those of other methods involving SPME-based approaches within a much shorter extraction time. Table 5 compares the figures of merit of the proposed method with that of a published method using off-line HF-LPME for the determination of HPAEs in water samples.[18] Clearly, our proposed method provides acceptable sensitivities with suitable dynamic linear ranges (LRs). In contrast, the acceptor phase used for that gas chromatography/mass spectrometry (GC−MS) method[18] was not suitable for HPLC determination, with low precision unavoidable because of problems related to inconsistency in the timing of the manual extraction, manual transfer of a low volume of volatile extraction fluid, and exposure of the extract to air. Because it involves on-line operation without manual handling of the sample, the proposed method can be used to extract HPAEs from complex matrixes with excellent precision. Notably, coupling of this method with HPLC can provide short sampling times (only 2 min), better stability, wider LRs, and sensitivity comparable to that of off-line HF-LPME coupled with GC−MS.

Application of the Proposed Method to Real Samples. Phthalate esters (PAEs) can be divided into three subcategories based on their physicochemical and toxicological properties: low-molecular-weight PAEs, transitional PAEs, and HPAEs. HPAEs are produced from alcohols with straight-chain carbon backbones of greater than or equal to C7 or benzyl alcohol in conjunction with a diester group having a total carbon backbone of greater than or equal to C7.[28] In 2011, the Food and Drug Administration (FDA) in Taiwan announced that DINP, a carcinogenic HPAE, was found at elevated levels in commercial drinks, including sport drinks.[29] We evaluated the applicability of our developed method by determining the levels of three HPAEs, DNOP, DINP, and DIDP, in sports drink samples collected from local markets in Kaohsiung city, Taiwan. We extracted the sports drink samples directly using the developed method, without any pretreatment. We could not, however, detect any of the target analytes in the sports drink samples after UA-PPP-HF-LPME; it is likely that they were not present or that their concentrations were below the LODs of this procedure. Figure 5 displays a chromatogram of spiked sports drink samples (5 ng mL^{-1} of DNOP; 10 ng mL^{-1} of DINP and DIDP) after extraction using our developed method. To assess matrix effects, we spiked the sports drink samples with HPAEs from a standard solution at a concentration of 0.5 or 5 ng mL^{-1} of each analyte (Supporting Information, Table SM1). The relative recoveries, defined as the ratios of the measured concentrations of the analytes in the sports drink samples to the measured concentrations of the analytes in pure water samples spiked with the same amounts of the analytes, were greater than 91.2% for all of the tested analytes; therefore, the matrixes of the sports drink samples had little effect on the extraction efficiency of the developed method. We conclude that this method is suitable for the determination of HPAEs at trace concentrations in sports drink samples.

Advanced Information. In the section Multiple Effect Evaluation, we note that the three-variable model has a value of R^2 of 96.6%, indicating that a high degree of variation in the extraction efficiency of the HPAEs can be obtained through varying a combination of the ultrasonication conditions, the temperature, and the flow rate. In this present study, we have determined five pieces of advanced information related to the three HF-LPME variables. First, we have, for the first time, reported the effects of PPP on the stability of the inner-fiber fluid. Other than our previous reports, no papers have described the problem or the extent of inner-fiber fluid leakage in HF-LPME systems. Second, we have demonstrated that ultrasonication is a universal technique that can improve the extraction kinetics for not only ionizable analytes (acidic

Table 4. Comparison of On-line UA-PPP-HF-LPME with Other Microextraction Approaches Coupled with HPLC for the Determination of HPAEs[a]

analyte	method	sample matrix	extraction time (min)	LRs (ng mL^{-1})	LOD (ng mL^{-1})	RSD (%)	ref
DNOP[b]	off-line PMME-HPLC-UV	cosmetics	13.9	3−5000	2.1	6.1	24
DNOP[b]	off-line SPME-HPLC-UV	environmental water samples	30	2.5−40	2.07	8.7	25
DNOP[b]	on-line PCFE-HPLC-UV	riverwater, wastewater	20	1−200			26
DNOP[b]	on-line in-tube SPME-HPLC-DAD	infusion solutions	8	1−500	1−10[b]	1.8−18.4[b]	27
DNOP, DINP, DIDP	on-line UA-PPP-HF-LPME-HPLC-UV	sports drinks	2	0.5−500	0.05−0.09	2.5−6.1	this method

[a]DAD = diode array detector; PMME = polymer monolith microextraction; SPME = solid-phase microextraction; PCFE = miniaturized polymer-coated synthetic fiber extraction. [b]Other compounds were also analyzed.

Table 5. Comparison of On-line UA-PPP-HF-LPME with Off-line HF-LPME for the Determination of HPAEs in Water Samples

method	extraction configuration	analyte	extraction time (min)	linear range (ng mL^{-1})	LOD (ng mL^{-1})	RSD (%)	ref
HF-LPME/GC–MS	off-line	DNOP	20	0.5–10	0.1	18	18
UA-PPP-HF-LPME/HPLC-UV	on-line	DNOP, DINP, DIDP	2	0.5–500	0.05–0.09	2.5–6.1	this method

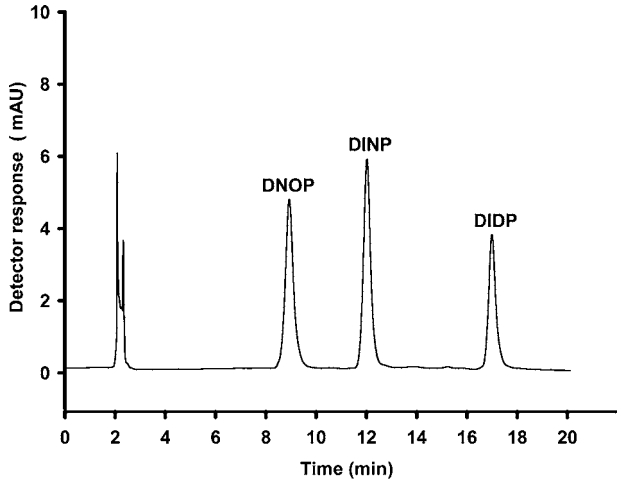

Figure 5. Chromatograms of HPAEs after extraction from a sports drink sample using UA-PPP-HF-LPME combined with HPLC-UV. Sports drink sample was spiked with HPAEs at concentrations of 5 ng mL^{-1} for DNOP, 10 ng mL^{-1} for DINP, and 10 ng mL^{-1} for DIDP.

phenols in our previous studies[10,11]) but also neutral compounds (HPAEs in this present study). Third, we have demonstrated that heating cannot be used as a routine technique to improve the extraction kinetics for neutral analytes, for example, the HPAEs tested in this present study; this finding confirms the importance of ultrasonication on improving the extraction kinetics of neutral analytes when using HF-LPME. Fourth, we have noted, for the first time, a significant negative interaction effect between ultrasonication and temperature. Fifth, through the assistance of the two techniques, PPP and ultrasonication, we have demonstrated that the extraction times for neutral HPAEs can be cut by more than 93% when using HF-LPME. We are currently implementing this new device in new applications, with special emphasis on its strong capabilities to overcome the drawbacks of conventional HF-LPME when analyzing environmental water samples.

■ ASSOCIATED CONTENT

Supporting Information

Additional figures and tables. This material is available free of charge via the Internet at http://pubs.acs.org.

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Funding

This study was supported by the National Science Council of Taiwan (grants NSC100-2627-M-037-001 and NSC101-2113-M-037-003-MY3) and by the Center of Excellence for Environmental Medicine of Kaohsiung Medical University.

Notes

The authors declare no competing financial interest.

■ ABBREVIATIONS USED

ANOVA, analysis of variance; DIDP, diisodecyl phthalate; DINP, diisononyl phthalate; DNOP, di-*n*-octyl phthalate; EME, electromembrane extraction; HF, hollow fiber; HPAEs, high-molecular-weight phthalates; HPLC, high-performance liquid chromatography; LLE, liquid–liquid extraction; LPME, liquid-phase microextraction; PPP, push/pull perfusion; SLM, supported liquid membrane; UA-PPP-HF-LPME, ultrasound-assisted push/pull perfusion hollow-fiber liquid-phase microextraction

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